Peer Review Workshop
for EPA's Draft
Toxicological Review of Hexavalent Chromium

Reviewer Post-Meeting Comments

July 6, 2011

Contents

Responses to Charge Questions

General Charge Questions

G1. Is the Toxicological Review logical, clear and concise? Has EPA clearly presented and synthesized the scientific evidence for noncancer and cancer hazard?

Reviewer	Comments
Byczkowski	Logical - yes; clear - mostly; concise - no, as there is a very large body of information covered by this Toxicological Review. While the scientific evidence of both noncancer and cancer hazards of oral exposures to Cr^{+6} has been appropriately reviewed and synthesized, the conclusions and numerical derivation of toxicity values are mostly based on strict (default) interpretation and literal application of U.S EPA guidelines, rather than on the current scientific understanding of the mode of action of Cr^{+6}.
Hamilton	In general, the report is a concise presentation of the vast primary literature for chromium toxicology and carcinogenicity and the writing is generally clear. However, there are specific sections with deficiencies as noted below in detail, and many other sections and specific comments that are not logical. In many cases a statement in one section is either not a logical extension of the data presented, or is in opposition to a statement elsewhere. Overall, the greatest concern is with the logic regarding the choice of a mode of action (MOA), which is the basis for many of the subsequent assumptions that are made, the default values that are chosen, and risk assessment modeling that follows from these choices. In this reviewer's strong opinion – and in the consensus opinion of the external reviewers who are experts in this area and who discussed this at the May 12, 2011 meeting – Cr(VI) is highly unlikely to act via a mutagenic mode of action in vivo. Rather, a careful review of existing information, as well as emerging studies all strongly indicate that the likely MOA involves a threshold mechanism that supports both the physiological uptake-reduction model of DeFlora and the cellular uptake-reduction model of Wetterhahn that were previously proposed. The current EPA draft document concludes that chromium(VI) acts via a mutagenic MOA by all routes of exposure, a conclusion that is illogical given the current state of knowledge of chromium biology and toxicology as already presented in this draft report, and also based on the recently emerging data from a series of 90-day rodent MOA studies sponsored by the American Chemistry Council (ACC). This is the most important and central point since the choice of a mutagenic MOA then drives all other considerations in this document. Specific areas of concern are outlined by chapter and section below in detail in a combined response to G1. and G2. **Chapter 1** EPA should include more definitive information about the literature it reviewed that contributed to this draft report. It currently states that the relevant literature was reviewed through September 2010 but the external reviewers all noted at the May 12 meeting a number of gaps in the literature being cited in the current draft. In addition, it would be helpful to know how many studies the EPA identified as being part of the chromium literature, how many it reviewed, how many it set aside or did not review, what criteria

	were used to include or exclude a study, etc. For example, a statement such as: *"The EPA identified 26,839 peer-reviewed scientific publications in PubMed from 1950 through September 2010 using the keyword 'chromium.' Of these, 9,456 were determined to be relevant to the current draft based on the criteria of covering aspects of chromium biology, toxicology, environmental chemistry and epidemiology."* **Chapter 2** On page 14 the draft states that,*"Natural occurrence of hexavalent chromium is rare …"* This statement should be qualified since geochemical surveys by the U.S. Geological Survey and others have indicated that there are background levels of naturally occurring Cr(VI) in most groundwater, and that these levels are typically in the range of 2-5 ppb but can be up to 3-5 times higher than that in certain areas. Likewise recent reports of Cr(VI) levels in soil and house dust indicate that there are natural sources, or at least sources that cannot be identified as being specifically anthropogenic, that contribute to the background levels and background exposures of people throughout the U.S. A more detailed literature review and discussion regarding background levels of Cr(VI) in soil, air and water, including more up-to-date information on such levels, should be included in this chapter to provide context for subsequent discussions regarding human exposure through drinking water. Keep in mind that, until recently, analytical methods to detect total chromium and to speciate chromium(VI) accurately were difficult, with higher detection levels than many natural sources contain. Further, speciation of chromium(VI) at very low levels has been very problematic until recently, so much of the older environmental data are inaccurate or had non-detect values where reanalysis has revealed widespread chromium(VI) background levels in the environment. This is of importance not only for understanding the potential contribution of these background levels to total chromium exposure but also in setting practical regulatory levels. Clearly it is of little value for the EPA to calculate a maximum contaminant level (MCL) or public health goal (PHG) that is lower that typical background levels since this would be virtually impossible to achieve by most public drinking water systems with limited resources, and because such background levels are unlikely to represent a significant health risk (see discussion below). **Chapter 3** This is an important chapter that would greatly benefit from reorganization and from synthesis of information. A number of studies are described here sequentially, and for several of these the draft document speculates on possible mechanisms. However, the order of presentation is not logical, and there is little in the way of more global synthesis of results and the conclusions that can be drawn – or those areas that remain controversial or poorly understood – while there are several areas where a statement in one section is contradicted by a statement elsewhere. Because the toxicokinetics of chromium is so central to its biology and toxicology, this is a critical chapter for its overall evaluation and therefore this chapter should be revised. Regarding organization, Section 3.3 is extremely important for understanding the studies described throughout the remainder of the chapter, providing context for extracellular versus intracellular reductive metabolism. It is suggested that this section should be moved to just before or immediately after the current Section 3.1. Likewise, Section 3.6 on Cr(III) and its nutritional benefit and essentiality is extremely important for

	understanding how the body normally absorbs, distributes and excretes chromium. From the standpoint of human environmental exposures, Cr(VI) is primarily, but not exclusively an anthropogenically derived form of chromium that is principally encountered and used in occupational settings, but humans and all other life forms have been dealing, both physiologically and biochemically, with Cr(III) and reduction of background levels of Cr(VI) for the entire history of life on Earth. It is recommended that Section 3.6 should be moved to the beginning of this chapter and greatly expanded. The current section on this important topic is extremely short, superficial, and inaccurately presents this subject as an area of controversy in the field. Cr(III) nutritional biochemistry has been extensively studied over the past fifty-plus years with a large and robust literature. Only two toxicologists are cited as the sources of the "current debate" about this, whose individual views have been well aired in their review articles but which represent views that are generally considered to be well outside the mainstream of toxicology and nutritional biochemistry. Citing their views so prominently in this short section does not balance well with the wealth of studies and numerous other investigators over decades who have concluded that there is a beneficial role of Cr(III) in human and animal nutrition and have demonstrated an underlying biochemistry and physiology that supports this role. This section should be greatly expanded and treated in a more balanced fashion since it sets the stage for understanding how Cr(III) is treated by the body in all aspects of toxicokinetics, and therefore also provides valuable insight into Cr(VI) toxicokinetics that largely explains much of the experimental literature on chromium disposition in intact animals and humans as discussed in more detail below. See for example reviews by W Mertz (J Nutr 1993, 123:626-633; Nutr Rev 1995, 53:179-185), RA Anderson (Reg Tox Pharm 1997, 26:S35-41), HC Lukaski (Ann Rev Nutr 1999, 19:279-302) and JB Vincent (J Am College Nutr 1999, 18:6-12) for summaries of this earlier literature. Some of this information is alluded to – for example, the end of the primary paragraph on page 44 beginning with *"Aitio et al. (1988) developed …"*, which represents an extremely important body of literature on Cr(III) toxicokinetics and which should be moved forward with section 3.6 and greatly expanded by citing other relevant literature. There are dozens of studies of Cr(III) uptake and kinetics. The current chapter leaves the impression that Cr(III) gastrointestinal (GI) uptake is uniformly low, and that chromium is not normally found above background levels in urine, therefore if one administers Cr(VI) and sees a dose-dependent increase in blood, urinary or tissue levels it is evidence of Cr(VI) uptake as Cr(VI). But there are a number of uptake studies of chromium picolinate and other natural and man-made Cr(III) complexes, as well as Cr(III) uptake in chromium-sufficient versus chromium-deficient diets, that defy this simple interpretation. For example, one study described on page 45 by Kerger et al. (1997) reported that *"ingestion of chromium picolinate resulted in significantly elevated urine concentrations such that participants routinely exceeded background."* Similar elevations have been reported for chromium picolinate in the nutritional literature. Likewise, other studies have shown an inverse correlation between Cr(III) levels in diet and the uptake, distribution, tissue storage and excretion of Cr(III), indicating that the body tightly regulates Cr(III) kinetics to maintain a steady-state body burden and availability of the nutritionally active form of Cr(III), and can actively take up Cr(III) from the GI when the body senses that it is deficient, or decrease uptake and increase excretion when internal stores are sufficient or exceeded.

	A related and key concept that is alluded to in this section but never addressed directly is the behavior of chromium in serum and red blood cells (RBC). It has been well established, beginning with RBC labeling studies going back to the 1950's in which RBC are incubated with Cr(VI) ex vivo, that Cr(VI) is readily taken up by RBC, rapidly reduced, and in the process forms highly stable chromium adducts on hemoglobin and other macromolecules which are very long lived, essentially remaining intact for the lifetime of the cell. In this way the half-life of RBC in humans and experimental animals was established (with human RBCs having a half-life of ca. 110-120 days) and this tool has also been used to look at RBC turnover. This is alluded to on page 27, where it states that *"The partitioning of hexavalent chromium from plasma into erythrocytes is significant. It has been used as a biomonitoring endpoint … and is responsible for the observed residence time of chromium in whole blood …"* Conversely, in the human and animal studies that were described in Chapter 3, it has been consistently shown that when Cr(VI) is administered orally or by gavage, there is a transient increase in both serum and RBC levels which rapidly return to baseline. Thus, it is highly unlikely that the chromium in the blood of these animals and humans was Cr(VI), or else the RBC would have been stably labeled. It was noted on page 45, for example, in describing the human studies of Paustenbach et al. (1996) where there was oral exposure to Cr(VI) that *"both plasma and RBC chromium concentrations returned rapidly to background levels within a few days, again suggesting that concentrations of 10 mg Cr(VI)/L or less in drinking water of humans appears to be completely reduced to Cr(III) prior to systemic distribution."* This is an extremely important experimental observation in humans – and one of the most important statements in this draft document -- that directly addresses the issue of whether Cr(VI) is taken up by the human GI as Cr(VI) and whether it survives as Cr(VI) in the circulation. Similar results were seen in the Sutherland et al. (2000) rat study in which the blood kinetics, and lack of chromium increases in brain or other distal tissues argued strongly that it was Cr(III) rather than Cr(VI) that was taken up by the gut. Were this not the case, they should have observed stable chromium labeling of the RBC and elevated chromium levels in distal tissues. But they reported the opposite, and this also strongly indicates in this key rodent study that Cr(VI) failed to survive as Cr(VI) in crossing into the bloodstream from a GI exposure. And elsewhere in Chapter 3, similar results are reported that would lead to the same conclusion, yet the text alludes to transient RBC uptake as possible evidence that Cr(VI) is being taken up from the GI tract as Cr(VI). This is a highly flawed, illogical argument that appears throughout this document. Likewise it is now well known that there are specific uptake, transport and storage mechanisms for nutritionally active Cr(III) that must be taken into account in any measurements of chromium in the blood or other tissues. In fact, none of the studies presented in Chapter 3 provide any direct evidence that any Cr(VI escapes the GI tract as Cr(VI) except perhaps where normal gastric reduction is bypassed or the bolus doses are so large as to completely overwhelm the reductive capacity of the gut, which is likely what occurred in the NTP (2008) study. This should be more clearly described in this chapter, perhaps with a new section prior to current section 3.5 that presents the theoretical PBPK model since this model also critically relies on how one interprets the in vivo data.

	Specific edits suggested below are based on these concerns (changes underlined): • Page 24, Line 24 – "…*for the chromium administered as hexavalent* …" • Page 25, Line 7 – "…*of the chromium administered as hexavalent* …" • Page 26, Line 3 – "…. *Generally, absorbed chromium is* …" • Page 26, Line 9 – "… *toxicokinetics of chromium*, …." • Page 26, Line 16 – "… *comparing administration of Cr(III)* ..." • Page 30, Line 2 – "… *bioavailability of chromium administered as* …" • Page 35, Lines 7-11 – two sentences beginning with "*The reason for the higher* …" This is highly speculative and should be deleted. This could all be Cr(III) rather than Cr(VI) using the arguments above. • Page 36, Lines 25-28 – end of sentence beginning with "… *indicating that a portion of the Cr(VI) escaped extracellular reduction* …" This is also highly speculative and is actually counter to the data presented, which clearly show transient blood levels that are indicative of Cr(III) distribution, not Cr(VI), as more appropriately alluded to in Lines 32-34 where is says "*Brain, ovarian, and whole-blood concentrations were below detection limits in all exposed groups. The lack of concentrations in whole-blood was attributed to rapid delivery of Cr to tissues and clearance of plasma Cr.*" The lack of stable blood chromium clearly indicates that it could not have been absorbed as Cr(VI) or else the RBC would have shown significant and stable elevations. • Page 38, Line 13 – "… *did not alter GI uptake appreciably at these concentrations*." • Page 41, Line 1 – "*Chromium is capable of crossing the placenta*." • Page 41, Line 15-17 – "*Absorption and elimination of chromium was evaluated …. Following ingestion by human volunteers of either trivalent or hexavalent chromium in single or multiple drinking water doses*." • Page 42, Lines 23-26 – This statement beginning with "*Because the Cr(VI) increases* …" is incorrect. Increases in RBC chromium that are stable over time, with the same half-life as the RBC, would be indicative of uptake of chromium as Cr(VI) by the RBC, but if the increase in RBC chromium is transient, as in this case, then it cannot be due to Cr(VI) in the blood but is most likely a Cr(III) complex that is carried by the RBC but not covalently bound. Evidence for this is cited elsewhere in this chapter (see middle of page 44 and top of page 46, for example) and in the broader Cr(III) literature. The draft report is illogical since it currently argues this issue both ways depending on the study being discussed. • Page 42, Lines 30-35 – These two sentences beginning with "*The higher bioavailability* …" are highly speculative and well outside the boundaries of the actual data presented and discussed above. If the report is going to speculate, it should pull all this into a separate section and synthesize it across all the studies that were cited.

Otherwise delete these speculations since they do not have a factual foundation.

- Page 49, last line – " … *chromium administered as Cr(VI) distributes* …"
- Page 58, Lines 2-3 – " … *greater percentage of chromium administered as Cr(VI) than Cr(III) is absorbed.*" Delete sentence reading "*This implies that some Cr(VI) escaped reduction* …" since this is not implied by the actual experimental data based on the above arguments.

Taken together, these studies, as argued above, lead one to only one logical conclusion: Cr(VI) is not taken up by the gut as Cr(VI), nor does it survive as Cr(VI) in the systemic circulation in these studies. It is only by greatly exceeding the normal doses and reductive capacity of the gut that one can see any signs of Cr(VI) surviving to reach other tissues. Therefore there is a clear biochemical barrier, a threshold, to Cr(VI) uptake and systemic exposure under normal physiological conditions. This must clearly be taken into account in any analysis of MOA and resulting risk assessment for this toxicant.

Chapter 4

Liaoning Province, China studies – At the beginning of this chapter (Pages 68-76) the EPA presents an extensive review and discussion of the various reports related to the study of populations in China near a site of chromium-contaminated drinking water from a nearby industry. Depending on the authors and methods used – particularly certain assumptions regarding age adjustment, use of an urban area as a control population, and exposure estimates -- the reports of this data set either find no statistical association between stomach cancer incidence or a modest elevation. The other epidemiology studies cited in this chapter report no statistical correlation between drinking water chromium exposure and cancer incidence. It is of considerable concern to this reviewer that the EPA has chosen in some places to highlight this one positive report and elevate it to the level of their major recommendations, such as on Page 239 of Chapter 6 (Major Conclusions) where they state that there is *"evidence of an association between oral exposure to hexavalent chromium and stomach cancer in humans."* Yet elsewhere, they state, appropriately, that *"this risk has not been established in other populations exposed to drinking water contaminated with hexavalent chromium. The epidemiology data are not sufficient to establish a causal association between exposure to hexavalent chromium by ingestion and cancer."* (Page 201, Lines 20-23; and similar language on Page 205, Lines 33-36)

It is inappropriate to "cherry-pick" a single study – indeed, a single treatment among three treatments of the same study – if this is going to influence their major recommendations regarding the human carcinogenicity of Cr(VI) via ingestion. Many of my other comments and edits relate to this concern, as well as previous concerns regarding the toxicokinetics of Cr(VI) via ingestion and the misinterpretation of those results with respect to mode of action and carcinogenic potential in animals and humans as detailed below. This reviewer feels strongly that the Liaoning Province study and its various treatments should be set aside, since the initial study (first reported in 1987) is highly flawed by today's epidemiology standards, there is a lack of information that would allow others to reevaluate this study in the manner that would most directly address the potential correlation, and the three subsequent treatments by original authors Zhang and Li (1997),

by Beaumont et al. (2008) and by Kerger et al. (2009) -- each of which makes slightly different assumptions in order to fill critical data gaps -- reached different conclusions. It is also important to point out that this population was exposed not just to extremely high levels of Cr(VI) but also to industrial effluent which contained high levels of a number of other chemicals of concern including sulfates, acids and other toxicants. Thus, even if an association was found, it is not possible to attribute this to Cr(VI) per se and could be the result of either other contaminants or a statistical anomaly based on the high rates of stomach cancer in China which are diet- and province-related. For these reasons this study and its series of treatments cannot be the basis for risk characterization or risk assessment, and should be set aside.

Section 4.4 also requires extensive revision and expansion. The first paragraph reports only briefly on the extensive literature examining occupational exposures and cancer, principally lung cancer. This section, and this literature, should receive a much more extensive treatment since these studies are our best data regarding human exposures to Cr(VI). It should be noted that in addition to inhaling extremely high levels of Cr(VI) prior to the advent of industrial hygiene practices in the 1960's and 1970's (levels as high as several mg chromium(VI) per cubic meter) these workers had extensive dermal exposures, and also had extensive ingestion exposure since it is well known that individuals exposed to high levels of dust swallow a large fraction of what they inhale either through direct deposition in the mouth, nose and throat, and via mucocilliary clearance of the pulmonary system. Thus, these occupational epidemiology studies are extremely important in understanding the toxicology of chromium(VI). Several key points will be made below citing a few critical studies, but the EPA should more thoroughly review this entire literature.

The U.S EPA's current risk assessment for chromium(VI) via inhalation is based principally on the studies by Mancuso of older worker populations through the 1960's prior to the advent of modern industrial hygiene practices. Similar risks were observed in other occupational studies of workers from the 1940's through 1970's who were exposed to much higher levels of chromium than are encountered in occupational settings today, such as those by RB Hayes et al. (Intl J Epi 1979, 8:365-374; Am J Indust Med 1989, 16:127-133), JM Davies (Br J Indust Med 1984, 41:158-168), T Sorahan et al. (Br J Indust Med 1987, 44:250-258), and R Kishi et al. (Am J Indust Med 1987, 11:67-74). Subsequent epidemiology studies of workers exposed to lower levels of chromium since the 1960's have not only provided estimates of risk at these high doses, but have also provided important information as to lower occupational levels at which no increases in lung cancer or other health effects were observed. These more modern studies have also taken into account other factors, such as accounting for cigarette smoking and other confounding variables, whereas many of the older studies did not control for these important confounders (see for example, KD Rosenman, Am J Indust Med 1996, 29:491-500). It should be noted that, where it was reported, virtually all the lung cancer cases in these occupational studies occurred in smokers (see for example Pastides et al., Am J Indust Med 1994, 25:663-675; T-C Aw, Reg Tox Pharm 1997, 26:S8-12). These studies not only provide better estimates of the actual health risks attributable to occupational chromium exposure, but also an estimate of a practical threshold below which we would predict either no effects, or risk of effects that are so low that it cannot readily be detected

	even in large populations of exposed people. Gibb et al. performed a follow-up study (Am J Indust Med 2000, 38:115-126) of a worker population in Baltimore MD that had previously been studied by Hayes et al. (Int J Epi 1979, 8:365-374) in examining the relationship between chromium(VI) exposure and cancer incidence. In the Gibb study, the workers were stratified according to different levels of cumulative exposure to chromium, allowing a more detailed examination of the potential dose-response. Cumulative exposure was expressed as $\mu g/m^3$-years (1,000 ng/m^3-years), integrating both the chromium level and the total time of exposure at that level. This is similar to smoking data that express cumulative dose as "pack-years" and is based on the observation that the risk of a 40 pack-year smoker who used 1 pack per day for 40 years is similar to that of a 40 pack-year smoker who used 2 packs per day for 20 years. In the Gibb study, the lowest quartile of workers had exposure to chromium between 0 and 1.5 $\mu g/m^3$-years (1,500 ng/m^3-years). This group had an observed/expected lung cancer ratio of 0.96, i.e., it was slightly less than expected from the comparison population (the general population of Maryland) that had no occupational chromium exposure. Pastides et al. examined a group of chromate production company workers in North Carolina (cited above), focusing on the possible differences in risk between cohorts of workers who were exposed to chromium under the older conditions and processes of the 1940's through 1960's and those who began work after 1971 in a modernized factory in which both the chemical process and the exposure levels to chromium had been modified. They found a slightly increased risk of lung cancer, proportional to exposure, in the older cohort working under the higher dose exposure conditions, as had been reported previously in other studies. However, the workers in the modernized factory had no excess of lung cancer, all cancers, heart disease, or all causes of death over an 18 year period. Personal monitors for the workers indicated that the chromium(VI) levels were all below 50,000 ng/m^3, and most were below 25,000 ng/m^3, with the majority in the range of 500-10,000 ng/m^3. Average duration of employment was 9.5 years, such that cumulative dose would have averaged 4,750-95,000 ng/m^3-years. Dividing the workers into two groups of exposure, i.e., those working less than 10 years versus those working more than 10 years, indicated no difference in mortality, further suggesting that these workers had no significant increase in cancer or other health risks from either the higher or lower chromium exposures. Similarly, Aw reported (cited above) that workers in the more modernized plants who were occupationally exposed to chromium since the 1960's showed no increase in disease risk, as was also noted by S Langard et al. (Br J Indust Med 1990, 47:14-19). WJ Blot et al. performed a large and comprehensive study (J Occup Environ Med 2000, 42:194-199) of a group of 51,899 workers of the Pacific Gas & Electric (PG&E) Company. A sub-set of 3,796 these workers had been exposed occupationally to chromium(VI), either as gas generator workers or trainees at the Kettleman CA station which used chromium as a rust inhibitor in cooling tower water at PG&E natural gas transfer stations from the 1950's through the 1980's. Examination of these workers for specific cancers, all cancers, specific non-cancer diseases, and all diseases indicated no increased incidence in any adverse health outcomes in relation to chromium exposure. In fact, the total cancer and lung cancer standardized mortality ratios (SMRs,

observed/expected ratios) were 0.64 and 0.81, and 0.55 and 0.57, respectively, for these two groups of chromium-exposed workers, which was less than those of the overall PG&E worker group and substantially less than those of the general California population against which they were compared. SMRs for all causes of death were also low (0.79 and 0.68, respectively). Likewise, JD Boice et al. performed a large and comprehensive study (Occup Environ Med 1999, 56:581-597) of a group of 77,965 workers at an aircraft manufacturing plant in California. A sub-set of 3,634 of these workers were exposed to chromates and other chemicals as part of airplane production for a total of 88,224 person-years of exposure and a mean of 24 working years per person of exposure. The SMR for total cancers was 0.93, and the SMR for lung cancer was 1.02. As with the Blot study, there was no association of any adverse cancer or non-cancer health outcome with chromium exposure in this group, nor did the overall worker population have an increase in overall or specific mortalities as compared to the general population despite exposure to a number of occupationally related chemicals.

Taken together, these occupational studies indicate that, although previous historical exposure conditions were associated with a modest risk of lung and respiratory cancer (average of 2- to 4- fold increased lifetime risk, as compared, for example, to a 10- to 20-fold increased risk for cigarette smokers), more recent occupational exposures at or below the current regulatory limits indicate that these represent levels that do not elevate cancer risk even for lifetime occupational exposures. Moreover, the previous exposures of concern in workers from the 1930's through the 1960's were at levels that typically exceeded 1,000,000 ng/m^3 and also involved exposure to the most carcinogenic forms of chromates, i.e., the insoluble or slightly soluble forms such as lead chromate, zinc chromate and calcium chromate. The newer lifetime occupational exposure limits -- at which no increase in cancer risk or other health effects has been observed – represent daily exposures that are hundreds to thousands of times higher than would occur in an environmental setting or via U.S. drinking water. There are two other major conclusions that can be drawn from these occupational exposure studies. First, although dermal exposure to chromium(VI) was extensive – particularly prior to the advent of industrial hygiene practices in the 1970's – there is no evidence for increased risk of skin cancer, even in workers where the chromate levels were high enough to burn "chrome holes" in their skin or nasal septum. These chrome holes healed and were not associated with increased skin cancer risk in these workers. This is relevant to the very high doses of chromium(VI) used in the NTP studies and a possible MOA. Second, taken together these occupational studies do not demonstrate an increased risk of GI cancers or other internal cancers, despite the fact that these workers swallowed a significant fraction of the dusts they were exposed to in the air. These data were recently summarized in a meta-analysis published in 2010 (NM Gatto et al., Cancer Epi 2010, 34:388-399).

Other specific edits and comments (changes underlined):

- Page 200, Lines 1-2 – Delete the phrase *"… and evidence of an association between oral exposure to hexavalent chromium and stomach cancer in humans"* which is based on a single study and EPA's selective treatment of this result as discussed above.
- Page 200, Lines 18-20 – *"This study found evidence <u>of a modestly increased incidence of stomach cancer mortality (OR 1.69, CI 1.12-2.44)</u> from 1970 to 1978 …."*

- Page 201, Lines 20-23 – "… *was reported in a re-examination of a single study in JinZhou ..*"
- Page 209, last section, Bioavailability – This section is highly flawed in logic and presentation as discussed under Chapter 3. Specific edits are as follows:
    - Page, 209, last two lines – "*Quantitative studies of GI absorption of chromium administered as hexavalent chromium have estimated …*"
    - Page 210, first line – Please note that hexavalent chromium was not measured, and without exception has never been measured systemically as Cr(VI) following GI absorption. It is an assumption that increased chromium uptake to the blood represents Cr(VI) uptake, but it could also represent other forms as discussed in Chapter 3 above. We know that certain forms of Cr(III) are much more readily taken up than others, and it is therefore possible, and perhaps quite likely, that reduction of Cr(VI) in the gut in the presence of organic molecules in the GI lumen leads to formation of complexes that are much more bioavailable than inorganic Cr(III) that is typically found in food, water and soil. You could also modify this phrase as a separate sentence to read *"This may indicate that not all hexavalent chromium is reduced by the gastric juices of the stomach, or that reduction of Cr(VI) in this environment leads to formation of organic chromium complexes that are more readily absorbed than inorganic Cr(III)."*
    - Page 210, Lines 7-12 - Delete the sentence beginning *"Thus, at oral doses within human exposure ranges …*" as per the argument above or modify to include alternative interpretations as suggested above.
    - Page 210, Lines 28-30 – End of sentence beginning with *"… and uptake of hexavalent chromium into the tissues … "* delete this phrase or modify it as per the argument above.
    - Page 210, last line – Add a sentence after the last sentence on this page reading *"However, none of those studies speciated the chromium that was absorbed systemically, and so the form(s) of chromium in the blood and other tissues is unknown following increased absorption of chromium following ingestion of hexavalent chromium. Therefore, it is not known whether chromium reaches the blood or distal tissues as chromium(VI) at doses relevant to human exposures. The lack of long-term labeling of RBCs by chromium in the animal and human studies argues that little, if any, chromium is absorbed as chromium(VI) under these exposure scenarios."*
    - Page 213, Section 4.7.3.5. Lines 3-5 – This statement is completely incorrect; there is no evidence for it as argued above. Delete this, or modify as follows *"Chromium absorbed following ingestion exposure to chromium(VI) may be in forms that can reach the systemic circulation and distal tissues, thereby potentially affecting tissues beyond those at or near the site of entry. However, the form(s) of chromium following such uptake is not known."*

	Chapter 5 This reviewer strongly objects to use of a linear low-dose approach for Cr(VI) risk assessment given the clear evidence for a threshold mechanism due to extracellular reduction of chromium at doses of relevance to human exposure via drinking water. The conclusion by EPA of a mutagenic action of chromium – most of which is based on cell culture data where chromium exposures and other parameters were extreme and where metabolism and intracellular exposure are far different than in vivo exposures – should not be the sole basis for use of this standard model which ignores the compelling toxicokinetic data summarized in Chapter 3 of this draft. More importantly, as discussed above for Chapter 4, Cr(VI) is unlikely to act via a mutagenic MOA in vivo, and requires extraordinary experimental manipulation to be positive in cell culture and in vivo mutagenicity studies. While it is clear that Cr(VI) can cause certain forms of DNA and chromosomal damage or other changes, it is not clear whether any of these is pre-mutagenic, and the in vivo data argue strongly against a mutagenic MOA under physiological conditions and normal routes of exposure. The document must more clearly differentiate between genotoxicity – i.e., damage to DNA or chromatin – and mutagenicity – or frank mutations that may result from DNA damage. Chromium(VI) can induce DNA damage but is a very weak mutagen at best, particularly in vivo. It is far more likely, and most consistent with all available data, that chromium(VI) acts via a non-mutagenic mechanism that involves a clear threshold – two threshold actually, one of which is extracellular and chemical involving reduction of chromium(VI) to chromium(III) and the other of which is biochemical and involves a threshold for cellular effects that lead to cell damage and cell death, resulting in turn in tissue proliferative responses that ultimately increase tumor risk via well known mechanisms of repeated tissue injury, compensatory cell proliferation and re-population. Given this most likely MOA based on a synthesis of several decades of chromium research, it is therefore inappropriate to use a linear low-dose extrapolation model for assessing risk via the ingestion route of exposure. It is clear from the animal and human studies that there is a threshold for in vivo effects that is based on the strong reducing capacity of the GI tract following oral exposures, and that at normal drinking water concentrations this will effectively protect from any in vivo exposure to Cr(VI) <u>as Cr(VI)</u>. Thus, a more appropriate risk assessment method would be to do dose-response modeling from the 2008 NTP study and the more recent ACC-sponsored 90-day MOA studies, and then use an approach similar to that for the RfD to calculate, with appropriate safety factors, a drinking water MCL that is protective based on threshold mechanisms. This should be done for Cr(VI) rather than the current MCL that is for total chromium, but it is likely that an MCL in the range of 50-100 ppb is going to be fully protective, including several uncertainty factors that separate it from the departure point of any likely human health effects for even the most sensitive individuals. Chromium is an excellent example of an opportunity to apply the concept of evidence-based risk assessment – which the EPA has claimed to be promoting for many years but has not, to date, actually applied in any meaningful risk assessment -- since there is a strong and compelling argument for use of a non-linear, threshold-based mechanism for chromium that logically leads to a real-world risk assessment that is based on that mechanism. Setting aside the 1987 Zhang and Li study and subsequent re-analyses as per

	the arguments above, there is not a single credible epidemiology study linking exposure to chromium via ingestion with cancer risk or any other long-term health effects, including in the extensive occupational epidemiology literature which includes several decades of extremely high-dose exposure cohorts. And even taking into account the Zhang and Li study, there is not a single peer-reviewed report linking any health effects to chromium(VI) at levels within a hundred-fold of likely environmental exposures via drinking water. The NTP 2008 animal data showed evidence of a cancer increase only at the highest doses, and the more recent ACC studies demonstrated hyperplasia consistent with a non-mutagenic MOA, which is also consistent with a threshold mechanism and which argues against developing a linear cancer slope factor from those data. **Chapter 6** There is considerable disconnect between the conclusions provided in Chapter 6 and the more considered and detailed discussions of the primary data in the previous chapters. The language should be modified to reflect this understanding such that the conclusions and their application to risk assessment of chromium(VI) follow logically from the scientific evidence. Specific edits: • Page 235, Lines 18-19 – " … *resulting in substantial, and in some cases complete reduction of hexavalent chromium to trivalent chromium depending on the concentration, dose, and precise route and method of exposure.*" • Page 235, Lines 21-26 – "*The extent of absorption of chromium from ingesting hexavalent chromium appears to be determined by both the solubility….. in the GI tract, but ingestion of both trivalent and hexavalent chromium results in systemic uptake of chromium. Trivalent chromium does not readily cross cell membranes except as part of certain organic complexes. Hexavalent chromium, if absorbed systemically, can easily* …." • Page 235, Lines 27-29 – *"Chromium absorbed systemically from the gut following ingestion of hexavalent chromium is distributed throughout the body. …. If hexavalent chromium is absorbed without extracellular reduction, it can cross cell membranes and, once inside the cell, …."* • Page 236, Line 3 – *"Chromium absorbed systemically following hexavalent chromium ingestion is eliminated primarily in the urine as trivalent chromium."* • Page 239, Lines 11-12 – delete the phrase *".. and evidence of an association between oral exposure to hexavalent chromium and stomach cancer in humans."* This is a significant statement to make, and as discussed above in reference to Chapter 4, the Zhang and Li studies (1987, 1997) should not be used to assess human cancer risk for oral exposure to chromium. • Page 240, Lines 12-14 – As noted for Chapter 5 above, there is considerable concern with the default choice of a linear low-dose extrapolation model for cancer risk.

Nordberg	EPA has presented and synthesized present knowledge about non-cancer and cancer hazards for hexavalent chromium. However, some further literature could be included in the document and paid attention to i.e, Langård and Costa Chapter 24 Chromium In: Nordberg GF, Fowler BA, Nordberg M and Friberg LT (Eds.) (2007) Handbook on the Toxicology of Metals, 3rd edition, Elsevier 487-510. Other chapters of interest e.g.,Chapter 10 Carcinogenicity by Ke, Costa and Kazantzis page 177-196. One chapter (14 by G. Nordberg and B A Fowler) deals with Risk assessment pages 281-301. A comparison between criteria for classification of carcinogenicity should be done between IARC, EU and USA. Hexavalent chromium is classified as a human carcinogen. This evaluation is also taken by USEPA for inhaled hexavalent chromium and related lung cancer. It should be highlighted that for some metals e.g., arsenic it has been reported in the scientific literature that oral intake i.e., via drinking water also can give rise to lung cancer though oral intake mostly is referred to cancer in the oral cavity or gastrointestinal system. My question is if the lung was studied in the NTP studies or any of the animal studies reported in the given report? Same question goes for the epidemiological studies that are cited? Table 3-7 page 30 reports levels of chromium in female controls both in kidney and in bone. It is not easy to find any comments on this in the document. Is there any analytical problem in this study?
Patierno	I will offer a response to this question in the form of general comments regarding specific sections of the Review in order of appearance in the text. In taking this approach my comments will also directly address questions (A)1-4 and (B)1-5. Page 7: The Environmental Protection Agency (EPA) should not be referencing a 2006 review article by Costa and Klein to site background on environmental chemistry. This review article was not a critical review of the environmental chemistry of chromium. Even if the general background in that review article is accurate, The Toxicological Review of Hexavalent Chromium (TRHC) should cite primary references from chemical or environmental journals or compendiums. Also, this paper is mis-labeled in the reference list as a 2008 paper. [Correct reference is "Toxicity and Carcinogenicity of Chromium Compounds in Humans" Crit. Rev. Tox.: 36(2):155-163, 2006]. Moreover, the premise of this review article [in essence that even very low exposures to any form of CrVI, including in drinking water, can cause virtually any type of cancer in virtually every organ, as well as plethora of assorted other diseases], and the preceding review article on which it was largely based ["Toxicity and Carcinogenicity of Cr(VI) in Animal Models and Humans" Crit. Rev. Tox.: 27:431-442, 1997], should not be universally accepted by the EPA without critical evaluation. Much of the epidemiological methodology applied in these papers is flawed. In these papers, the author(s) repeatedly and selectively tabulated whatever instances could be found in any of the many epidemiologic studies of chromium, of an elevated Standard Mortality Ratio (SMR). These were presented with no mention of the fact that most of these instances

	were small, non-statistically significant elevations (likely to be random fluctuations due to the large breadth of the studies), which were either ignored or discounted by the original authors because of confounding factors. The paper also failed to take into account that many of the small, non-statistically significant elevations in some cancers in one selected study, were counter-balanced by either no elevation or decreased SMRs in other studies. This "tabulation" approach does not constitute a true meta-analysis and is also statistically incorrect. There are also additional reasons that the EPA should be circumspect about citing either of these articles. The 2006 article, and its preceding counterpart published in 1997, were written and published at a time when the senior author was actively engaged as an expert witness for the plaintiffs in high-profile hexavalent chromium lawsuits. This involvement was not disclosed in the 1997 article, which was focused on attempting to implicate low dose exposure to CrVI in a broad array of human cancers. In the Acknowledgements section of the 2006 article there is partial disclosure that production of the paper was paid for in part by Baron and Budd. In fact, Baron and Budd is one the law firms with whom the senior author was under contract with as an expert witness for the plaintiffs in an active lawsuit. This article specifically tried to implicate CrVI as a human drinking water carcinogen even at very low doses, as well as suggesting that exposure to CrVI causes a broad array of other diseases, including neuropsychiatric problems, for which there is no support. If the EPA is going to site these review articles it is critical that EPA conduct an independent critical review of every paper sited in these review articles. In the latter scenario it is certain that EPA will reach a different conclusion. I am not of the opinion that a scientist who serves as an expert witness should have to disclose all litigation-related work in all scientific publications, particularly not in reports of original laboratory research into mechanisms of action, or even in review articles that give an unbiased evaluation of the existing literature, especially as it relates to basic mechanisms of action. Indeed, in the world of chromium toxicology it is hard to find experts who have not participated in some sort of chromium-related litigation. However, these two articles do not merely describe original laboratory research or present an unbiased review of the literature (note that part of the 2006 review article is a recapitulation of an already published journal article on UV light and chromium exposure). These two particular articles are essentially position/opinion papers, with speculative declarations that even very low dose exposures to soluble CrVI can cause virtually any kind of cancer (and other disease) in virtually any organ, a theory of obvious benefit to any plaintiff's case in chromium-related litigation, but one that is not supported by either epidemiological studies or in vivo animal studies. Page 20-21: Although the draft TRHC frequently describes each specific study in this section and offers a conclusion/interpretation, the TRHC discussion of the Donaldson and Barrera paper ends with a reference to Table 3-1 with no summary. There is important information in Table 3-1 that strongly supports the capacity of gastric juice to rapidly reduce CrVI to CrIII. Note that the uptake of Cr in intestinal rings was virtually identical, whether the starting material was CrIII without gastric juice or CrVI plus gastric juice at pH 1.4. The text at the bottom of page 21 and the top of page 24 seems to be nuanced to cast doubt on the body of work of DeFlora, by using the word "suggested" (second line from

	the bottom of pg 21), and then suggesting that the values of reducing capacity given by DeFlora "should be considered with some caution". This "caution" is based on speculation found in the paper cited (Zhitkovich, 2005), and reiterated in the 2006 Costa and Klein article (from where the draft TRHC apparently drew its language). This speculation is addressed in the supplementary materials under "Public Comments". The TRHC and the EPA should not cast doubt on the body of work by DeFlora, based on unsubstantiated speculation. Page 24-5: The TRHC should recognize and illustrate the main point of the absorption studies cited: no matter whether the original starting material is CrVI or CrIII there is limited absorption and little retention of either: fecal recovery in rats was 98% for CrIII and 97.7% for CrVI (pg 24) and in humans was 99.6% for CrIII and 89.4% for CrVI. Pretreatment of CrVI with gastric juice completely inhibited absorption of CrVI after direct perfusion into the small intestine. On pg 27 another study indicates that 99% of CrVI is recovered in feces using rats gavaged with CrVI. On pg 28 another study indicates that maximal uptake after gavage of rats with CrVI occurred in liver and was only 1%. Absorption in other organs was in the range of 0.1 to 0.2%. It is important to note that in all of these absorption studies, including drinking water studies, the increased tissue distribution was only observed after chronic administration of more than 5 ppm. Many of the studies used greater than 100ppm. The main point that there is very little absorption and retention of Cr even after administration of CrVI. Page 26: It is incorrect to state that absorbed "hexavalent" chromium is distributed throughout the body. Few studies actually speciated the Cr found in organs distal to the route of administration and even extremely large doses of CrVI, large enough to saturate reduction in the stomach and GI tract, do not deliver much more than trace amounts of CrVI to most distal organs because of the vast reducing capacity of blood components. The vast majority of Cr reaching distal organs arrives as CrIII. The TRHC should make this absolutely clear. Page 34: The TRHC should provide an accurate summation of Table 3-8 which compares tissue chromium after ingestion of a very large, gastric reduction-saturating dose (12.9 mg/L) of either CrIII or CrVI. The only "organ" that showed consequential increased levels of Cr after CrVI compared to CrIII was blood. Most of the other organs exhibited only trace amounts of Cr, even after this huge dose of CrVI, except for the intestine which showed significant and nearly identical increased Cr concentrations after both CrIII and CrVI. This supports the conclusions of the supplementary data in the Public Comments showing that some sections of the intestine (jejunum for example) are sites of Cr accumulation, regardless of whether the source material is CrIII or CrVI. Page 35: Note that there is no increased accumulation after 8 weeks of exposure compared to 4 weeks of exposure, even at the enormous dose of 130 ppm (mg/L). Page 36: The NTRC should not interject the commentary statement: "indicating that a portion of the CrVI escaped intracellular reduction in the GI tract and became bioavailable for systemic distribution". Like almost every other study this study measured total tissue Cr and did not speciate tissue Cr, and the NTRC therefore cannot speculate on what the form of Cr was that reached the tissues. Note in Table 3-8 the accumulation of Cr in the intestine and blood after CrIII.

	Page 37: Note the obvious threshold of increased bone and kidney concentrations after 10 ppm compared to all lower doses, as well as estimated body Cr burdens. Note that in liver there is a significant Cr burden even after ZERO CrVI exposure. Note the strangely compressed scale of the Y axis: even the increase in females at 10 ppm is only an increase from 0.3 to less than 0.5. These data demonstrate the exact opposite of the conjecture on page 36: there is a clear threshold of accumulation indicating saturation of reductive capacity. Pages 38-39: Virtually every study shows the same thing. The NTP study used a "low dose" that is already higher than the 10 ppm in the Sutherland study. What is being referred to here as a "dose-dependent" increase is already supra-saturation of gastric reductive capacity. What these studies really show is how little Cr is absorbed, even in tissues that are directly exposed (glandular stomach and forestomach), even after massive doses are administered. Page 41: It is inappropriate to make such an unqualified statement as found at the top of this page: "Hexavalent chromium is capable of crossing the placenta". This is only true in the highly contrived circumstances referenced below the statement wherein pregnant mice were given an IV injection of a massive dose of CrVI (10mg/kg). Page 42: The TRHC does a good job describing the bioavailability and kinetics of Cr absorption in humans after CrIII, CrVI in OJ and CrVI. It correctly acknowledges that the CrVI-OJ was completely reduced and that even the full dose of CrVI was insufficient to overwhelm the reducing capacity of blood. The potential explanations offered are correct but need to add another possibility. Often overlooked is the fact that not all CrIII is alike. Anyone who works with CrIII in the laboratory knows that it undergoes aging in aqueous solution, even visibly changing color with time after solubilization. It is possible that CrIII generated from newly reduced CrVI (as in the CrVI-OJ) may have some different biological parameters than straight CrIII made up in water and allowed sit for a couple of days. In fact overall absorption of newly formed CrIII may be higher than aged CrIII, possibly as a function of its ability to form complexes with biological ligands that may alter its absorption potential. Page 49 bottom: It is inaccurate to state that "CrVI distributes to other tissues, notably the blood, kidney, and liver." Except for the cases of treatment with extreme doses, or use of pathways like intra-intestinal instillation or IP injection, the vast majority of Cr that arrives at distal tissues is CrIII. Once again, it is critical that TRHC make that fact clear, otherwise it gives the appearance of non-objectivity. Page 50, last paragraph: The TRHC should not simply reiterate speculation that is found in the papers it cited (Zhitkovich 2005, Costa and Klein 2006), in suggesting that the mutagenicity of Cr may be underestimated in cultured cells because of lower levels of intracellular ascorbate when cells are cultured in absence of added ascorbate. Indeed, it is just as likely that the mutagenicity of CrVI in cultured cells is grossly overestimated, because the lack of ascorbate in the extracellular medium allows CrVI to persist in the extracellular medium thereby maximizing its uptake as the hexavalent oxyanion. At the very least the TRHC should discuss both possibilities and not give the appearance of bias. Page 58: It is inaccurate to state that model simulations "imply" that some CrVI escaped reduction in the stomach. This is circular reasoning. The "input" data that went into

	formulating these models was based on experiments wherein massive doses of CrVI were administered, doses that would clearly exceed reductive capacity. It is not appropriate to then state that the model simulation "implies" that some CrVI escaped reduction, as though the model now supports a novel biological observation. It would be completely expected for the model to predict that scenario since it would logically emanate from the very data that was used to formulate the model. It is critical that the TRHC indicate that these models do not apply to, or accurately predict, the toxicokinetics of low, environmentally relevant doses of CrVI. The discussion in the THRC does become more balanced on page 64 where the non-linear aspects of CrVI uptake, reduction and bio-distribution are given some weight. Page 66: This section (3.6) needs to be completely rewritten as it lends undue weight to an opinion expressed in only one or two papers, at least one of which was written under financial inducement by a law firm with a vested interest in characterizing all Cr, including CrIII, as a potential hazard (see preceding comments). The TRHC needs to not indiscriminately cite speculation found in review articles without more rigorous analysis. Except for those few biased citations it is almost universally accepted that CrIII is an essential element. Page 68: Section 4.1.2, last sentence: This is nearly the ultimate example of how critically important it is for the TRHC to do its own critical analysis of the literature. The paper cited, (Bick et al, 1996) should not be cited under any circumstances and in fact it should be retracted from the scientific literature. Two of the authors, Walter Lack and Thomas Girardi, were two of the lead lawyers for the plaintiffs in several high-profile chromium lawsuits, now immortalized by the Hollywood movie "Erin Brockovich". They listed their "academic" credentials as the Department of Hematology at the University of Tasmania in Australia. The other three authors (Costa, Bick and Teitlebaum) were paid expert witnesses for the plaintiffs in the same case, which was active at the time. None of this was disclosed in the paper. The two cases of Non-Hodgkin's lymphoma discussed in this case report were plaintiffs in the active lawsuit and the information was supplied by the lawyers. Moreover, at best this report is merely a case-report (not even a case-control study), merely reporting that two people in Hinckley CA, at that time, had been diagnosed with Non-Hodgkin's lymphoma. In contrast, the draft TRHC does not yet, but should reference the recent work of Dr. John Morgan, an epidemiologist for the California Cancer Registry. He has been tracking cancer incidence in the town of Hinckley CA (the "Brockovich" town) from 1996 to present. He recently published data showing that from 1996 to 2008, not only is there no excess of total cancer or any specific cancer in Hinckley, there are actually fewer cancers than expected. Pages 71-80: The draft TRHC conducts a very thorough depiction of the different interpretations of the Liaoning Province situation. What seems to get lost in the details is the larger picture. This is a Province of a country wherein the background rates of both stomach and lung cancer are high even in non-chromium exposed comparison groups, indicating the presence of other contributing risk factors. This is a situation where exposure is characterized in terms of high dose, long term "yellow water", yet despite this potential significant exposure, the question of whether there is an additional modest increase in risk for stomach cancer hangs on whether a particular industrial area is

	included in the comparison group or not. There is much controversy surrounding the reports of cancer risk in this Province, but after discussing the controversy the draft THRC aligns itself with the method of re-analysis of Beamont et al. The THRC should then also cite the commentary by Allan Smith [Epidemiology 19:24, 2008] which accompanied the Beamont article: although Smith is sympathetic to Beamont's attempt to re-analyze the data, he also describes the extensive weaknesses of the approach. This is not the kind of data that a regulatory decision should be based on. Page 81: The NTP toxicology studies on subchronic oral exposure (Section 4.2.1) are technically well done. The principle issue that needs to not be lost in the detail is that the lowest dose was 62.5 ppm, an enormous concentration of little or no environmental relevance. This is a "yellow water" situation to the extreme. Despite these enormous doses most of the observations did not exhibit a consistent pattern of dose or duration dependence. It is also important to recognize that these enormous doses of CrVI actually serve to deliver an enormous amount of CrIII to the organs and cells in question. Remembering that CrIII is not without biological activity (acting as a co-factor in insulin action), it is entirely possible the some of the observed effects are due to the physiological effects of massive CrIII overload. The extensive new data provided by ToxStrategies, described in the Public Comments, needs to be incorporated into the TRHC. Page 84: Again, a consistent relationship between severity and dose was not observed. This implies the presence of effects caused by indirect mechanisms, likely chronic inflammation and/or tissue damage only observed at the highest doses (see below). Urinalysis shows effects due to decreased water intake due to poor palatability of the yellow water. This dehydration alone is capable of rendering epithelial tissues more fragile. Changes in organ weights were only observed at doses above 500ppm (pg 86). Page 87-108: The results are described repeatedly as "without clear dose-response relationship". Indeed, minimal to mild histiocytic cellular infiltration was observed in all groups including the control animals. Even less toxicity was observed in mice compared to rats; in fact even at 1000 ppm for 3 months there was no evidence of any hepatotoxicity, only mild changes in some hematological indices that were attributed to changes in body weight (probably caused by massive CrIII overloading and its potential effects on insulin and glucose metabolism). What needs to be emphasized here is that the lowest dose used in any of these studies is at or above saturation of gastric reductive capacity and yet still very little toxicity was observed except at the two highest doses (and often only at the one highest dose) (Tables 4-12, 4-13, 4-14). At the lower end of these very high doses, only inconsistent observations were made and when "toxicity" was reported it was generally ranked minimal to mild. Only the index of Liver (fatty change) was ranked as moderate, but that was identical to the ranking of that same index in the Controls. The main point here should be that these are massive doses and they are eliciting minimal effects. This important concept should not be lost in the mass of detailed results. Pages 109-120: The NTP carcinogenesis studies in rats and mice show that there is no carcinogenic response except at the two highest doses that also produce chronic tissue damage at the sites of carcinogenicity. The dose-response is definitively non-linear, as is the absorption data described above. Given that the lowest dose is already above the

	reductive capacity of the oral cavity and stomach, these data provide strong evidence of the protective effects of the reductive capacity of blood components. It should be noted that the NTP's published report by Stout et al [Hexavalent Chromium is Carcinogenic to F344/N Rats and B6C3F1 Mice after Chronic Oral Exposure, Environmental Health Perspectives 117: 716, 2009] presents an inaccurate Discussion of potential mechanism of action, drawn heavily from the 2006 Costa and Klein article, especially in criticizing the work of DeFlora. In point of fact, the results of the NTP assay, and the extensive additional data found in the Public Comments generated by a group of investigators around the country funded by ToxStrategies, give nearly definitive proof that the work of DeFlora is correct. Even the lowest dose of the NTP assay exceeds the reductive capacity of the oral cavity and upper digestive tract. Yet little toxicity and no carcinogenicity is observed except at the two highest doses. The argument by Stout et al that the NTP doses were below gastric reduction-saturation, based on a supra-linear (decreasing response with dose) rather than sub-linear (increasing response with dose) dose response is incorrect. If the doses were below saturation of reductive capacity, as the dose increased the ratio of unreduced CrVI to reduced CrVI (CrIII) in the stomach would increase (due to depletion of reductive capacity), and absorption would show an increasing rate of response (opposite of what was observed) because of an increased percentage of the total Cr that would be in the unreduced hexavalent state. Yet both absorption and toxicity exhibit a decreasing rate of response with dose in the NTP assay. This would actually be expected at supra-saturation doses: once the reductive capacity of the oral, digestive and blood components is exceeded, the organs receiving the highest amount of CrVI will sustain inflammatory tissue damage provoking tissue regeneration. It is unlikely that such tissue damage would display dose dependence since it only occurred at the two highest doses of the assay and it is a complex, disseminated biological response. It is likely then that a combination of three factors contribute to the high dose carcinogenic response: (i) tissue damage with regenerative cell profieration, (ii) regenerative cell proliferation in the presence of macromolecular damage, and (iii) regenerative cell proliferation occurring in the presence of massive CrIII loading, which may affect insulin-dependent proliferative signaling. Pages 122-149: For these studies on the potential reproductive toxicities caused by CrVI one can only hope that the TRHC and EPA will remember the 16th century adage of Paracelcus "all substances are poisons, the right dose differentiates a poison from a remedy". These studies show reproductive toxicity at huge doses of CrVI, often given using invasive administration procedure (IP or IV injection), with little relevance to environmental exposure levels. Pages 176-178: The in vivo studies showing DNA damage or mutagenicity in cells peripheral to the point of administration of CrVI were only positive when massive doses of CrVI were administered by gavage, direct instillation, or intravenous injection. Although some studies claim to find mutations in the absence of cytotoxicity these are highly contrived systems: for example eye spots in offspring of pregnant female mice given huge doses (62.5 ppm) of CrVI in drinking water. All studies of mutagenesis in cultured mammalian cells, including human cells, demonstrate that mutagenesis is only observed at doses that produce some degree of cytotoxicity and replication arrest. It should also be clarified in the TRHC that DNA damage and mutagenicity should not be

	equated: while mutagenicity may result from DNA damage, the relationship is not simple or linear and is further complicated by DNA repair. Also, it is unclear whether all forms of DNA or chromatin alterations (collectively termed DNA damage) are pre-mutagenic. For example, in silico studies on DNA-protein crosslinks suggest that under certain circumstances CrIII can serve as binary crosslinking agent between small peptides and DNA. However, in in vitro studies in cultured cells and in in vivo studies, it is not clear what is actually being measured by assays for DNA-protein crosslinks. This phenomenon may in fact only indicate that chromatin isolated from certain cells exhibits a higher degree of condensation during isolation, rendering chromatin proteins more difficult to extract. What appear to be DNA-protein crosslinks can be actually be observed in cells treated with agents that do not participate in or catalyze formation of an actual binary crosslink. Pages 202-214 (section 4.7.3.2): Many of the preceding comments directly address and provide major qualifications to the MOA discussed in this section, including the interpretation of reductive capacity found in the Stout et al report of the NTP assay. It is clear that the EPA is faced with a unique situation in assessing the MOA of Cr(VI) at it relates to low-dose risk assessment. It is abundantly clear from all the science that the effects of Cr(VI) at the massive doses necessary to produce tissue toxicity and carcinogenesis in rodents, have no bearing on the effects of low-dose, environmentally-relevent exposures. This is consistently borne out by epidemiological, animal and cell experimentation. This is especially pertinent in relation to whether or not Cr(VI) should be considered with a mutagenic MOA. I have spent more than 25 years studying the genotoxic properties of Cr(VI) and I have frequently contributed to the plethora of studies showing DNA damage and what we thought was associated mutagenesis. There is no doubt that Cr(VI) can be forced to be genotoxic and "mutagenic" under experimentally contrived systems and at high doses that evoke major amounts of cell death. However, in hindsight many of us "DNA damage and repair" scientists have come to appreciate several important factors: (i) DNA damage is only observed at very high dose that kill a lot of cells, (ii) Cr(VI) is at best a very weak "mutagen", requiring very high doses that kill most cells and experimental "backflips" to select for survivors, and (iii) what we thought was "mutagenesis" is actually selection for stochastic cell survivors of massive toxic insult. Dr. Rossman's group has shown that the base sequence of the genes used for mutation detection and selection is intact and that the changes in gene expression enabling selection are epigenetic, not mutagenic. Our group has shown that what we really selected for at toxic exposures are cells that are resistant to apoptosis, and Dr. Zhitkovich's group has shown that the "mutant" cells were actually surviving cells that were selected for changes in specific forms of DNA repair. Again, this only occurs at doses that kill a lot of cells, not dis-similar to the high-dose rodent assays wherein tumors were only observed at doses that produced chronic and fairly severe tissue damage. This harkens to what is sometimes viewed as a landmark study of lung cancer and occupational exposure to high doses of Cr(VI) by Gibb et al. Occupational exposure to in the chromate production industry was categorized into 4 quartiles. The lowest two quartiles are huge levels of exposure by "environmental" standards, orders of magnitude beyond the even the highest known environmental exposures. The lowest quartile of exposure was essentially a No Effect

	Level (no elevated risk) and the slightly elevated risk ratio in the second quartile was not statistically significant. Interestingly, of the total of 120 lung cancer cases found in chromium-exposed workers, 116 were also smokers. The EPA may be under certain historical regulatory precedents and pressures to deem Cr(VI) with a mutagenic mode of action simply because there are published studies that have "Cr(VI)" and "mutation" equated in the title (some of these papers are my own), but this decision would not be based on science. At high, tissue damaging doses one can get tumors to form and those tumors will have mutations in specific genes because that is the molecular etiology of how that particular cancer develops. It will have no relation to any chemically-specific mutations caused by Cr(VI) because Cr(VI) is an exceedingly poor mutagen. Even at the low end of very high doses there is NO MOA because there is NO toxicity, no mutagenecity, and no carcinogenesis. Extrapolating linearly from events observed at the two highest doses of the NTP assay, to anything close to reality for environmental exposure, is simply not scientific. If ever there was a textbook case to be made for a "threshold carcinogen", it is Cr(VI).
Rossman	In general, this was a clearly written document. However, my area of expertise is in the mutagenic and epigenetic mechanisms of action of carcinogens, and I found many more problems in those areas than in the rest of the document. I will discuss these in section B2. Here I will mention some errors in the rest of the text. p. 38, end of 1st paragraph reads, "Uptake in guinea pigs did not appear to generally differ from that of rodents". The guinea pig (*Cavia porcellus*) is a rodent. p. 46, section 3.3, refers to transport of the hexavalent chromium oxyanion (for clarity, should this read chromate/dichromate?) by sulfate and phosphate transport systems (should be pleural). It is claimed that this allows accumulation in cells at higher concentrations than the extracellaular concentration. Neither the transport systems nor the evidence for higher intracellular accumulation are referenced. Actually, what allows higher accumulations is the fact that Cr(6) is reduced in the cell to Cr(3), which cannot get out, so what accumulates is Cr(3). p. 201, 1st full paragraph: It is claimed that the key precursor events leading to chromium-induced mutagenicity have been identified in animals. This is not so. It is not even true for mammalian cells in culture. Some ideas have been derived from cell culture studies (but with Cr-damaged shuttle vectors). Almost nothing is known about mutagenicity in animals, and nothing at all is known about the genetic changes occurring in animal tumors or in the target tissues. p. 202. Section 4.7.3. For reasons that will become clearer in my response to B2, this section is very flawed. I will mention just one point here: The confusion between "mutagenic" and "genotoxic" must be cleared up throughout this section (as well as throughout Section 4.5.). The thinking on this issue is very sloppy. A mutagenic mode of action is just that: it requires mutagenesis.

Salnikow	In September 2010, the U.S. Environmental Protection Agency prepared the "Toxicological Review of Hexavalent Chromium" to assess health risks associated with hexavalent chromium exposure. This document will appear on the Agency's online database, the Integrated Risk Information System (IRIS). The existing IRIS file for hexavalent chromium, prepared in 1998, does not consider hexavalent chromium to be carcinogenic by the oral route of exposure. The purpose of the new document is to update the IRIS regarding noncancer and cancer health effects associated with oral exposure to hexavalent chromium after considering the latest scientific evidences. The prepared draft of the "Toxicological Review of Hexavalent Chromium" provides a detailed analysis of the data obtained in several studies carried out in 2008, 2009 by the National Toxicology Program along with other studies, formulates a mutagenic mode of carcinogenic action, and suggests that the reduction of orally administered hexavalent chromium in guts, even in low doses, is incomplete. The draft also calculates a cancer slope factor for humans. Recognizing the importance and relevance of this document, this reviewer needs to address some shortcomings of the document. In general this is a dense document cataloguing many diverse and sometimes controversial studies in the area of hexavalent chromium toxicology and carcinogenesis. Of course the limitations in available experimental data obtained from existing animal models, ongoing investigations regarding the mode of action (MOA) of hexavalent chromium, and uncertainties in epidemiological data make it difficult to prepare a comprehensive document that will fully address public health concerns. Chapter 3 should be reorganized and more emphasis should be given to the role of chromium III in toxico- and pharmacokinetics as well as in biological effects produced by hexavalent chromium. Hexavalent chromium is generally considered a much more potent mutagen and carcinogen than trivalent chromium. Lack of carcinogenic effects observed with trivalent chromium compounds can be explained by poor permeability of cell membrane for this ion. However, considering that the end product of intracellular reduction of hexavalent chromium is trivalent chromium, which may accumulate in tissues, it is important to consider what role intracellularly deposited trivalent chromium may play in chromium toxicity and carcinogenesis. The majority of studies indicate that after intracellular reduction of hexavalent chromium to trivalent chromium, it can form various damaging DNA adducts. These adducts can inhibit the enzymatic activity of DNA polymerases, simultaneously increasing the rate of replication and the processivity of the DNA polymerase, and thereby decreasing its fidelity and causing more frequent errors. The frequency of errors increases with a dose-dependent increase in mutation frequency *in vitro* (Snow, 1991; Salnikow and Zhitkovich, 2008). Unfortunately, it is not clear how applicable these studies are to understanding the effects of trivalent chromium on DNA synthesis and cellular metabolism in vivo because the experiments were done in either in test tubes or in artificial model systems with concentrations far exceeding those obtained through environmental exposure (Snow, 1991; Dai *et al.*, 2009). Numerous attempts have been done to study the distribution and retention of chromium (III) in vivo. Onkelinx studied tissue retention of $^{51}CrCl_3$ in groups of female Wistar rats of various ages (35, 60, and 120 days) after a single intravenous injection of trace amounts of $^{51}CrCl_3$ (Onkelinx, 1977). The study showed that total excretory clearance is the sum of three components: urinary clearance (fu), fecal clearance (fd), and a residual clearance (fs), corresponding to an apparently irreversible deposition of chromium into long term body reservoirs.

	Consistent with the model, ^{51}Cr was found to accumulate with time in several organs such as bone, kidney, spleen, and liver after a single intravenous injection of $^{51}CrCl_3$. These data are supported by those obtained by O'Flaherty (O'Flaherty, 1996) and others indicating that the retention of chromium III by bone, liver, kidney, and spleen is prolonged. Also, to make the toxicological review concise, I suggest eliminating Table 4-21"In vitro genotoxicity studies of hexavalent chromium in nonmammalian cells" and Table 4-24 "In vivo genotoxicity studies of hexavalent chromium in *D. melanogaster*" because these are irrelevant to the MOA of hexavalent chromium in mammalian systems. There are numerous errors throughout the text of the Draft. Some of them are shown at the end of these comments as Errata.
Wise	The Toxicological Review is logical, clear and concise. However, overall the document is inconsistent and thus, EPA has not presented and synthesized the scientific evidence for noncancer and cancer hazard in a clear manner. Some sections, primarily the ones focused on animal data are clearly presented and synthesized. These sections present the primary literature and discuss the merits of each study with balance and insight. Other sections, however, particularly those involving *in vitro* cell culture data and underpinning the mode of action are much less appropriately considered, are not well-presented, and do not synthesize the underlying data very well. Determining a mode of action is a key part of the risk assessment. The Toxicological Review would be a stronger document if it fully analyzed and synthesized the primary literature to ascertain the possible modes of action for Cr(VI). The strengths, weaknesses and data gaps for each could have been highlighted and discussed, and then a rationale for the chosen mode of action presented. However, as presented this approach is not apparent. Instead, as presented, the document gives the impression that the mode of action was pre-determined from a select set of review articles and the best case for that mode of action presented. Decisions appear to have been made to agglomerate all of the genotoxicity data into positive or negative proof of mutagenesis rather than more careful consideration of individual lesions. Confounding factors, such as ascorbate levels, are cautioned against, but inadequately and inaccurately presented and unevenly applied, which undermines confidence that the primary data were adequately considered and contributes to a perception that decisions were predetermined. This perception is strongly reinforced by poor management and citation of the underlying literature and a heavy reliance on a few select review articles that unfortunately miscite the primary literature. As a result, many sections of the Toxicological Review lack clarity, accuracy, synthesis and rigor and the rationale for the choice of mode of action seems predetermined and forced. Each of these factors is elaborated on in more detail below. 1. <u>Unnecessary agglomeration of genotoxicity data</u> The Toxicological Review essentially combines all of the lesions related to genotoxicity (stated on page 212 as "*…including DNA adduct formation, DNA damage, gene mutations, chromosomal aberrations, and micronuclei formation"*) into one bundle and refers to them as "mutagenicity". This decision is typically based on the presumption that

	the various genotoxic lesions will ultimately manifest as mutations in the primary DNA sequence. Hence, it is generally recognized that a crosslink or a strand break is not inherently a mutation, but that it may eventually manifest as one and thus, it is a mutagenic event. Based on the aggregation of all of these data, the Toxicological Profile declares Cr(VI) to be a mutagen and proposed a mutagenic mode of action. This approach is consistent with older practice and perception of these genotoxicity assays and it may be a useful approach for a chemical with a limited data set. However, the genotoxicity data for Cr(VI) is a rich data set and deserves a more sophisticated consideration. The Cr(VI) literature often distinguishes in its presentation between mutagenic and genotoxic lesions. The Toxicological Review does not carry forward that distinction and does not explain the rationale for ignoring it. However, it is an important distinction because not all of these lesions are likely to be mutagenic after Cr(VI) exposure. Aggregating these lesions oversimplifies the interpretation of the data and masks the fact that much of the primary data suggest that in actuality, Cr(VI) is a very weak mutagen. Discussing each class of lesion on its own merit with a more careful consideration of the primary literature would have better framed the strengths and limitations of the genotoxicity studies and brought this discussion into a clearer light. The fundamental problem underlying this section is a failure to clearly consider the primary literature to see that Cr(VI) is a weak mutagen when defined as an agent that can directly change the primary DNA sequence. Cr(VI)-induced mutations have indeed been observed in bacteria, cultured cells and animal studies. However, in most cases, one has to experimentally force the mutations to occur by using a high dose, a forced experimental system or a non-physiological exposure route. There is no real synthesis of this literature beyond listing outcomes in a table. The Toxicological Review also considers the results as simply positive or negative. That certainly is one approach; however, it misses the opportunity to consider the data more thoroughly. Some consideration should be given to potency and its potential impact and the robustness of the underlying assays. If the experimental data show that Cr(VI) induces a 2-fold increase in mutations, then the Toxicological Review would call that outcome positive. However, if in that same assay an established mutagen induced a 50-fold increase in mutations, then does Cr(VI) still appear to be a mutagen? Or does it suggest a different mode of action, particularly when the frequency of mutations have not been reported to increase in Cr(VI)-induced human tumors? Does the fact that ascorbate is higher in rodents make these mutations a rodent-specific event? A more careful and thoughtful presentation of the underlying data would have better informed the consideration of this mode of action. In addition, before lumping all of these genotoxic endpoints together as all mutagenic outcomes, a careful review and discussion of the primary mutagenesis literature is needed. That review needs to determine, for Cr(VI), which of the various lesions (e.g. DNA adducts, DNA crosslinks, DNA strand breaks, gene mutations, chromosome damage, etc.) actually occur in cells (for example as discussed below the adducts may not actually form in cells) and to what extent they occur. Then, the review needs to determine which, if any, of these lesions actually lead to gene mutations. The discussion below illustrates that there are reasons to doubt that the various lesions are all mutagenic outcomes. After this

	analysis, those lesions that do form in cells and that do produce mutations could more reasonably be combined into a category of "mutagenicity". Perhaps, the data will indicate Cr(VI) is a mutagen, but, perhaps, the data indicate that one only gets mutations in the DNA sequence when systems are forced experimentally to do so at very high concentrations, due to species specific factors or by non-physiological exposure routes. If the latter were true, this possibility would suggest that mutations are not likely to occur in humans, raising direct implications for the mode of action decision. A more thorough treatment of the primary mutation data is needed to clarify these important points. There is concern that the discussion of some lesions is overstated while others are mentioned but not discussed. The section explaining DNA adducts is greatly overstated and also does not fully consider the primary literature. The section presents a case that implies the status and impact of the various potential adducts are known in cells and *in vivo*. The Toxicological Review even provides a structure of a Cr-DNA adduct. The major concern is that when the primary literature is fully considered, it becomes apparent that these adducts are all based on cell-free systems and no one has been able to clearly identify any specific adducts in cells, whole animals or humans beyond observing tangles of DNA, protein, and Cr that are considered to be DNA-DNA or DNA-protein crosslinks. The primary literature has only measured adduct levels in cells by isolating DNA and then measuring the amount of Cr associated with it or by nonspecific P32 postlabelling. These measures cannot ascertain how or if Cr is bound to the DNA, only that it is associated with it in some way. Some studies have synthesized adducts in cell free systems and applied them to cells, but that does not mean those specific adducts form in the cell. Thus, it is unknown if specific DNA adduct events occur in cells, whole animals or humans. Nonspecific adducts have been detected by postlabelling, but specific adducts remain elusive. To lump these studies together as clear evidence of mutagenicity gives them a weight of evidence that seems premature and inaccurate. Overall, this section is very misleading in its portrayal of the status of adducts as more understood than they actually are. Oxidative damage is also included as evidence of mutagenicity. However, discussion is missing to establish whether this oxidative damage is a direct effect of Cr(VI) causing oxidative damage to DNA and thus, potentially a mutagenic event, or if this damage is actually indirect, resulting from overall oxidative stress to cells caused by high doses of Cr(VI) depleting intracellular antioxidants. Cr(VI)-induced strand breaks are cited as another type of mutagenic event. These lesions are discussed as post-replication-induced breaks. However, the discussion fails to question and discuss whether or not these are actually frank DNA breaks. As the Toxicological Review indicates, the studies have focused on gamma-H2A.X focus production as the measure of breaks. Data indicate that chromatin remodeling may also induce the production of gamma-H2A.X so they may not be frank DNA breaks after all. This possible explanation is missing from the section. DNA-protein crosslinks are included as a type of mutagenicity in the tables and description despite the fact that the document states on page 186 that it is unknown if they

	are mutagenic: "*Tests for the mutagenicity of these crosslinks have proved inconclusive (reviewed in Macfie et al., 2010), but the bulkiness of these lesions indicates the potential for genotoxicity…*" The most consistent genotoxic outcome is the production of damage to metaphase chromosomes manifested as aberrations, sister chromatid exchanges, and micronuclei. The fact that Cr(VI) induces these events was presented but not discussed. This lesion may be the key lesion as it is the most consistent and yet the mechanisms that may underlie it are ignored and not discussed. Chromosome damage could be a mutagenic lesion as assumed. Alternatively, it could be the consequence of epigenetic changes in the cell resulting from Cr binding to centrosomes in the mitotic spindle assembly apparatus or from bypass of the spindle assembly checkpoint. Cr(VI) has been shown to affect centrosomes and the spindle assembly checkpoint and perhaps it causes uneven pulling leading to breaks and errors in chromosome number. Cells with broken chromosomes may undergo apoptosis, while those with increased chromosome number may go on and survive as highly aneuploid cells. Cr(VI) has been shown to induce highly aneuploid cells that can clonally expand and survive. This outcome would not be consistent with a mutagenic mode of action. These concerns above are only magnified by the problems with uneven consideration of confounders and in poor management of the underlying literature described below. Together these factors give the impression of excluding or avoiding different syntheses of the data. More care and balance are needed to discuss and consider the genotoxicity data separately and evaluate if they are mutagenic markers. 2. <u>Uneven application of experimental confounders</u> The perception of bias caused by bundling all of the genotoxicity endpoints together is magnified by an apparent uneven consideration of experimental confounders in the document. It appears that the Toxicological Review does not fully consider and present all of the relevant *in vitro* cell culture data that inform possible modes of action. Instead, selected examples of primary literature that reinforce one point of view are presented. This approach undermines the synthesis of the literature and because of a marked unevenness in presentation creates a perception of bias that should not be part of the analysis. For example, on page 184-185, the Toxicological Review gives the reader the impression that ascorbate-trivalent chromium-DNA adducts have been found to be highly mutagenic. However, the section did not describe the experimental detail that indicate the cells used in the study were abnormal and genetically modified so that they could not carry out proficient DNA repair or apoptosis, or that the cells were not actually treated directly with Cr(VI). These omissions stand in stark contrast to the experimental criticisms the Toxicological Review applies to other cell culture studies. For example, on page 47, the document states: *"Caution should be used in interpreting cell culture data, as the cell culture medium could play a role in hexavalent chromium reduction, confounding the extent of intracellular hexavalent chromium reduction."*

It then cites a couple of examples where Cr(V) was detected in extracellular cell culture medium. The use of the word "caution" leads the reader to conclude that many *in vitro* studies may be flawed due to this reduction. No explanation is offered as to why this outcome is a problem. No discussion is provided that points out whether this same type of reduction might be expected to occur outside of cells in the body and thus actually be normal. The practical reality is that reducing agents are present in the extracellular fluid and thus, some extracellular reduction probably occurs in the extracellular fluid. This factor is probably not appropriate as a cautionary one and may actually reflect physiological conditions. But, no balanced discussion is provided for the reader to decide if this factor is indeed a concern.

A similar unevenness occurs during the presentation of the relative importance of ascorbate. The Toxicological Review states on page 50:

> *"An additional important note on these biotransformations regards the interpretation and reliability of data from in vitro assays. In vivo, the intracellular levels of ascorbate are quite high (about 1 mM). In contrast, the levels of ascorbate in tissue culture media are quite low since generally it is not added to the media so that the only source is supplemented fetal bovine serum (FBS). With 10% FBS, the level of ascorbate in tissue cultured cells is only about 50 μM which is 20 times lower than that which is found in vivo (Zhitkovich, 2005). Therefore, experiments on mutagenesis and other toxic effects of hexavalent chromium in tissue culture may underestimate its mutagenic, genotoxic, and cell-transforming activities (Zhitkovich, 2005; Costa and Klein, 2006)."*

No further discussion is presented. There is no presentation of the primary literature in cell culture showing the impact or lack of impact of this difference, just speculation that it might cause some underestimation. There is no presentation of the primary literature to establish what the ascorbate levels are in the relevant tissues of concern. There is just this one review article (Zhitkovich 2005) with some comment from a secondary review article (Costa and Klein 2006) cited. Closer inspection shows that the Costa and Klein review is actually citing the same Zhitkovich review so in the final consideration, this entire section relies only on Zhitkovich 2005 which, as discussed below in section 3.D., miscites the primary and secondary literature and draws a conclusion the primary literature does not closely support.

Thus, the Toxicological Review draws attention to the possible presence of extracellular metabolism and lack of intracellular ascorbate as confounding factors, which it may have used to exclude some cell culture studies. But, by contrast, it makes no mention and expresses no concern about studies done in abnormal compromised cells treated only indirectly with Cr(VI). This discrepancy makes the document and its treatment of the underlying literature seem uneven.

Moreover, in its discussion of the impact of ascorbate, the Toxicological Review does not discuss the potential impact of ascorbate differences on the bacterial mutation studies or the possible impact of ascorbate differences on the animal genotoxicity data. For example, in Table 4-23 on page 172, the Toxicological Review indicates a positive effect for mutagenicity in mice after intratracheal instillation. Mice, however, have more ascorbate in their lung tissue. If one accepts the speculation in the Toxicological Review that

	ascorbate–trivalent chromium–DNA adducts form and are highly mutagenic, then the elevated mutations in this study might simply be due to the elevated ascorbate levels in this species suggesting a species specific effect. Such a possibility would explain why there are mutations in rodents but not in human tumors. Regardless of which conclusion is correct, the point is that the Toxicological Review does not appear to apply this confounder it stresses in the *in vitro* work evenly to all studies reinforcing a perception of selective bias. Similarly, the Toxicological Review presents the primary research studies by Quievryn et al., 2003; Voitkun et al., 1998 as showing adduct effects, but both studies used the cell culture medium the Toxicological Review expresses concerns about and neither study addressed the ascorbate concern, but these aspects are not mentioned in the document. The absence of discussion of these confounders in these experiments give the impression that the Toxicological Review does not seem to apply its confounding criticisms evenly. This inconsistent presentation of experimental expectations and application of confounding factors creates a perception of uneven evaluation of the primary literature. Considered together, the language and approach suggest a strong bias against *in vitro* studies and the cautionary language should be removed to eliminate that bias. The discussion about ascorbate needs to be more balanced and thorough and the information better synthesized. The ascorbate section could be removed or if the EPA feels the issue needs to be considered, it should be fully vetted with a discussion of how differences in ascorbate might affect the interpretation of the bacterial mutagenesis studies and the rodent data. The discussion would need to also include the strengths and limitations of the primary literature. The relative merits of data from a primary normal human cell line without vitamin C supplementation versus data from a tumor-derived cell line with ascorbate supplementation would need to be presented and discussed. The various underlying phenotypic issues in cell lines would also need to be considered as a mitigating factor. Similarly, the technical limitations of ascorbate supplementation in culture would need to be considered including how long it is retained by the cell and the impact of its diffusion out of the cell and into the extracellular medium. This discussion would need to include a full evaluation of ascorbate levels in tissues of interest in humans and animals and whether those levels are intracellular or extracellular or both. It should include a full discussion of any data that show whether or not there is an actual impact of different ascorbate levels on outcomes inside the cell from cell culture studies. It should also include a discussion about the fact that ascorbate in the cell becomes depleted over time after Cr(VI) exposure and whether the relevant exposure is when ascorbate levels are normal or depleted. 3. <u>Poor management and citation of the literature</u> The above two concerns are further magnified by the presentation and management of the literature in the sections involving *in vitro* cell culture data and underpinning the mode of action. There is a tendency in the document to overstate the findings of the selected primary literature included in the document, raising questions about whether the primary literature was properly evaluated and weighed. There is inconsistent application of the phrases "*in vitro*" and "*in vivo*" resulting in substantial confusion regarding the underlying

literature and reinforcing a perception of inaccuracy in the document. There are flaws in citations, extensive direct quoting and long stretches of general paraphrasing of a small number of review articles raising questions about the heavy reliance on those articles and points of view. Finally, there is often a failure to check the underlying primary research studies cited in these review articles reinforcing the perception that the document relies on the review and not the underlying primary research data. Considered together, these aspects raise questions about the process of how the conclusions were drawn, create confusion about whether the primary data were fully reviewed or whether the view of the authors of those few review articles was simply adopted, and raise significant questions about the credibility of the overall evaluation. Each concern is explained in more detail below.

A. Overstating the findings of the selected primary literature.

There are concerns that the Toxicological Review over-generalizes its presentation of the primary literature, particularly with respect to *in vitro* cell culture studies. One example of this problem is seen in its discussion of the literature concerning DNA adducts where the Toxicological Review states on pages 184-185 that:

> *"Although the ascorbate–trivalent chromium–DNA adducts are recovered less frequently in vitro due to the low concentrations of vitamin C present in commonly used tissue culture media (Zhitkovich, 2005), these adducts have been shown to be the most mutagenic of all the ternary adducts (Quievryn et al., 2003)." … "They have been detected in vitro in Chinese hamster ovary cells following exposure to hexavalent chromium, and account for up to 50% of all chromium–DNA adducts. The ternary adducts have been found to cause mutagenic and replication-blocking lesions in human fibroblasts in vitro (Quievryn et al., 2003; Voitkun et al., 1998)."*

Thus, the reader is led to believe that ascorbate-trivalent chromium-DNA adducts have been found in cells and these adducts have been shown to be highly mutagenic. Careful examination of the two cited references reveals that the statement in the Toxicological Review quote listed above that states:

> *"They have been detected in vitro in Chinese hamster ovary cells following exposure to hexavalent chromium…"*

is incorrect. Detection of adducts in Chinese hamster ovary cells was not actually presented as data in either paper or mentioned in the text of either paper. The claim is unsubstantiated as presented, which makes it potentially misleading.

Furthermore, when one considers the experimental detail in Quievryn et al., 2003 and Voitkun et al,, 1998, one learns that the adducts were synthesized in a cell free system. A sequence of DNA was treated with Cr(VI) and ascorbate in a cell free system. Then, the damaged DNA sequence was administered to cells. The cells then converted the damaged DNA sequence to a mutation that was revealed when the sequence was recovered and sequenced.

The detail also shows that the cells used were not normal human cells, but rather a SV40 immortalized cell line. SV40 is known to silence p53 activity, among other cellular and

molecular changes, thus these cells were unable to carry out proficient DNA repair or apoptosis, as these are normally p53-dependent events.

Thus, the studies did not show that these adducts were normally present or able to form inside the cell. They could not account for the fate of the adduct structure after the transfection process and are only assuming it remained intact. The studies did not show that mutations would have occurred normally inside the cell as a consequence of Cr(VI) exposure or as a consequence of these lesions. Moreover, they do not show that these events would have happened in a repair proficient or apoptosis-proficient cell. It could be that the only reason mutations were seen is that the cells' ability to repair or eliminate them through apoptosis was artificially turned off beforehand.

An alternative interpretation of the studies could be that one can experimentally force a cell to generate a mutation in response to a Cr adduct if repair and apoptosis are silenced. Indeed, a step forward, but not one that establishes that adducts form or are mutagenic in cells.

It is unclear why these two studies were chosen to show that Cr induces adducts in cells. It is remarkable that given the emphasis the Toxicological Review places on the importance of physiologically relevant cell cultures, that it would fail to mention or discuss the integrity of the cell line itself, which in these studies were not robust cells. The document seems to be saying that there is a problem with cell culture studies that have some extracellular metabolism of Cr(VI) or that might not have enough ascorbate, but there are no problems with studies in cells with compromised DNA repair and cell death pathways.

There are more examples of this type of exaggeration of the implications of the primary literature in the Toxicological Review. These exaggerations obscure the meaning and applicability of the data and should be corrected. These exaggerations also undermine the integrity of the document and raise questions about its accuracy and process. Other studies in the primary literature may reemerge as more relevant if treated more evenly and these studies should be reconsidered and possibly presented.

B. <u>Flaws in citations, extensive direct quoting and long stretches of general paraphrasing</u>

One concern is that the Toxicological Review actually appears to directly quote sources without indicating the comments are quotes of the original source. For example, in the Toxicological Review on page 46, lines 13-15, the writing states:

> *"Studies on the reduction of Cr(VI) by extracts of rat liver, lung, or kidney have found that ascorbate accounted for at least 80% of Cr(VI) metabolism in these tissues (Standeven et al., 1991,1992)."*

which is the exact same sentence that occurs in the Toxicological Review's Zhitkovich 2005 reference. That reference states on its page 5:

> *"Studies on the reduction of Cr-(VI) by extracts from rat lung, liver, or kidney have found that ascorbate accounted for at least 80% of Cr(VI) metabolism in these target tissues (45, 46)."*

Then on the same page, lines 20-22, the Toxicological Review states:

> *"Depending on the nature of the reducing agent and its concentration, this process can generate various amounts of unstable Cr(V) and Cr(IV) intermediates (Stearns et al., 1994)."*

which is the exact same sentence that is in Zhitkovich 2005. That reference states on its pages 5-6:

> *"Depending on the nature of the reducing agent and its concentration, this process can generate various amounts of unstable Cr(V) and Cr(IV) intermediates (14-16)."*

Neither of these sentences are indicated as being exact quotes of the original source and neither one is attributed to the original source. This omission is a concern as it is important to know when the document is choosing to quote from a source directly. These examples are not the only occurrences of this type of error and the entire document needs to be checked to identify other such problems.

In other instances, the Toxicological Review only changes a couple of words in a direct quote and fails to indicate it is a direct quote, which is also unacceptable. For example, the Toxicological Review states on page 50:

> *"Therefore, experiments on mutagenesis and other toxic effects of hexavalent chromium in tissue culture may underestimate its mutagenic, genotoxic, and cell-transforming activities (Zhitkovich, 2005; Costa and Klein, 2006)."*

The underlying reference by Costa and Klein 2006 states on its page 157:

> *"Thus, experiments on mutagenesis and other toxic effects of hexavalent Cr in tissue culture may underestimate its mutagenic, genotoxic, and cell-transforming activity (Zhitkovich, 2005)."*

The quote in the Toxicological Review and the Costa and Klein review differ by only substituting a "Therefore" for a "Thus" at the beginning and "activities" for "activity" near the end. Changing two words does not avoid the need to offset this sentence as a direct quote. As written, it is sufficiently in the original authors' words that it is considered a direct quote.

Similarly, in the Toxicological Review on page 46, the writing states:

> *"Ascorbate is also the fastest reducer in the in vitro reactions, and its rate of reduction at 1 mM exceeds that of cysteine and glutathione by approximately 13 and 61 times, respectively (Zhitkovich, 2005; Quivryn et al., 2001)."*

which is the exact same sentence that is in the Toxicological Review's Zhitkovich 2005 reference that states on its page 5:

> *"Ascorbate is also the fastest reducer of Cr(VI) in the in vitro reactions, and its rate of reduction at 1 mM concentration exceeds that of cysteine and glutathione approximately 13 and 61 times, respectively (48)."*

Again, this language is a direct quote and needs to be offset in quotation to make that clear.

This type of error also occurs with some frequency in the document and needs to be addressed.

Next, there are numerous instances when the Toxicological Review extensively paraphrases a review article and the meaning of the original passage is altered to another meaning that was not originally intended resulting in some overstatements and inaccuracies. For example, the Toxicological Review states on page 185:

> *"Reduction of hexavalent chromium in vitro produces a large proportion of binary trivalent chromium−DNA adducts, but these have not been detected in vivo. It has been theorized that the formation of the ternary adducts described above occurs far more frequently due to the high concentration of ligands capable of complexing with trivalent chromium before it can bind to DNA. (Zhitkovich, 2005)."*

Given its general use of "*in vitro*" to mean "in cell culture", the Toxicological Review appears to be stating that binary trivalent chromium-DNA adducts occur in cell culture but not in *in vivo* studies. However, the underlying Zhitkovich review reference actually states that the binary adducts have been detected in a test tube and not in cell culture. Specifically, it states (bold added here for emphasis):

> *"Reductive metabolism of Cr(VI) in vitro usually generates a large number of binary Cr(III)-DNA adducts (22, 37, 53), but the presence of these DNA modifications* ***in cells*** *has not yet been established. The formation of binary Cr-DNA complexes in cells is expected to be strongly inhibited due to the abundance of intracellular ligands capable of rapid coordination to Cr(III) prior to its binding to DNA."*

This error occurs quite often and creates confusion about what the underlying literature is indicating.

There is a reference to "Salnikov and Zhitkovich, 2009" in a couple of places and no citation for this reference is provided. In the citation list, there is one reference listed as "Salnikow, K; Zhitkovich, A. (2008)" and another just below it as "Salnikov, K; Zhitkovich, A. (2008)" that looks to be exactly the same reference. These details should be straightened out and the entire reference section rechecked.

The occurrence of these various errors in citations undermines confidence in the Toxicological Review and raises significant concerns about process. It gives the impression that review articles formed the basis for the evaluation, rather than primary sources and, with the extensive quoting and paraphrasing, that some articles were simply integrated into the document. The use of these review articles in the document needs to be revised and addressed.

C. <u>Confusion due to inconsistent application of the phrases "in vitro" and "in vivo".</u>

The data in the Toxicological Review essentially fall into four groups. There are cell-free system studies, cell culture studies, whole animal studies and human studies. To describe these data, the phrases "*in vitro*" and "*in vivo*" are used. To most readers "*in vivo*" is

thought to refer to studies in the body and so include whole animal and in some instances human studies. By contrast, "*in vitro*" is thought to refer to cell culture studies. There are inconsistencies in the use of these terms in the Toxicological Review as some of the underlying references use "*in vivo*" to mean in cell culture and "*in vitro*" to mean in cell free systems. The Toxicological Review has often failed to clarify the underlying studies and carried the underlying language forward into the review.

Two examples of this problem are presented in the preceding criticism, where the Toxicological Review elevated the cells in culture to an "*in vivo*" status, but there are many more occurrences in the document. There are two explanations for this outcome. One possibility is that in some places the Toxicological Review uses "*in vitro*" to mean in cell culture and in others to mean in cell free systems and in some places it uses "*in vivo*" to mean in whole animals and in others to mean in cell culture. The second is that the authors of the Toxicological Review did not realize that the underlying literature meant for "*in vivo*" to mean in cell culture and "*in vitro*" to mean in cell free systems. Regardless of which reason applies, as written the use of the terms is confusing and in some cases, such as the one explained above, misleading. The EPA needs to decide on a definition for these terms, present it, review the underlying literature to be sure they reflect what is meant and then apply them consistently in the document.

D. Failure to check the underlying primary research studies in review articles.

There was a failure to fully consider the underlying primary research articles in the review articles that are extensively cited. This failure creates a perception that the authors did not read beyond that review article, raising questions about process and whether primary data was evaluated at all. Where the document depends on a review article for its source, the underlying primary literature should be checked to confirm the integrity of the statements. One example of this problem is seen in the passage below from page 50 of the Toxicological Review:

> *"An additional important note on these biotransformations regards the interpretation and reliability of data from in vitro assays. In vivo, the intracellular levels of ascorbate are quite high (about 1 mM). In contrast, the levels of ascorbate in tissue culture media are quite low since generally it is not added to the media so that the only source is supplemented fetal bovine serum (FBS). With 10% FBS, the level of ascorbate in tissue cultured cells is only about 50 μM which is 20 times lower than that which is found in vivo (Zhitkovich, 2005). Therefore, experiments on mutagenesis and other toxic effects of hexavalent chromium in tissue culture may underestimate its mutagenic, genotoxic, and cell-transforming activities (Zhitkovich, 2005; Costa and Klein, 2006)."*

Thus, the Toxicological Review wants the reader to question cell culture studies if they do not contain intracellular levels of ascorbate in the mM range. However, the Zhitkovich review article that this section is entirely based upon miscites the primary literature on this matter and it appears the Toxicological Review did not check it. Specifically, as seen in the passage below from the Zhitkovich 2005 review, the claims about physiological levels of ascorbate being in the millimolar range rely on primary research papers that are its references 53 and 54. The Zhitkovich review states:

	"Under standard tissue culture conditions, A549 and many other human and rodent cells either lack detectable ascorbate or contain it only at micromolar levels (physiological levels are in millimolar range) (53, 54) due to low concentrations of this vitamin in fetal bovine serum and its absence in the most commonly used types of growth media (DMEM, RPMI 1640, F10, F12)." These two references are: *"(53) Quievryn, G., Messer, J., and Zhitkovich, A. (2002) Carcinogenic chromium(VI) induces cross-linking of vitamin C to DNA in vitro and in human lung A549 cells. Biochemistry 41, 3156-3167.* *(54) Salnikow, K., Donald, S. P., Bruick, R. K., Zhitkovich, A., Phang, J. M., and Kasprzak, K. S. (2004) Depletion of intracellular ascorbate by the carcinogenic metals nickel and cobalt results in the induction of hypoxic stress. J. Biol. Chem. 279, 40337-40344."* Examination of these two references shows, however, that neither one offers any data or evidence of physiological ascorbate levels being in the mM range. Salnikow et al. measures the amount of ascorbate loss in cultured cells treated with nickel and cobalt. They do measure ascorbate levels in untreated control cells to determine the background level of their experimental system. But their study does not measure any levels of vitamin C in any physiological setting. Nor does the citation make any reference at all to any study that does. Quievryn et al., treats the human carcinoma cell line A549 with ascorbate and dihydroascorbate and then measures the amount of vitamin C inside the cell under these experimental conditions. But the study does not measure any levels of vitamin C in any physiological setting. The discussion section does make a comment that: "*Human cells in vivo contain a millimolar concentration of Asc (43)…*". However, that reference 43 is "*43. Meister, A. (1994) J. Biol. Chem. 269, 9397-9400*", which is a review article concerning glutathione and ascorbate in rodents. It contains no mention of ascorbate in human cells. Thus, the EPA expresses a significant concern about *in vitro* cell culture studies based on a single review article that miscites the primary and secondary literature. This oversight implies that in the preparation of this Toxicological Review, the EPA did not access the primary literature and confirm the secondary and tertiary review articles. If one were to look at primary literature for ascorbate levels, one would find that these claims are overstated. Ascorbate can reach mM levels in the body, but they are not universally mM levels. For example, Slade et al., report lung ascorbate levels of 2.91 to 62.35 mg/100 g (Slade, R., Stead, A.G., Graham, J.A., and Hatch, G.E. (1985) Comparison of lung antioxidant levels in humans and laboratory animals. Am. Rev. Respir. Dis. 131(5), 742-6). This measure can be converted to a range of 165 uM to 3.5 mM. However, these are not intracellular levels, but rather the product of tissue homogenization and so a mixture of extracellular and intracellular sources. Thus, there is clearly variability in levels that span the uM and mM range, indicating that this factor may not be so essential.

	E. Some exaggerations about Cr transport in cells. Of less concern, but certainly in need of being addressed is some of the inaccurate language concerning Cr transport into the cell. In several places, cells are described as being impermeable to Cr(III). This characterization is too strong and inaccurate. Cr(III) will enter cells. It is just a slower uptake process than Cr(VI) uptake as Cr(III) moves by simple diffusion and it requires a higher dose to create the concentration gradient to get in the cell. This language should be adjusted. Also, there is language implying Cr(VI) is actively transported into cells. Cr(VI) does enter rapidly by facilitated diffusion, but it is not an active transport process. These comments should be adjusted. F. Some typographical errors. There is also a mention on page 150 that states: 'As discussed in detail in Section 4.4.2 (Intracellular Reduction)…" It is actually section 4.5.2. In sum, these factors all combine to give the appearance that the mode of action was not fully and consistently considered. To make the document and its conclusions much stronger and more accurate and the rationale behind its decisions more transparent, the following steps should be taken: 1) The EPA needs to separate the genotoxicity literature into discrete endpoints and consider them individually. This consideration should be based on the primary literature, which should be presented in a more careful and coherent fashion so that the reader can understand the strengths, weaknesses and data gaps. 2) Based on that analysis the EPA should choose which lesions are the key lesions and explain the rationale for that choice. 3) Once the key lesions are chosen, the EPA should consider the possible mechanisms of action that may cause those lesions and determine if there are data to support those mechanisms of action. 4) Once the key lesions are identified and the likely mechanisms described, the EPA should explain its rationale for the one chosen to be the mode of action. Of course, non-genotoxic modes of action should also receive similar analysis and presentation. This approach would help the EPA determine the most robust mode of action based on the primary literature. In addition, the EPA should decide what factors are truly confounders of concern and then apply them evenly to all of the literature, reduce its use of secondary and tertiary review articles and improve its management and citation of the literature.
Zhitkovich	In general, I found the Draft to be well prepared and balanced in its presentation of various aspects of chromium-6 toxicology and carcinogenesis. It has a logical structure, leading a reader from the basics of redox chemistry of chromium-3 and chromium-6 and their interactions with biosystems to the detailed description of *in vivo* studies on bioavailability, tissue disposition and finally, toxic and carcinogenic effects. Weaknesses and strengths of the key *in vivo* studies along with the reasons for the inclusion or exclusion of specific findings were also clearly presented. Different sections vary somewhat in their degree of emphasis on the importance of one or another mechanistic aspect of Cr(VI) toxicology, which is also reflective of divergent opinions in the field. As typical for any large document covering a complex topic, the Draft contains some information that is not up-to-date and would benefit from additional editorial work.

	Suggested modifications and corrections: 1) Section 2.1 "*Environmental Sources and Occurrence*" appears to draw a large amount of information from decades-old literature. The analytical approaches for the detection of both total Cr and Cr-6 underwent major improvements in 1980s and the older references to the amount of Cr-6 in various environmental media and biological samples should be looked at with a healthy degree of skepticism and scrutinized for potential overestimations. My specific concerns are related to the included values for Cr-6 levels in soil, freshwater and seawater. All three sets of values are too high for the typical samples from the noncontaminated areas/sites. 2) Table 2-1 "*Industrial uses of hexavalent chromium compounds*" is missing uses of sodium/potassium chromate and dichromate. The addition of information on sodium dichromate is particularly important in light of its testing for carcinogenicity by the NTP. 3) p.14, last para: Cr(III) oxidation to Cr(VI) by atmospheric oxygen can also occur in the presence of calcium oxide (Pillay et al. 2003). 4) Table 2-4 "*Detection limits for methods…*" reports outdated values. The EPA's Method 218.6 for Cr(VI) in water has a detection limit which is ~100 times lower than detection limits listed in Table 2-4. A recent modification of this method affords detection of Cr(VI) at the 0.003 ppb level (Application Update 179 from Dionex). The detection sensitivity of flame AAS is also underestimated. Based on the discussion of work by Levine (2007) on p.16 and other available literature, the detection limit for total Cr in water samples by ICP-MS reported in Table 2-4 is probably lower by a couple orders of magnitude. 5) p.25, lines 5-6: in Donaldson and Barreras (1966), urinary excretion for orally administered Cr(VI) and Cr(III) were 2.1 and 0.5%, respectively (not 2.1 versus 1.5%). 6) Table 3-5 and the discussion of these results appear contradictory. 7) Table on p.39 is confusing: it reports daily doses of sodium dichromate dihydrate in the NTP-2008 study but the ratio mice:rats looks incorrect based on the data in the top two rows. It is also unclear why Cr(VI) consumption was compared between male rats and female mice and not between animals of the same sex. 8) Finley et al. (1996) delivered a Cr-6 dose of 0.005 mg/kg/day, not 0.005 mg (p.45). 9) Section 3.3 describes Cr(VI) reduction by microsomal enzymes in detail on three pages. This degree of attention may create an erroneous impression about the importance of the specialized enzymatic processes in Cr-6 metabolism. There is a strong consensus in the field that Cr(VI) reduction in mammalian cells is primarily accomplished nonenzymatically by ascorbate and small thiols such as glutathione and cysteine. As acknowledged in other sections of the draft, ascorbate alone accounts for reduction of 80-95% Cr(VI) depending on the tissue (Standeven and Wetterhahn, 1991,1992). A combined contribution of ascorbate and thiols is responsible for more than 95% Cr(VI) reduction. These estimates from tissue preparations were confirmed by the measurements of individual reduction rates (Quievryn et al. 2003). It is clearly important to present mechanistic aspects of Cr(VI) reduction but the detailed focus should be on ascorbate and non-protein thiols, not enzymatic systems with a minimal contribution to the overall

	Cr(VI) metabolism *in vivo*. The absence of Cr(V) intermediate during Cr(VI) reduction by ascorbate is especially important. 10) Last sentence on p. 50: The description of vitamin C accumulation by cells is not entirely correct. Cellular accumulation of vitamin C via uptake of its oxidized form dehydroascorbic acid is a *physiological* mechanism that functions in all mammalian cells. It is particularly active in human cells, which leads to very efficient recycling and much lower daily requirements for vitamin C in humans compared to rodents (Nualart et al. 2003, Montel-Hagen et al. 2008). These differences between humans and rodents are relevant for the interspecies extrapolation. 11) Figure 3-6 needs to be modified: a) Depiction of the cation channel with the comment "No effect" could be interpreted as indicating some nontoxic delivery route for chromium. Unlike some other toxic metals, cation channels play no role in uptake of Cr ions and the cation route should be deleted from this Figure. b) Although some Cr(III)-ligand complexes can exhibit a limited ability to enter cells, there is no evidence that they can react with DNA and cause mutagenic/genotoxic ternary Cr-DNA adducts, as shown in the Figure. The Figure should be modified by removing this nonexistent route of DNA damage. c) The route for the formation of DSB by mismatch repair needs to be revised. As demonstrated in a recent study by Reynolds MF et al (2009), ternary Cr-DNA adducts are directly bound by mismatch repair proteins followed by DSB formation in G2 phase without stalling replication forks in the preceding S-phase. 12) Summary Table 4-20 should add the *+(M) designation* for mutagenesis of sodium dichromate in laboratory animal, as demonstrated by Cheng et al. 2000 (this study was later cited in Table 4-23). 13) Table 4-23 "In vivo genotoxicity studies… Mutations section" is missing references to two positive mutagenesis studies in vivo by Itoh and Shimada (1997, 1998). 14) Section 4.7.3.1. *Hypothesized Mode of Action*: a) Ref. to Salnikow et al. (1992) in support of ternary complexes is inappropriate and could be replaced by Voitkun et al. (1998). b) Neither Zhitkovich (2005) nor Voitkun et al. (1998) dealt with "intrastrand DNA-DNA crosslinks". A study by Lloyd et al. (1998) is the only original report describing putative intrastrand crosslinks, which were generated in a buffer solution with massive concentrations of hydrogen peroxide. Tested under the same reaction conditions, essential metals copper and cobalt were even more potent inducers of these presumed crosslinks. There is no evidence for the formation of these crosslinks by chromium-6 in cells or in acellular systems containing its main biological reducers. 15) Tables 4-22 and 4-23 failed to include any references to studies reporting the formation of chromium-DNA adducts in cultured mammalian cells and *in vivo*. This is a critical omission as the presence of chromium-DNA adducts demonstrates a direct DNA-

damaging mechanism for Cr(VI) genotoxicity. The formation of DNA adducts was briefly discussed in other sections of the Draft.

16) Discussion of a negative report on DNA damage by DeFlora et al. (2008) on pp.206-207:

> This study found no evidence of DNA damage in forestomach, glandular stomach and duodenum of female SKH-1 mice after a 9-month long exposure to 5 and 20 mg/L Cr(VI) in drinking water. Based on the study by DeFlora et al. (2008), a high safety threshold argument was also made in some of the submitted public comments. The Draft argued that a shorter duration of exposure (9 months vs. 2 years in the NTP study) made the DeFlora 2008 study "infeasible" for the comparison. With the exception of mutations and a potential accumulation of unrepaired damage in a population of the long-lived crypt stem cells, there are no other obvious factors suggesting that the formation of DNA damage by Cr(VI) in the entire duodenum during the first half of the 2-year exposure would be significantly different from damage occurring at the end of the 2 years.
>
> However, the study by DeFlora et al. (1998) is uninformative for other reasons. The authors assayed tissues for two forms of DNA damage: DNA-protein crosslinks and 8-oxo-dG (the Draft incorrectly described 8-oxo-dG as a DNA adduct; it is actually a base oxidation product). Both types of damage showed no increases above background in tissues of exposed animals; however, these negative results were predictable based on the technical limitations of their analytical methodologies. Since Cr(VI) tumorigenesis occurred in the duodenum, I will limit my discussion to this tissue.

1) DNA-protein crosslinks:

A positive control consisting of mouse duodenal cells treated *ex vivo* with 1.6 mM Cr(VI) (83.2 mg/L) generated a 2.5-fold response. Such a low responsiveness was clearly insufficient to detect DNA damage for exposures with 4.2- and 16.6-times lower Cr(VI) levels in the 20 mg/L and 5 mg/L test groups, respectively, even in the unlikely scenario of no reduction and no dilution of Cr(VI) with stomach juices before reaching the duodenum. Although chronic exposures frequently leads to the accumulation of unrepairable damage, a dramatic increase in DNA-protein crosslinks during chronic Cr(VI) exposures would be not very likely. DNA-protein crosslinks are repairable lesions in mammalian cells *in vivo* and culture (Tsapakos et al. 1983, Sugiyama et al. 1986, Zecevic et al. 2010) with the possible exception of human peripheral blood lymphocytes (Quievryn and Zhitkovich 2000). Furthermore, ongoing proliferation and shedding of cells in the duodenal villi would result in continuous dilution of damage and loss of previously exposed cells.

2) 8-oxo-dG measurements:

A positive control generated by exposure of mouse duodenal cells to 1.6 mM Cr(VI) (83.2 mg/L) produced a 3.8-fold increase in the levels of 8-oxo-dG. It is doubtful that this assay sensitivity was sufficient to detect significant increases in 8-oxo-dG levels for even undiluted/unreduced 20mg/L and 5mg/L Cr(VI) concentrations that were used in the treatment groups. 8-oxo-dG as a biomarker of DNA damage has one critical limitation – a

	short lifetime due to its rapid removal by base excision repair. Repair of 50% 8-oxo-dG occur within 30 min and is complete within 2 hr (Lan et al. 2004). Not only would this short lifetime prevent any accumulation of 8-oxo-dG during chronic exposures, but it would also make it very difficult to detect this lesion even after recently ingested water with a sufficiently high dose producing positive responses under *ex vivo* conditions.
Zhu	This EPA's Review is well organized overall and for most part well presented. The literature review is extensive and thorough. However, the Review does not contain a set of clearly-stated criteria under which the literature was searched, critiqued, and synthesized. Specifically, was each published study judged with respect to design (including sample size), exposure assessment, choice of dose metrics, choice of endpoints, adequate dose-response data, dose-response modeling, and positive findings? Whereas some of these criteria may have been used in the Review, the lack of a systematic approach may have compromised the consistency and transparency of this review process. In its independent review of the EPA's IRIS Documents on Formaldehyde and Dioxins, for example, the National Academies of Science and National Research Council have strongly advocated the adoption of a systematic review approach to EPA's IRIS risk assessment process (NAS 2006, 2011). The present Review of Hexavalent Chromium once again demonstrates the need for adopting a systematic review approach.

G2. Please identify any additional studies that would make a significant impact on the conclusions of the Toxicological Review.

Reviewer	Comments
Byczkowski	It seems that up to date (around the year 2009), all important studies (and some unimportant too) have been already covered by this Toxicological Review. However, I strongly suggest that the results of study presented at the workshop by ToxStrategies, Inc., and especially the physiologically based pharmacokinetic (PBPK) modeling by Summit Toxicology, LLP., should be included in the revised Toxicological Review of Hexavalent Chromium document.
Hamilton	See G1. above.
Nordberg	To my understanding there are new experimental studies with more for this purpose fitted doses. It could be worthwhile to await the outcome of these studies to find out whether more appropriate values for both NOAEL and LOAEL will be reported and include these in an appendix if feasible. Any further data obtained in the cited NTP studies should be included and presented to the reader. It would be of interest to know if there are any data on lung cancer or other effects from the NTP studies. During the workshop a number of ongoing studies were presented and it was suggested that they be paid attention to. It is always an advantage to get more and more information and research is always going on.

	In my opinion it is however important to set recommendations for exposure to toxic agents in order to protect humans from developing adverse health effects. It is a human right to be protected from unwanted exposure which also will cause unnecessary worry during the time from alert to protection. People expect regulatory agencies to make evaluations and set exposure limits. Studies underway even if published in peer review scientific journals should be carefully evaluated and scrutinized by EPS´s working group to determine if presented data is reliable e.g., based on a number of factors such as, just to mention a few, how large are the studies and what is the power o the study, analytical procedures that include quality control so data is validated and to be trusted. Based on experience it takes time before data will be available even for ongoing studies. I recommend that IRIS, EPA sets a recommendation based on information presented in the draft document. In case important information which can change any evaluation shows up in time, such data can be included in the final document as an appendix or addendum. It is important in Risk Assessment to keep in mind that any recommendation set for exposure levels values needs to be reevaluated over time because by new techniques e.g., rapid development of usage of "omics" has to be considered. In view of said it is important to draw conclusions now and on data available now and not to wait.
Patierno	The TRHC should absolutely consider the extensive new data being provided by ToxStrategies and presented in part in the supplementary section under Public Comments.
Rossman	Some of these will be presented in section B2, as they pertain to mode of action. It is extremely important that the new information supported by American Chemical Council (performed by ToxStudies and others) should be considered before the final document is completed. They address a number of missing data sets. It is already clear that proliferative increases occur in the mouse duodenum at doses of Cr(6) lower than those that cause tumors. Also, there is evidence for cytotoxicity at these lower concentrations that may be driving the proloferative responses. The fact that Cr is an essential element needs to be addressed. What are the implications for a threshold? It is possible that dietary Cr(6) is significant and should be evaluated. All parts of grain contain Cr(6) and 10% of the Cr in bread is Cr(6) (Mishra et al., Food Chem. Toxicol. 33:393-397, 1995; Soares et al., J. Agric. Food Chem. 58:1366-1370). River waters have a median Cr value [which is probably Cr(6)] of 10 ppb (range <1-30), and even rainwater has a range from 0.14-0.9 (ATSDR, Chromium, Draft for Public Comment, online). A recent meta-analysis of cancers of the G.I. tract among those occupationally exposed to Cr(6), concludes that these workers are not at greater risk than the general population (Gatto et al., Cancer Epidemiol. 34:388-399, 2010). Inhalation exposure usually leads to G.I. exposure, suggesting a possible threshold if the ingested dose can be estimated.

Salnikow	Key issues that should have an impact on the conclusions of the draft of the Toxicological Review of Hexavalent Chromium are: 1) the use of appropriate animal models, 2) an understanding of the chromium carcinogenic MOA, including genotoxic (mutagenic) and non-genotoxic (epigenetic) mechanisms and their interrelations, 3) the co-carcinogenic effects of hexavalent chromium, and 4) the role of iron metabolism in chromium carcinogenesis. Unfortunately, studies to address these issues are either not done or are in the early stages of research. Thus, it is too early to draw any conclusive decisions on risk assessment of hexavalent chromium in drinking water. Are used animal models appropriate for risk assessment? Although the NTP studies provide evidence that oral exposure to hexavalent chromium induced tumors in rodents, the main argument against these studies is that the toxic and carcinogenic effects could be achieved/observed only at high chromium concentrations, which significantly exceed human exposure levels. Also, it is noted that biological effects were seen only at chromium concentrations that overwhelmed the cellular defense systems (reducing capabilities). Ascorbate is a major reducing agent of hexavalent chromium in biological fluids and tissues (see review (Zhitkovich, 2005; Salnikow and Zhitkovich, 2008)). Humans cannot synthesize ascorbate in the body because of a mutation in the L-gulono-γ-lactone oxidase gene coding for the final enzyme in ascorbate metabolism and thus ascorbate is supplemented through the diet. Unlike humans, laboratory mice and rats, used for carcinogenicity assays, are capable of synthesizing ascorbate endogenously. Because ascorbate regulates many cell and tissue functions that are critical for cancer development, the changes in the level of ascorbate should be considered in animal models of choice (Salnikow and Kasprzak, 2005). It is impossible to deplete tissue ascorbate levels by metal exposure in wild-type rodents because the enzyme producing ascorbate will be up-regulated when the level of ascorbate drops below a critical point. To avoid this problem for *in vivo* testing of the toxic and carcinogenic effects of heavy metals, which efficiently destroy ascorbate, an appropriate model is the use of mice or rats that like humans cannot synthesize ascorbate (Kasprzak *et al.*, 2011). Two rodent model systems unable to synthesize ascorbate are available: Gulo-/- mice (Maeda *et al.*, 2000), and a similar rat strain (Mizushima *et al.*, 1984). Our preliminary data show that when Gulo-/- mice were supplemented with ascorbate in drinking water their blood and tissue ascorbate levels were undistinguishable from that in wild type mice. However, ascorbate levels were significantly decreased by metal exposure in Gulo-/- mice but not in wild-type mice, in which the enzyme responsible for ascorbate production was activated in response to metal exposure (ascorbate depletion) (Kasprzak *et al.*, 2011). In this model system we found that the reduction in ascorbate levels increased acute toxicity induced by Ni_3S_2 in Gulo-/- mice and that Gulo-/- mice were more susceptible than wild-type mice to nickel-induced carcinogenesis. Additionally, in tumor transplantation assays, Gulo-/- mice had shorter tumor latency than wild-type mice. After the lag period established tumor growth rates were comparable in Gulo-/- and wild-type mice. Although cancer initiation and development is a very complicated process our results indicate that ascorbate is a potentially important part of the molecular mechanisms of metal carcinogenesis and acute toxicity. Ascorbate is involved in diverse biological activities. Ascorbate is essential for the function of numerous 2-oxoglutarate-dependent hydroxylases. This group of hydroxylases

	includes the asparaginyl and prolyl hydroxylases, FIH-1 and PHD1, PHD2, PHD3, which are responsible for HIFα hydroxylation (Epstein *et al.*, 2001; Mahon *et al.*, 2001; Hewitson *et al.*, 2002; Lando *et al.*, 2002); the collagen prolyl-4-hydroxylases (Myllyharju, 2003), which is critical for extracellular matrix formation; and a new class of histone and DNA demethylases that remove methyl group through hydroxylation (Shi, 2007). We already pointed out that the level of ascorbate is critical for metal carcinogenesis mainly by affecting epigenetic pathway (Salnikow and Zhitkovich, 2008). More recently a link between changes in ascorbate concentration and DNA demethylation of human embryonic stem cells (hESCs) has been identified (Chung *et al.*, 2010). Thus, ascorbate levels have the potential to directly impact the differentiation of hESCs and the reprogramming of somatic cells. Given that ascorbate has diverse cellular functions and ascorbate levels are critical to interpreting carcinogenic effects of heavy metals, the animal models described in the Toxicological Review are not the most appropriate. The results obtained in NTP 2007 studies are consistent with the idea that ascorbate is an important factor to consider. In preliminary toxicokinetic studies in which animals were exposed to chromium in drinking water for 21 days chromium concentrations in the blood of the guinea pigs (which are unable to synthesis ascorbate) was greater than chromium concentrations in the blood of the rats or mice suggesting greater absorption (less reduction) of chromium in guinea pigs. http://ntp.niehs.nih.gov/ntp/htdocs/ST_rpts/tox072.pdf I suggest that new studies similar to the NTP studies with several dietary concentrations of ascorbate and lower chromium does (i.e., those more relevant to environmental exposures), be done in ascorbate-deficient rats or mice or both animal models. Additionally, because of more efficient depletion of ascorbate in tissues of Gulo-/- animals by chromium these animal models will show whether Cr(III) and Cr(VI) kinetic that were developed using the wild type animals (O'Flaherty, 1996; O'Flaherty *et al.*, 2001) will be applicable to Gulo-/- animals. This will allow for adjustment of kinetic models, if needed, and identification of new or confirming known compartments of chromium retention.
Wise	Although this review is focused on oral exposures, some insight may be gleaned from the inhalation exposure data. Specifically, the data on mutations in lung tumors for Cr(VI)-exposed workers should be considered. These data show a lack of mutations in those tumors suggesting that mutagenicity as considered as a primary change in the DNA sequence is not a key event in the mechanism of action. They are consistent with the fact that one only sees these types of mutations in mammalian experimental models when one forces them by applying very high doses. These studies are: Katabami M, Dosaka-Akita H, Mishina T, Honma K, Kimura K, Uchida Y, et al. Frequent cyclin D1 expression in chromate induced lung cancers. Hum Pathol 2000; 31 : 973-9. Kondo K, Hino N, Sasa M, Kamamura Y, Sakiyama S,Tsuyuguchi M, et al. Mutations of the p53 gene in human lung cancer from chromate-exposed workers. Biochem Biophy Res Commun 1997; 239 : 95-100. Ewis AA, Kondo K, Lee J, Tsuyuguchi M, Hashimoto M, Yokose T, et al. Occupational cancer genetics: Infrequent ras oncogenes point mutation in lung cancer samples from chromate workers. Am J Ind Med 2001; 40 : 92-7.

	Hirose T, Kondo K, Takahashi Y, Ishikura H, Fujino H, Tsuyuguchi M, et al. Frequent microsatellite instability in lung cancer from chromate-exposed workers. Mol Carcinog 2002; 33 : 172-80. Takahashi Y, Kondo K, Hirose T, Nakagawa H, Tsuyuguchi M, Hashimoto M, et al. Microsatellite instability and protein expression of the DNA mismatch repair gene, hMLH1, of lung cancer in chromate-exposed workers. Mol Carinog 2005; 42: 150-8. Kondo K, Takahashi Y, Hirose Y, Nagao T, Tsuyuguchi M, Hashimoto M, et al. The reduced expression and aberrant methylation of p16INK4a in chromate workers with lung cancer. Lung Cancer 2006; 53 : 295-302. Ewis AA, Kondo K, Dang F, Nakahori Y, Shinohara Y, Ishikawa M, et al. Surfactant protein B gene variations and susceptibility to lung cancer in chromate workers. Am J Ind Med 2006; 49 : 267-73. There are also studies of Cr(VI)-induced neoplastic transformation of cells in culture. These need to be considered and included. In particular, reports by Xie et. al., show that cells must acquire a DNA double strand break repair phenotype to undergo transformation indicating escape from repair may be a key event in the mode of action. These studies are: Patierno SR, Banh D, Landolph JR. Transformation of C3H/10T1/2 mouse embryo cells to focus formation and anchorage independence by insoluble lead chromate but not soluble calcium chromate: relationship to mutagenesis and internalization of lead chromate particles. Cancer Res 1988; 47 : 3815-23. Xie H, Holmes AL, Wise SS, Huang S, Peng C, Wise Sr JP. Neoplastic transformation of human bronchial cells by lead chromate particles. Am J Respir Cell Mol Biol 2007; 37: 544- 52. Xie H, Wise SS, Wise Sr. JP. Deficient repair of particulate chromate-induced DNA double strand breaks leads to neoplastic transformation. Mutat Res 2008; 649 : 230-8. The document only considered mismatch repair, but there are important data showing that other DNA repair pathways must be overcome to induce genotoxicity and carcinogenesis. Double strand breaks and their repair, in particular, are important. The following studies should be added to the repair/DNA double strand break discussion: Xie, H., Holmes, A.L., Young, J.L., Qin, Q., Joyce, K, Pelsue, S.C., Peng, C., Wise, S.S., Jeevarajan, A., Wallace, W.T., Hammond, D. and Wise, Sr., J.P. Zinc Chromate Induces Chromosome Instability and DNA Double Strand Breaks in Human Lung Cells. Toxicology and Applied Pharmacology, 234: 293–299, 2009. Xie H, Wise SS, Holmes AL, Xu B, Wakeman T, Pelsue SC, et al. Carcinogenic lead chromate induces DNA double-strand breaks and activates ATM kinase in human lung cells. Mutat Res 2005; 586 : 160-72. Xie H, Wise SS, Wise Sr. JP. Deficient repair of particulate chromate-induced DNA double strand breaks leads to neoplastic transformation. Mutat Res 2008; 649 : 230-8. Stackpole MM, Wise SS, Goodale BC, Duzevik EG, Munroe RC, Thompson WD, et al. Homologous recombination protects against particulate chromate-induced genomic instability in Chinese hamster cells. Mutat Res 2007; 625:145-54.

	Camrye E, Wise SS, Milligan P, Gordon N, Goodale B, Stackpole M, et al. Ku80 deficiency does not affect particulate chromate-induced chromosome damage and cytotoxicity in Chinese hamster ovary cells. Toxicol Sci 2007; 97: 348-54. Bryant HE, Ying S, Helleday T. Homologous recombination is involved in repair of chromium-induced DNA damage in mammalian cells. Mutat Res 2006;599:116-23. Grlickova-Duzevik EG, Wise SS, Munroe RC, Thompson WD, Wise Sr. JP XRCC1 protects cells against particulate chromate-induced chromosome damage and cytotoxicity in Chinese hamster ovary cells. Tox Sci 2006a;92(2):409-15. Grlickova-Duzevik E, Wise SS, Munroe RC, Thompson WD, Wise Sr JP. XRCC1 protects cells from chromate-induced chromosome damage, but does not affect cytotoxicity. Mutat Res 2006; 610(1-2):31-7. Vilcheck SK, Ceryak S, O'Brien TJ, Patierno SR. FANCD2 monoubiquitination and activation by hexavalent chromium [Cr(VI)] exposure: Activation is not required for repair of chromium(VI)-induced DSBs. Mutat Res 2006;610:21-30. Savery LC, Grlickova-Duzevik E, Wise SS, Thompson WD, Hinz JM, Thompson LH, Wise Sr. JP. Role of the Fancg gene in protecting cells from particulate chromate-induced chromosome instability. Mutat Res 2007, 626(1-2):120-127. There needs to be a stronger and clearer discussion about aneuploidy as a potential mechanism. These studies should be added to that discussion (some are in the document already): Holmes, A.L., Wise, S.S., Pelsue, S.C., Aboueissa, A., Lingle, W., Salisbury, S., Gallaher, J. and Wise, Sr., J.P. Chronic exposure to zinc chromate induces centrosome amplification and spindle assembly checkpoint bypass in human lung fibroblasts. Chemical Research in Toxicology, 23(2): 386-395, 2010. Guerci A, Seoane A, Dulout FN. Aneugenic effects of some metal compounds assessed by chromosome counting in MRC-5 human cells. Mutat Res 2000; 469 : 35-40. Seoane AL, Guerci AM, Dulout FN. Malsegregation as a possible mechanism of aneuploidy induction by metal salts in MRC-5 human cells. Environ Mol Mutagen 2002; 40 : 200-6. Holmes AL, Wise SS, Sandwick SJ, Lingle WL, Negron VC, Thompson WD, et al. Chronic exposure to lead chromate causes centrosome abnormalities and aneuploidy in human lung cells. Cancer Res 2006; 66: 4041-8. Wise SS, Holmes AL, Xie H, Thompson WD, Wise Sr JP. Chronic exposure to particulate chromate induces spindle assembly checkpoint bypass in human lung cells. Chem Res Toxicol 2006; 19 : 1492-8. The following two studies should be added to the clastogenicity results, particularly in light of one reviewer's comments that telomerase may be important as the second paper suggests telomerase does not affect Cr genotoxicity:

	Xie, H., Holmes, A.L., Wise, S.S., Gordon, N. and Wise, Sr., J.P. Lead chromate-induced chromosome damage requires extracellular dissolution to liberate chromium ions but does not require particle internalization or intracellular dissolution. Chemical Research in Toxicology, 17(10): 1362-1367, 2004. Wise SS, Elmore LW, Holt SE, Little JE, Antonucci PG, Bryant BH, et al. Telomerase-mediated lifespan extension of human bronchial cells does not affect hexavalent chromium-induced cytotoxicity or genotoxicity. Mol Cell Biochem 2004; 255: 103-11. Finally, there are misleading comments about DNA-DNA crosslinks. The Toxicological Review states they are unlikely to form *in vivo*. When one studies the underlying review cited as evidence by Salnikow and Zhitkovich, it becomes apparent that by *in vivo* they mean cells in culture or whole animals. Thus, the review implies that Cr -DNA-DNA crosslinks would not be predicted to occur in cells or whole animals, however, data from Josh Hamilton and Karen Wetterhahn show Cr DNA-DNA crosslinks do form *in vivo*. The inclusion of Josh Hamilton's study showing DNA-DNA crosslinks *in vivo* would correct the inaccurate conclusion in the Toxicological Review that these lesions do not occur *in vivo*. I cannot locate that paper in the time frame available, but Josh Hamilton is a reviewer and should be able to provide it.
Zhitkovich	The Draft included information from all major studies that have a significant impact on the main conclusions. It does not list or discuss every study published on Cr(VI) but there was also no systematic exclusion. In Section 4.4, I would recommend adding an important report by Gibb et al. (2000), which is the largest epidemiological study of cancer risk due to inhalation exposure to Cr(VI). While the omission or inclusion of this study does not change the overall conclusion about Cr(VI) carcinogenicity to humans via inhalation, Gibb et al. (2000) provided strong evidence of chromate dose-dependence for lung cancer risk and its independence of the common confounder, tobacco smoking.
Zhu	NA

Chemical-Specific Charge Questions

(A) Oral Reference Dose (RfD) for Hexavalent Chromium

A1. A two-year drinking water study of sodium dichromate dihydrate in rats and mice (NTP, 2008) was selected as the basis for the derivation of the RfD. Please comment on whether the selection of this study as the principal study is scientifically supported and clearly described. Please identify and provide the rationale for any other studies that should be selected as the principal study.

Reviewer	Comments
Byczkowski	The two-year drinking water study by NTP (2008) seems to be the most comprehensive from all available chronic bioassays, and thus, it is suitable as the basis for derivation of the RfD. However, there is still some concern regarding the selection of the NTP study for developing of RfD (this document states - C.f. P. 85, L# 1: "...*Urinalysis showed dose-related decreased volume and increased specific gravity, consistent with decreased water intake. NTP (2007) suggested that decreased water intake was due to decreased palatability*..." and then, P.89. L# 34: "...*Drinking water consumption was reduced*...") Thus, the reduced drinking water consumption, and consequently at least partial dehydration, may have increased the osmolality of gastrointestinal fluid, which could be a significant confounder in the chronic toxicity study, even though (according to the quotation on P. 120) the Technical Report by NTP (2008) attempted to dismiss such a concern.
Hamilton	No concerns regarding A1. And A2. This reviewer would point out that the hyperplasia that was chosen as this endpoint, while appropriate for this RfD, is also appropriate for considering the carcinogenic MOA as well, as argued above and below and taking into considering the recently reported ACC studies that should be considered in this regard.
Nordberg	EPA suggests an oral RfD of $9x^{10-4}$ mg/kg-day. The epidemiology studies in Liaoning province, China (p 68-76 in draft report) reported increased incidence of cancer after intake of drinking water contaminated with hexavalent chromium. It is stated in the document to be the only reported human data. That study supports the statement of hexavalent chromium in drinking water to be carcinogenic. It should be explained to the reader why sodium dichromate dehydrate was chosen for oral exposure study. Data in the literature indicates that bioavailability and bioaccesibility depends on species of the compound and also exposure media. Are there any data on hexavalent chromium species in the drinking water in the general environment? The NTP studies that are reported have used doses that are much higher than reported present concentration in drinking water in the general environment. Thus values for LOAEL is identical to lowest administered dose. It has not been possible because of applied doses to

	set a NOAEL.
Patierno	The two-year drinking water study of sodium dichromate dyhydrate in rats and mice (NTP, 2008) is the most thorough and technically well-conducted study available. It is likely the best study available for selection. However, the interpretation of and conclusions drawn from that study need to be re-evaluated in light of the issues raised in my preceding comments and the additional data shown in the Pubic Comments and coming available from a multi-institutional study sponsored by ToxStrategies.
Rossman	This does seem like the best and most complete study to use.
Salnikow	Outside of my area of expertise.
Wise	This study is the proper study based on the available data. The study is flawed because only very high doses were considered in the study, thus, there is concern that it may not reflect events at lower doses. The EPA is in the unique position that a study that repeats the one above and extends it to lower doses is almost completed. The EPA should wait for the final results of that study to make the most informed analysis.
Zhitkovich	The NTP-2008 is the best available study of chromium-6 toxicity via oral exposure and its choice for the calculation of the RfD is scientifically sound and was clearly explained in the Draft.
Zhu	The Review offers EPA's rational for selecting the NTP's two-year drinking water study of sodium dichromate dihydrate in rats and mice (NTP, 2008). EPA's justification includes the lack of reliable epidemiological data, solid design of the NTP's experiment, its controlled exposure regimens, the sensitivity of the endpoint, the availability of dose-response data, and consistency with hypothesized genotoxicity MOA. These justifications are acceptable. There are still merits to include other studies, particularly the 3-month sodium dichromate dehydrate drinking water exposure of rats and mice (NTP 2007) in calculating RfDs. Inclusion of additional and all qualified studies is especially beneficial for better quantifying uncertainties and variations arising from different studies due to different study designs, strain/species of animals, and exposure regimens. As in a systematic review, studies meeting selection criteria should all be included for review and for analysis. Selecting a final RfD then becomes a risk management decision.

A2. Diffuse epithelial hyperplasia in the duodenum of female mice was selected as the critical effect for the RfD. Please comment on whether the selection of this critical effect is scientifically supported and clearly described. Please identify and provide the rationale for any other endpoints that should be selected as the critical effect.

Reviewer	Comments
Byczkowski	Diffuse hyperplasia, in itself, is considered to be a physiological (normal) response to several stimuli, as the cells of a hyperplastic growth remain sensitive to normal regulatory control mechanisms. Such a physiological proliferation of cells may be secondary to several pathological factors (e.g., the increased osmolality, changed pH, infection by Helicobacter, etc). Still, the proliferation in "diffuse hyperplasia" is a normal process - although, it may be generated in response to abnormal condition (in contrast to neoplasia, where the proliferation in itself becomes abnormal). On the other hand, the hyperplasia is a common early preneoplastic response to potentially carcinogenic stimuli. Considering its rather ubiquitous and nonspecific character, it is remarkable that even no single case of epithelial hyperplasia was found during the two-year study by NTP (2008) in duodena of as many as 100 male and female control mice. Taking this result at a face value, it may be assumed that the diffuse epithelial hyperplasia in duodena could be an early biomarker of oral exposure to Cr^{+6}, at least in the two-year NTP (2008) bioassay study. However, another end point - the pathological changes in the liver were noted in the same study with significantly increased frequency at the lowest dose employed. Even though they may be considered somewhat less "specific" than the epithelial hyperplasia (because they were observed at low frequency also in controls), but perhaps they could be more relevant to the systemic toxicodynamics of Cr^{+6} - appearing further away from the portal of entry then the hyperplasia and apparently being less sensitive to the potentially confounding effects of the reduced drinking water consumption.
Hamilton	See A1. above.
Nordberg	Perhaps some information on possible effects on the lung should be comment on. The document should give information about solubility of different chromium (VI) species should be given and specifically for the chromium species that have been used in the quoted studies. Soluble salts are mentioned on page 54 under 7 but soluble in what media is not mentioned. The reference WHO/IPCS (2006) Environmental Health Criteria 234, Elemental speciation in Human Health Risk Assessment, WHO, Geneva is recommended to be included.
Patierno	The selection of Diffuse epithelial hyperplasia in the duodenum of female mice as the critical effect for the RfD should be re-evaluated in light of the issues raised in my preceding comments. It must be considered in the context of the non-linear, dose-related issues discussed above regarding saturation of reductive capacity and definitive threshold data for toxicity and carcinogenicity.

Rossman	This seems like an appropriate choice, but it's outside my area of expertise.
Salnikow	Outside of my area of expertise.
Wise	This endpoint is a proper endpoint based on the available data, but is not necessarily a toxic outcome. To allow for better understanding, RfD's for other endpoints should be done and presented including some continuous endpoints. The EPA may conclude this endpoint is the critical effect, but this approach makes the analysis more transparent, open and clear.
Zhitkovich	The incidence of diffuse epithelial hyperplasia in the duodenum of female mice was the most sensitive histological response observed in Cr(VI)-exposed groups and therefore, it was appropriately selected as the critical effect for the RfD.
Zhu	EPA considered seven non-cancer endpoints for deriving RfDs (Table 5-1): chronic liver inflammation in female rats, histiocytic cellular infiltration in the liver of female mice, diffuse epithelial hyperplasia in the duodenum of the male and female mice, histiocytic cellular infiltration in the mesenteric lymph nodes of male and female mice, and cytoplasmic cellular alteration of acinar epithelial cells in the pancreas of female mice. All seven are quantal response from the NTP's 2-year chronic exposure study. The selections were largely driven by the dose-response data these effects exhibited. After dose-response modeling and the estimation of benchmark dose (BMD) for each of these select effects, diffuse epithelial hyperplasia in the duodenum of the female mice was chosen as the critical endpoint simply because it yielded the smallest BMD and its corresponding lower confidence limit (BMDL). It must be noted that the dose-response model for this critical effect was done only after deleting the two highest doses. As a result, the dose-response modeling relied on only three dose level (including the control), leaving little room for any flexible dose-response forms other than the "linear" multi-stage model with a polynomial of 1 degree of freedom. Instead of relying on a select "critical" effect, EPA could report a range of RfDs based on a set of qualified and select effects. As a result, EPA will be able to a range of RfDs, projecting the uncertainty and variation of RfDs arising from man y sources and affording risk management the opportunity to make an informed choice of a final RfD (NAS, 2010). This is important as EPA is moving towards enhancing analysis of uncertainty and variation in risk assessment. To this end, EPA could have benefited greatly by including additional endpoints from this principle study as well as other qualified studies. Potential candidates include histiocytic cellular infiltration in the duodenum of female rats and male mice, histiocytic cellular inflammation in pancreatic lymph nodes of male rats, and histiocytic cellular infiltration in the liver of female rats. EPS considered only the quantal responses in this Review for the purpose of computing RfDs. It is unclear why effects of continuous measurement scale were not considered. Many of these effects show unequivocal dose-response (e.g. Tables 4-12 and 4-13) and seem to be relevant to the hypothesized MOA of hexavalent chromium. The availability of EPA's software BMDS for dose-response modeling and benchmark dose computation makes it practical and useful to consider continuous effects for RfD derivation as well.

A3. Benchmark dose (BMD) modeling was applied to the incidence of diffuse epithelial hyperplasia in the duodenum of female mice to derive the point of departure (POD) for the RfD. Has the BMD modeling been appropriately conducted and clearly described? Is the benchmark response (BMR) selected for use in deriving the POD (i.e., a 10% increase in the incidence of diffuse epithelial hyperplasia) scientifically supported and clearly described?

Reviewer	Comments
Byczkowski	The benchmark dose modeling was applied in accordance with the *U.S. EPA Benchmark Dose Technical Guidance Document (2000)* in the prescribed manner. However, the modeled BMD, derived from the diffuse epithelial hyperplasia, was supported only by the three dose-points (including zero dose). In contrast, the pathological changes in the liver were consistently fitted, for both female rats and mice with Log-logistic model which included all the five data points (including controls), and thus, they could be considered to be robust dose-response adverse effects for derivation of BMD. So, the pathological changes in the liver may be used as alternative end points for derivation of BMD.
Hamilton	This reviewer is not an expert on modeling and cannot comment on A3 in detail.
Nordberg	Yes.
Patierno	See answer to A2 above.
Rossman	This does seem like the best and most complete study to use (but it's a bit outside my area of expertise).
Salnikow	Outside of my area of expertise.
Wise	The BMD modeling has been appropriately conducted and clearly described. To allow for better understanding, BMR modeling at 5% and 1% should be done and presented. This approach makes the analysis more transparent, open and clear.
Zhitkovich	BMD modeling and the calculations of the POD both appear to be appropriately performed.
Zhu	EPA should be commended for conducting BMD modeling for multiple effects with different model forms. For the modeling of the incidence of diffuse epithelial hyperplasia in the duodenum of female mice, EPA should provide a more detailed discussion on the limitation of the dose-response modeling (See A2). Uncertainties due to model choice, variation in the shape of seemingly equally well-fit models also can be quantified to a degree by considering multiple benchmark response levels (BMR) for each model. EPA used only BMR=10%. It makes perfect sense to also consider BMR=5% or even BMR=1% when such a choice is supported by the data. This is the general recommendation of EPA's own guideline (EPA, 2000). By doing so EPA would be able to quantitatively demonstrate uncertainty and variations due to the choice of different models

	and different BMR levels. Additionally, EPA should briefly but clearly define the BMD concept and methodology in an appendix to improve the readability for readers unfamiliar with the process.

A4. Please comment on the rationale for the selection of the uncertainty factors (UFs) applied to the POD for the derivation of the RfD. Are the UFs scientifically supported and clearly described? If changes to the selected UFs are proposed, please identify and provide a rationale.

Reviewer	Comments
Byczkowski	While the uncertainty factors (UFs) were applied in accordance with the U.S. EPA guidelines, the use of the UF = 10 for extrapolating the toxicity value based on effects in the portal of entry of Cr^{+6} in animals to predict GI effects in humans, seems to be problematic. Thus, both the reductive capacity of human gastrointestinal fluids and the antioxidant protection of human tissues exceed those in mice. So, adequately nourished and hydrated humans should be less vulnerable than mice to the adverse GI effects of oral exposure to Cr^{+6}, rather then 10 times more sensitive, as the U_A=10 may suggest.
Hamilton	No concerns regarding A4. Uncertainty factors are policy decisions, not scientific ones, and we can neither prove nor disprove any of the assumptions on which they are based nor can we accurately determine when and how such factors might be applied. Based on previous EPA doctrine, these seem to be consistent with previous applications.
Nordberg	The reason for chosen UFs is clearly and properly described. It is known that the reduction of hexavalent chromium to chromium three is influenced by vitamin C. This can perhaps be used in setting UFs and thus not only choose the standard UFs of 10 between species and 10 for interindividual differences. Humans can not synthesize vitamin C and are thus depending on vitamin C supplementation. The tested animals i.e., the mouse and the rat both produce vitamin C themselves. In this context a laboratory animal that resembles the human by being depended on vitamin C supplementation might be used in future studies. The concentration of vitamin C in tissues and organs are very important in evaluation of carcinogenic metals. It is likely to be involved in the mechanism in causing cancer and plays a role in the MOA.
Patierno	The Uncertainty Factors must be re-evaluated in the context of the non-linear dose-response data, the clear evidence of thresholds for toxicity and carcinogenicity and the fact that these high-dose, supra-saturation experiments cannot be extrapolated linearly to low or vanishingly small doses.
Rossman	This is outside my area of expertise.
Salnikow	Outside of my area of expertise.

Wise	The rationale for the selection of the uncertainty factors (UFs) applied to the POD for the derivation of the RfD are appropriate. It was suggested that an UF for children and those with different conditions be used. People with different conditions (e.g. antacid use) are already contemplated in the UF applied for interindividual variation. Currently, such a factor for children is not included in the EPA guidelines. It could be that the same interindividual variation may apply in that case as well.
Zhitkovich	Two UFs were applied: UF=10 for interspecies extrapolation to humans and UF=10 for interindividual variability in the human population. The interspecies UF was used because there is no available information to quantitatively assess the true differences in chromium-6 toxicokinetics and toxicodynamics between humans and laboratory rodents. There are, however, two biological factors that point to a potentially greater sensitivity of humans relative to mice. The first is related to the fact that telomerase was shown to suppress genetic damage by chromium-6 (Glaviano et al. 2006). All mouse cells express telomerase while only stem cells retain telomerase expression in human tissues. The second factor is the difference in ascorbate metabolism. Human cells actively recycle ascorbate (Nualart et al. 2003, Montel-Hagen et al. 2008), resulting in ~100 times lower requirements for this vitamin by humans relative to rodents. A more economical use of vitamin C by humans also results in lower ascorbate concentrations in the extracellular fluid (for example, as reported for bronchoalveolar lavage fluid by Slade et al. 1993), which would more rapidly detoxify chromium-6 via extracellular reduction. However, No specific information about ascorbate concentrations in the extracellular environment of the duodenum and jejunum of mice and humans is currently available. The interindividual variability in sensitivity to chromium-6 was not studied and the application of the safety coefficient (UF) is definitely appropriate in this case. However, the proposed UF=10 likely underestimates the range of the interindividual variability. The Draft has a brief discussion on the common presence of genetic polymorphism in DNA repair genes as one source of interindividual differences. Four major DNA repair pathways (mismatch repair, nucleotide excision repair, base excision repair and homologous recombination) are known to impact the extent of genetic damage and cytotoxicity by Cr(VI), and the use of UF=10 to account for interindividual differences in the overall DNA repair would assume a quite low degree of variability for each repair process (overall 10-fold variation would result from a very narrow 1.8-fold variation in each process: $1.8^4 = 10.5$). Chromium-6 toxicity can be affected on three levels: 1) differences in extracellular detoxification, 2) differences in cellular uptake and 3) differences in cellular/genomic defense mechanisms. A 5-fold variation at each stage would give a potential 125-fold variation in the general population. A study by Donaldson and Barreras (1966) showed that individuals with pernicious anemia had 4-times higher systemic uptake of chromium-6 due to its lower detoxification in the stomach. Widespread use of antacid medications has a clear potential to diminish reduction rates of chromium-6. No systematic studies on potential variations in chromium-6 uptake have been performed yet, but two human lung carcinoma lines, H460 and A549, displayed a 5-fold difference in chromium-6 accumulation (Macfie et al. 2010). A caveat of using information from these two cell lines is that they are malignant and therefore it is not possible to determine whether their

	differences were present in the initial normal cells or whether it is a side effect of different transformation processes. The Draft correctly stated on p.214 that there is no information about susceptibility of children to chromium-6 toxicity. In this case, it would be clearly appropriate to use additional UF=10 to account for a potential early life susceptibility. If EPA considers it unnecessary, then the exclusion of this UF should be justified in Section 5.1.3. My recommendation would be to use UF=100 to account for the combined effects of the interindividual variability in susceptibility and early life exposures.
Zhu	The use of uncertainty factors (UFs) in this review is well described and is consistent with EPA's guidance documents for RfDs. Exposure in earlier stage of life was discussed.

(B) Carcinogenicity of Hexavalent Chromium

B1. Under EPA's 2005*Guidelines for Carcinogen Risk Assessment* (www.epa.gov/iris/backgrd.html), hexavalent chromium is *likely to be carcinogenic to humans* by the oral route of exposure. Is the cancer weight of evidence characterization scientifically supported and clearly described?

Reviewer	Comments
Byczkowski	Even though the U.S. EPA (2005) guidelines were applied appropriately, there is no direct evidence of dose-dependent carcinogenicity by orally administered Cr^{+6} in humans (C.f. P. 236, L# 33: "*...EPA concluded that the exposure-response analyses presented by Zhang and Li (1997), Beaumont et al. (2008), and Kerger et al. (2009) are not based on the quality of data that is needed to support a conclusion regarding the presence or absence of a dose-response among the observed cancer rates in these villages. The other epidemiologic studies did not find a significant correlation between hexavalent chromium concentrations in drinking water (or proximity to the source of hexavalent chromium soil contamination) and cancer...*") The classification of hexavalent chromium as "*likely to be carcinogenic to humans by the oral route of exposure*" is based on carcinogeniesis observed only at the highest dose levels employed in animal studies. Therefore, it may, or may not be relevant to humans at environmentally relevant exposure concentrations.
Hamilton	Regarding B1. and as outlined in the detailed comments under G1. and G2., this reviewer is concerned that the evidence for carcinogenicity is not strong in animals and is not supported by human epidemiology. As also noted in comments under G1. and G2., there is considerable concern with statements by EPA in sections of this draft, particularly under Major Conclusions in Chapter 6, regarding evidence for human carcinogenesis based on a single human epidemiology study that in turn is a re-analysis of another study that lacks critical information that would be useful in fully assessing relative cancer risk. However, based on the current criteria for selection of this designation, it appears to be

	consistent with EPA doctrine since there is evidence of increased tumors in animals under certain exposure conditions.
Nordberg	Though it is an American document prepared for US I would recommend also to consult and cite documents published by the United Nations Organizations such as International Agency for Research on Cancer (IARC) and World Health Organization (WHO). Recommended literature is IARC Monographs on the Evaluation of Carcinogenic Risks to Humans Volume 49 (1990), Lyon, France and Chromium, Nickel and Welding 1-677 pages and IARC Monographs on the Evaluation of Carcinogenic Risks to Humans Volume 100 which is in preparation and further information on www.iarc.fr Hexavalent chromium is classified as a human carcinogen. It is not clear to the reader why classification can be different upon different route of exposure.
Patierno	The cancer weight of evidence characterization is not scientifically supported. The conclusion that hexavalent chromium is "likely to be carcinogenic to humans by the oral route of exposure" is not scientifically supportable given the issues raised in my comments above. The non-linear dose data in both the NTP studies and the data preliminarily discussed in the Public Comments clearly demonstrate that the toxicities and carcinogenesis observed at these extremely high, obviously supra-saturating doses, cannot and should not be extrapolated to lower doses. See detailed comments above.
Rossman	Given the fact that there is not enough human data to firmly establish carcinogenicity, but there is animal data, "likely to be" is reasonable but "possibly carcinogenic at high dose" would be more accurate.
Salnikow	Hexavalent chromium has been classified by IARC as carcinogenic to humans (group 1) via inhalation route of exposure based on results obtained in human and animal studies. However, when animals were exposed to hexavalent chromium in drinking water the carcinogenic effects were observed only at very high doses, which are irrelevant to human exposure. These results seems to cast doubt on carcinogenicity of hexavalent chromium via oral route of exposure and yet as I already pointed out in G2 the reason for only high chromium doses producing carcinogenic effect may be stemming from the inappropriate animal models which have higher protective ascorbate levels as compared to humans. Although, more human and animal studies are required to make an informed conclusion the ability of hexavalent chromium to produce tumors makes it likely to be carcinogenic by oral exposure. Another important consideration is that hexavalent chromium is a co-carcinogen and consumption of water with other toxic or carcinogenic compounds will result in unraveling chromium carcinogenic effects at much lower doses. Additionally, people with chronic inflammation of digestive tract could be more susceptible to chromium-induced carcinogenesis.
Wise	The general lack of accuracy in the document in its handling of citing, paraphrasing and considering the underlying literature is of some concern in this presentation. There is a lot of cell culture and animal data showing genotoxicity and clastogenicity, however, the

	motivation for these studies were largely inhalational exposure-induced cancer. It seems if they can support one route, they could support the other, but it functionally means the data underpinning the oral route of exposure is the one NTP study. That NTP study is flawed because only very high doses were considered in the study, thus, there is significant concern that it may not reflect events at lower doses. The EPA requirement for a "likely to be carcinogenic to humans" classification defined as "…appropriate when the weight of the evidence is adequate to demonstrate carcinogenic potential to humans but does not reach the weight of evidence for the descriptor "Carcinogenic to Humans." It is understandable why an initial assessment of "likely to be carcinogenic" was chosen as the guidance states: " Supporting data for this descriptor may include: … an agent that has tested positive in animal experiments in more than one species, sex, strain, site, or exposure route, with or without evidence of carcinogenicity in humans". The next possible descriptor is "suggestive evidence of carcinogenic potential". It is indicated as "…appropriate when the weight of evidence is suggestive of carcinogenicity; a concern for potential carcinogenic effects in humans is raised, but the data are judged not sufficient for a stronger conclusion." It is also said to cover "…evidence associated with varying levels of concern for carcinogenicity, ranging from a positive cancer result in the only study on an agent…". Thus, there is conflicting guidance for Cr(VI) on these descriptors. On the one hand, there is a study of multiple species and genders possibly qualifying it for a descriptor of "likely to be carcinogenic to humans". On the other hand there is only one study showing this outcome making it suitable for a descriptor of "suggestive evidence of carcinogenic potential". In studying the data and descriptors, it appears to be premature to conclude that the weight of the evidence is nearly adequate for demonstrating carcinogenic potential to humans. Moreover, in considering the spirit of the guidelines for the two descriptors, it is clear that "likely to be carcinogenic" descriptor is contemplating that the data concerning multiple species, genders, strains etc. will come from multiple studies not just one. It is also clear that the "suggestive evidence of carcinogenic potential" descriptor is intended for a database with flaws. This database is flawed by the lack of multiple studies and the fact that the one study available relied on very high doses. Accordingly, a descriptor of "suggestive evidence of carcinogenic potential" is more appropriate at this time. However, the EPA is in the unique position to soon have another study that repeats the NTP study is almost completed. The EPA should wait for the final results of that study to make the most informed analysis. If it too shows tumors at all doses, then the stronger descriptor would be justified.
Zhitkovich	The classification of hexavalent chromium as "likely to be carcinogenic to humans" via the oral route of exposure is supported by evidence of its tumorigenicity in the oral cavity of female and male rats and in the small intestine of female and male mice. An increased incidence of stomach cancers in the JinZhou area (China), which was contaminated with high concentrations of chromium-6 in drinking water, is supportive of the selected classification. Even if the ecological study from China is excluded, the weight of evidence

	from animal studies is adequate to designate hexavalent chromium as "likely to be carcinogenic to humans" via the oral exposure.
Zhu	The descriptor of "likely to be carcinogenic to humans" for hexavalent chromium is consistent with EPA's Guidelines for Carcinogen Risk Assessment. EPA gave a clear description of the hypothesized MOA.

B2. A mutagenic mode of carcinogenic action by all routes of exposure is proposed as the primary mode of action for hexavalent chromium. Please comment on whether this determination is scientifically supported and clearly described. Please comment on data available for hexavalent chromium that may support an alternative primary mode of action.

Reviewer	Comments
Byczkowski	The positive laboratory results of mutagenicity tests do not prove genotoxicity and are not necessarily biologically relevant to humans exposed *in vivo* to the environmentally relevant concentrations of Cr^{+6}.The mutagenicity results from humans occupationally exposed to Cr^{+6} (Table 4-25) were inconsistent (this document states - C.f. P. 178, L# 14: "*...In general, associations between hexavalent chromium exposure and mutagenicity in workers are uncertain...*") and actually, the results presented in the document did not prove the direct genotoxic mode of action of Cr^{+6} i*n vivo*. An alternative, indirect mode of action seems plausible to this reviewer. As discussed in Section 4.5.2., the intracellular products of one-electron reduction of Cr^{+6} *in vivo*, causing oxidative stress and free-radical damage to cellular macromolecules, seems to be responsible for initiating carcinogenicity at relatively low dose levels. Erosion of GI tract mucosa with inflammation that followed at high Cr^{+6} dosage (discussed in section 4.2.1) apparently caused promotion in the lesions, which eventually progressed to benign and malignant tumors. Importantly, such a mode of action implies a threshold phenomenon, *i.a.* due to antioxidant protection of cells.
Hamilton	There is considerable evidence that Cr(VI) is genotoxic in cell culture and in vitro, and under certain extreme conditions it can also be shown to be mutagenic. However, there is far less support that Cr(VI) is genotoxic or mutagenic in vivo by the oral route of exposure at doses of relevance to humans; conversely, there is considerable evidence that there are protective threshold mechanisms that significantly impact the ability of Cr(VI) to reach target tissues and cause DNA damage under physiological conditions. In addition, while alternative mechanisms are briefly discussed, these are essentially dismissed without extensive treatment. As noted in the document, there is not a large literature on alternative mechanisms, but this is largely because, since the discovery of the genotoxic potential of Cr(VI) some forty years ago, most of the field has only focused on this one aspect, and I suspect it would be very difficult to get peer-reviewed funding to study non-genotoxic mechanisms for this toxicant. It is important to note, however, that there are no reports of increased skin cancer under occupational exposure settings, despite the fact that workers until the past few decades were directly exposed to Cr(VI) on the skin to the

	extent that they formed "chrome holes" that eventually healed. Yet this direct application and clear signs of chromium reduction and toxicity directly on the skin produced no increased skin cancer risk. Likewise, the occupational exposure literature only recently investigated the role of smoking status in chromium-related lung cancer risk. The increased risk of lung cancer associated with Cr(VI) exposure is modest considering the exposure levels and duration of exposures that span decades, and virtually all of the cancer cases in the epidemiology studies were seen in smokers, suggesting an interaction but one that is very modest. This in turn suggests alternative mechanisms such as inflammation, oxidative damage, damage-induced proliferation, and other mechanisms not directly tied to the ability of Cr(VI) to enter cells and damage DNA. These should considered and explored in more detail, since they are the basis for many of the assumptions regarding the risk of cancer from oral exposure to Cr(VI).
Nordberg	Again this might be linked to differences among hexavalent chromium species regarding for example solubility in body fluids. Somewhere in the document it should be pointed that iron can reduce hexavalent chromium to chromium three. This has been done in some products. This is touched by on page 48 line 18. The document describes in detail the possible MOA. IARC 1990 stated "… chromium (VI) compounds on the basis of the combined results of epidemiological studies, carcinogenicity studies in experimental animals, and several types of other relevant data which support the underlying concept that chromium(VI) ions generated at critical sites in the target cells are responsible for the carcinogenic action observed" However, also alternatives like DNA- methylation and other epigenetic mechanisms should be considered, because for many metals DNA-methylation is recognized as a possible mode of action also addressed as mechanism for carcinogenicity. Effects on cell signalling and gene expression may also serve as mechanisms involved in carcinogenesis of metallic compounds. See further discussion in review by Davidsson et al, Chapter 5 in Nordberg et al (eds) Handbook on the Toxicology of Metals, p79-100
Patierno	The determination of a mutagenic mode of action by all routes of exposure should be re-evaluated before proposing it as the primary mode of action. Nearly all indices (NTP studies, inhalation studies, mammalian cell mutagenesis studies etc) indicate that carcinogenicity of CrVI is only observed under exposure conditions that evoke cellular toxicity, inflammatory tissue damage, and tissue regeneration. The DNA damage and presumed mutagenicity (actually epigenetic or stochastic selection of cells that survived toxicity) of CrVI is only observed at doses that also cause cell death and tissue damage. In vivo, these effects are only achieved at very large doses that clearly overwhelm the reductive capacity of the oral cavity, stomach and blood components resulting in a sharp threshold of carcinogenesis only at the two highest NTP doses. I have spent more than 25 years studying the molecular mechanisms of CrVI genotoxicity and mutagenesis and I have a deep appreciation for its capacity to interact with cells and alter DNA and DNA replication and transcription. However, just because CrVI is capable of causing DNA damage and what we thought was "mutagenicity" (see above) in carefully contrived experimental systems, does not mean that it does so under physiologically and

	environmentally relevant conditions. It is much more likely that the chronic tissue damage, with accompanying inflammation and subsequent proliferative regeneration, possibly in the presence of unrepaired DNA damage, all of which are only observed at the highest doses, is the principle mode of action.
Rossman	By definition, the "mode of action" (MOA) of a carcinogen is "a sequence of key events and processes, starting with interaction of the agent with a cell, proceeding through operational and anatomical changes, and resulting in cancer formation" (USEPA, 2005). For mutagenesis to be a carcinogenic MOA, the agent must at the very least cause heritable mutations in mammalian cells. The mutations should be induced in a concentration range with low toxicity (preferably similar to concentrations seen in human exposures), and the mutations should be induced in the target tissues in animal experiments and in humans. Human and animal tumors should also show genetic alterations consistent with the types of mutations induced by the agents, and these should be early events. The information about Cr(6) is lacking for much of these criteria. In fact, the human tumor data support an epigenetic mechanism more than a mutagenic one. Genotoxic is not the same as mutagenic, and sections 4.5 and 4.73 must be completely rewritten, as they consistently confuse these terms. Standard genotoxicity assays were not designed to inform specific modes of tumor induction. With the exception of mutagenesis, these other assays (non-mutagenic assays) do not measure heritable events, but rather measure evidence of DNA damage or its repair. Non-mutagenic assays include chromosome aberrations, micronuclei, comet assays, DNA lesion measurements, and DNA repair assays. These assays are useful for hazard identification or as biomarkers of exposure. They provide only supportive evidence that mutagenesis might be a MOA. DNA damage *per se* does not inform us about eventual heritable change, which is the true issue. Most (but not all) mutagens cause heritable changes in DNA sequences by causing damage to DNA (pre-mutagenic lesions) that is converted to mutation after cell division. Table 4-21 should be deleted, as results in bacteria are not relevant to tumorigenic MOA. A simple statement that Cr(6) is mutagenic in bacteria should suffice (referencing a review such as Klein CB. "Carcinogenicity and genotoxicity of chromium" In: Toxicology of Metals (Chang LW, ed). Boca Raton, FL:CRC Press, 1996, pp.205–219. Table 4-22 represents positive results in a group of assays that measure both mutagenic and non-mutagenic endpoints (including a paper on epigenesis, Klein et al., 2002, which should be removed, as this is not a genotoxic event; neither is disruption of mitosis, which can have many causes). Table 4.22 has neither information on the concentrations inducing positive results, nor on the toxicity of those treatments. This table is also deficient in the most important results in mammalian cells, i.e. mutagenesis. All of the studies reported are mouse lymphoma cell studies, yet later in the document (page 203), reference is made to mutations at the HGPRT locus in "Chinese Hamster ovary cells (V79 and AT3-2)" V79 is not a Chinese hamster ovary cell, it is a Chinese hamster lung fibroblast. In fact, one such study using V79 cells (Sugiyama et al., Mutat. Res. 260:19-23, 1991) shows a modest positive effect only in a narrow concentration range. A CHO line (AA8) showed a small increase (3.4 fold) at a dose

	giving 75% survival (Brooks et al., 2008). There is a fuller discussion of chromate mutagenicity (with references) in: Klein CB. "Carcinogenicity and genotoxicity of chromium" In: Toxicology of Metals (Chang LW, ed). Boca Raton, FL:CRC Press, 1996, pp.205–219. This article points out that chromium compounds are mutagenic in a narrow dose range, possible because of persistent toxicity after treatment (e.g. residual toxicity was seen a week after treatment of V79-derived G12 cells). Chromosome aberrations and DNA strand breaks can occur as a result of cytotoxicity. Dead cells do not become tumors. Unless assays for cytotoxicity are performed, it is not possible to know whether DNA damage occurs in cells that can replicate to form clones. Traditional cytogenetic assays rely on short-term cell survival to generate the mitotic figures necessary for analyses; the long-term viability of these treated cells cannot be determined. Thus, the relevance of this kind of data for carcinogenic MOA is questionable. To measure cytotoxicty, the gold standard is clonal survival, a method that is common in gene mutation assays, but not in other genotoxicity assays. Short-term survival assays, such as MTT, neutral red, and trypan blue, as well as measurements of mitotic index that are commonly used in cytogenetic assays, fail to detect early or delayed apoptotic events. Trypan blue detects only necrosis. MTT and neutral red assays can be delayed to allow time for apoptosis to develop, at which point the results approach clonal survival (Komissarova et al., Toxicol. Appl. Pharmacol. 202:99–107, 2005). As mentioned above, Cr(6) causes delayed residual toxicity (Klein et al. Environ. Health Perspect. 102 (suppl 3):63-67, 1994), and thus clonal survival or at least apoptosis at later times after exposure, are essential in establishing cytotoxicity levels. Normal human fibroblasts show ~80% loss of clonality after a 25h exposure to 2 µM sodium chromate [Vilcheck et al., Environ. Health Perspect. 110 (Sup5):773-777, 2002]. Micronuclei can result from DNA damage or from malsegregation of chromosomes. It has been recommended that this assay should be performed under conditions of high survival (an increase of >90% in number of viable cells) and that markers for apoptosis and necrosis be included [Kirsch-Volders, et al. (2003) Report from the in vitro micronucleus assay working group. Mutat. Res. 540:153-163]. In the case of Cr(6), at lower concentrations, most of the micronuclei are kinetecore-positive, meaning that they arise from malsegregation and not DNA strand breaks (Seoane and Delout, Mutat. Res. 490:99-106, 2001; Figgitt et al., Mutat. Res. 688:53-61, 2010). Those that are kinetecore-negative (arising from chromosome breaks) occurred only at the highest concentrations. Thus, Cr(6) induces aneuploidy rather than DNA damage at lower concentrations (Holmes et al., 2010; Figgitt et al., Mutat. Res. 688(1-2):53-61, 2010). Aneugenesis is caused by alterations in proteins, not DNA, and has thresholds. It should also be noted that the mouse lymphoma assay (MLA), chromosome aberration assay (CA) and micronucleus assay (MN) give a large number of false positives, even compared with the Ames test. Chemicals that are non-carcinogenic after thorough testing in both male and female rats and mice are often positive in these assays (Kirkland et al., Mutation Research 584 (2005) 1–256). The Comet assay detects single and double strand DNA breaks as well as alkali-labile sites. Nucleotide excision repair (NER) and base excision repair (BER) of adducts can create breaks as intermediates. Single strand breaks are quickly repaired and are not regarded as significant premutagenic lesions. During apoptosis, DNA fragmentation into

segments of 180 base pairs occurs, whether or not the apoptosis was induced by a genotoxic event (Choucroun et al. 2001, Mutat. Res. 478:89-96; Henderson et al.,.1998, Mutagenesis 13:89-94.) Necrotic cells also display DNA damage (Fairbairn et al., 1996 Scanning 18:407-416.). In order to avoid false positive responses, Henderson et al. (1998) suggests that the concentration of test substance should produce >75% viability.

In summary, standard genotoxicity assays from hazard identification exercises cannot be used to establish a mutagenic MOA, because these assays do not measure heritable events and because the doses used in such assays are usually too high.

Other MOA's have not been adequately considered. These include, for example, selection for Cr-resistance, resistance to apoptosis, and aneuploidy. The evidence for a mutagenic MOI is weak. Mutations can result from DNA damage, but can also be a secondary effect of the loss of mismatch repair, aneuploidy, and other types of genomic instability (in other words, it is a later effect). With the exception of the mouse lymphoma system, Cr(6) is only weakly mutagenic in mammalian cells, rarely giving more than a 3-fold increase in mutant fraction over background levels (in endogenous genes), and in a very narrow (and toxic) dose-range with a strong threshold (reviewed in Nickens et al., 2010).

In some cases the "mutations" have been shown to be epimutations resulting from altered DNA methylation (Klein et al., 2002). Since none of the other studies on mammalian cells looked for epigenetic inactivation, this calls into question whether the "mutants" seen are really mutants. These are important considerations for the MOA of Cr(6), since cells grown in the presence of Cr(6) show selection for cells with inactivated mismatch repair (MMR) genes. These cells are Cr(6)-resistant and could be the result of either mutation or epigenetic inactivation (reviewed in Salnikow and Zhitkovich, 2008). Cells with epigenetically inactivated MLH1 (a MMR gene) were seen in human lung A549 cells exposed to Cr(6) (Sun et al., Toxicol. Appl. Pharmacol. 237:258-266, 2009). MMR-deficient cells are mutators (having a high spontaneous mutation rate) and show microsatellite instability. An important consideration for MOA is the fact that chromium-induced lung cancer cells also show epigenetically-inactivated MMR genes (Takahashi et al., 2005). Also against the idea of a mutagenic MOI is the fact, discussed in Salnikow and Zhitkovich (2008), that Cr-induced lung tumors in humans lack p53 mutations, in contrast to lung tumors associated with other agents such as tobacco smoke, and the fact that the few mutations found do not correspond to the types of mutations caused by Cr in *in vitro* systems. The fact that Cr is essential also implies that oral Cr(VI) could supply the necessary Cr, again implying a threshold at nontoxic doses. Also, experiments from the Costa laboratory (Davidson et al.,Toxicol. Appl. Pharmacol. 196:431-437, 2004; Uddin et al., Toxicol. Appl. Pharmacol. 221:329-338, 2007) showing that chromate in drinking water is a cocarcinogen with solar UV, and the implications of this finding, are not discussed.

Other problems (by page):

Page 176, top of the page, is a good example of the confusion between mutagenicity and other endpoints. The statement "Hexavalent chromium-induced mutagenicity has been demonstrated following oral exposure" is misleading. There is only one mutagenicity assay showing positive effects on eyespots (presumed deletions) in offspring of female rats given drinking water with 62.5 mg Cr(6)/L. The deletions were not confirmed, so the

	eyespots might be epigenetic events (as Klein found with so-called mutants). All of the other assays are for non-mutagenic endpoints, and tend to be negative for drinking water exposure, but positive for gavage (a more toxic type of exposure). Page 178, 4.5.1.2, it is claimed that mutagenicity has been evaluated in humans experimentally exposed to hexavalent chromium. No such studies appear in Table 4-25. The paragraph mistakenly refers to mutagenicity many times. Page 186: It is not ER (excision repair) that is responsible for removal of bulky lesions, but NER (nucleotide excision repair) that is. Reynolds et al. 2004 is missing in references. Page 187, bottom. It doesn't make sense that mice given 0 mg/kg Cr(6) should have a significant level of apoptosis (compared to what?). Page 188: The Dai et al 2009 paper does not measure mutation frequency in human cells. Page 190: 3rd paragraph: The mutational spectrum of chromate is not clear. See the review by Klein referenced above. Page 202: Key Events, #3: The authors skip from discussing mechanisms of DNA damage by Cr to "overall genomic instability which can lead to mutations if not adequately repaired". Genomic instability can occur as a result of other factors besides DNA damage, and genomic instability is not repaired (DNA damage can be, but the repair often leads to apoptosis). As discussed above, toxic exposure would play a role in the selection of Cr-resistant and/or apoptosis-resistant cells. There is no obvious tie-in here between DNA damage and mutagenesis as a key event, since cells resistant to Cr could have arisen by epigenetic silencing (and this may also be a mechanism in resistance to apoptosis). In a sense, this point is recognized in #4, but in postulating apoptosis as a key event, the authors do not seem to realize the implications, i.e. that a toxic dose is needed for carcinogenicity. They suggest that selection for resistance to apoptosis is due to mutations (either pre-existing or Cr-induced) but there is no evidence for this. Besides altered DNA methylation, other mechanisms for the appearance of Cr(VI)-resistance in exposed cells include epigenetic effects via altered histone modification (Sun et al., Toxicol. Appl. Pharmacol. 237:258-266, 2009) as well as reduction of Cr(VI) transport via down-regulation of sulfate ion transporter activity, and resistance to apoptosis via altered gene expression (upregulation of survival pathways and down-regulation of apoptotic pathways) (discussed in Nickens et al., 2010) p. 204-210: What is described as "mutagenicity" is not in all cases. p. 206, end of paragraph 1: It is claimed that there is evidence that Cr(6) induces mutagenicity in tissues at the site of entry and systemically at doses relevant to human exposure. Where is the evidence for this? p. 206, bottom: Again, it is important to note that DNA damage can lead to apoptosis as well as mutation, so it does not necessarily support a mutagenic MOA. p. 207: De Flora et al., 2008, did not look for mutagenesis, they looked for DNA damage.

	p. 208: Neither O'Brien et al., 2005 nor Eastmond et al., 2008 is in references. p. 209: Low (non-toxic) concentrations would not provide selective pressure for Cr-resistant, mismatch repair deficient cells. Especially in vivo, toxicity would be the driving force to stimulate the outgrowth of such cells. p. 209, 2nd. paragraph: This is a lot of speculation (should be, may be). p. 211, bottom: It is claimed that the study by NTP found no evidence of tissue damage or necrosis. Did they look for apoptosis? It is also claimed that most available studies found Cr-induced genetic damage at doses below those that inducing cytotoxicity. This has not been demonstrated, since clonal survival (the only assay that will detect delayed toxicity) was not performed in these studies. p. 212, end: "…giving rise to mutagenicity (including DNA adduct formation, DNA damage, gene mutations, chromosomal aberrations and micronuclei formation" This is nonsense, as is the next sentence. There is no evidence for mutagenicity at the target tissue at all. p. 213: More confusion between mutagenicity and other endpoints. It is not true that other hypothesized MOA's have not been demonstrated. There is actually more tumor evidence for an epigenetic MOA. Thus, the weight of evidence favors the alternative. This is not a numbers game. Epigenetic studies are relatively new, compared with DNA damage and other "genotoxic" studies, so there are fewer studies. p. 214 top: Has EPA concluded this? Then what is the purpose of reviewing this document? p. 238, bottom: More confounding of mutagenesis and other endpoints. <u>Additional papers showing epigenetic effects of Cr</u> Schnekenburger et al., (2007) Chromium cross-links histone deacetylase1-DNA methyltransferase1 complexes to chromatin, inhibiting histone remodeling marks critical for transcriptional activation. Mol. Cell Biol. 27(20): 7089-101. Sun et al. (2011) Comparison of gene expression profiles in chromate-transformed BEAS-2B cells. PloS ONE 6(3): e17982. Doi:10.1371/journal.pone.0017982. Ali et al. (2011) Aberrant DNA methylation of some tumor suppressor genes in lung cancers from workers with chromate exposure. Mol. Carcinogenesis 50(2):88-99.
Salnikow	**MOA. Genotoxic effect of hexavalent chromium.** The draft of the Toxicological review provides a substantial body of information regarding the mutagenic potential of hexavalent chromium and concludes that hexavalent chromium is carcinogenic by a mutagenic MOA. Indeed this topic has been studied extensively. The results of in vitro and in vivo studies provide substantial evidence for the mutagenic activity of hexavalent chromium, which is mediated through the generation of the highly reactive chromium intermediates penta- and tetravalent chromium, reactive oxygen species, and trivalent chromium formed during the intracellular reduction of

	hexavalent chromium. These chromium and oxygen species can react with DNA, leading to oxidative DNA damage, chromium-DNA adducts, DNA strand breaks, and chromosomal aberrations. Despite these studies, the significance of chromium-induced DNA damage in the mechanisms of chromium carcinogenicity is not clear. If DNA damage/mutations are important and a causative factor in chromium-induced carcinogenesis, then these mutations should be frequent in chromium-induced tumors. However, very few publications exist in this respect. In animal studies, De Flora et al. (De Flora et al., 2008) found no evidence of DNA-protein crosslinks and DNA adducts in the duodenum following drinking water chromium exposures. Other available hexavalent chromium drinking water exposure studies that measured mutagenicity in mice also could not detect evidence of micronucleus induction in the blood or bone marrow (Mirsalis et al., 1996; De Flora et al., 2006; De Flora et al., 2008). Analysis of lung cancers from chromate-exposed workers revealed that p53 mutations are not very frequent, with only six missense mutations identified in 4 (20%) of the 20 chromate lung cancer samples (Kondo et al., 1997). There were fewer mutations in the patients with lung cancers who had been exposed to chromate than in those who had not (20% vs about 50%). This study also revealed that there was no association between p53 mutations and the period spent working in chromate factories. It is conceivable that chromium causes genotoxic effects, damage DNA, but this damage is efficiently repaired and do not play any role in carcinogenic effects of chromium. Thus, in order to confirm or refute a possible role of chromium genotoxic/mutagenic effects in chromium-induced carcinogenesis, comprehensive analyses of mutations in oncogenes and tumor suppressor genes in experimentally induced tumors in animals should be done first, followed by more detailed sequence analyses of chromium-exposed human tumors. A feasible study that could be done in a short period of time (assuming that tumor samples collected in the NTP studies are stored and frozen) is **exon only global sequencing** of DNA from chromium-induced rat tumors versus spontaneous tumors (several spontaneous tumors of different origin were observed in NTP 2008 study). These studies will allow comparison of tumor driving mutations versus passenger mutations. **MOA, Epigenetic effects of hexavalent chromium.** Global changes in the epigenetic landscape are a hallmark of cancer. The initiation and progression of cancer, traditionally seen as a genetic disease, is now realized to involve epigenetic abnormalities along with genetic alterations. Recent advancements in the rapidly evolving field of cancer epigenetics have shown extensive reprogramming of every component of the epigenetic machinery in cancer including DNA methylation, histone modifications, nucleosome positioning and non-coding RNAs, specifically microRNA expression (Sharma *et al.*, 2010). Epigenetic effects have also been observed following hexavalent chromium exposure (Salnikow and Zhitkovich, 2008; Arita and Costa, 2009). Increased DNA methylation was observed in the promoter region of the tumor suppressor gene p16 and the MMR gene hMLH1, indicating that chromium can induce epigenetic effects (Takahashi *et al*., 2005; Kondo *et al.*, 2006). Gene transcription has also been shown to be affected by exposure to hexavalent chromium in vitro via epigenetic mechanisms. Sun et al. (Sun *et al.*, 2009) found alterations in the levels of histone methylation in human lung A549 cells exposed to hexavalent chromium, indicating the capability of these exposures to lead directly to changes in gene expression.

	Taken together, these studies suggest that epigenetic mechanisms may contribute to the carcinogenicity of hexavalent chromium. However, it is not clear whether epigenetic changes produced by chromium exposure are acting alone or linked to chromium genotoxic effects and that both genetic and epigenetic changes are essential for tumor appearance and evolution. More research is needed in this area including the identification of changes in DNA methylation (analyses of frequency of inherited silencing of tumor suppressors in tumors and in miRNA expression patterns following chromium exposure in animal models and humans before any conclusions can be drawn regarding the role of epigenetics in the carcinogenic effects of hexavalent chromium. **MOA. Co-carcinogenic effects of chromium.** Co-carcinogenic effects of hexavalent chromium were reviewed recently (Salnikow and Zhitkovich, 2008). The majority of occupational and probably all environmental exposures to hexavalent chromium occur as co-exposures with other carcinogens. The most common examples of co-exposures occur among stainless steel welders, and among hexavalent chromium-exposed workers who are also smokers. Two reports from the Costa Lab (Davidson *et al*., 2004; Uddin *et al.*, 2007) provided strong experimental data demonstrating that hexavalent chromium can act as a potent co-carcinogen for UV-induced skin tumors. In both studies, the presence of hexavalent chromium in drinking water caused dose-dependent increases in the frequency of skin tumors in UV-irradiated hairless mice. Hexavalent chromium alone produced no tumors, indicating that it acted a strong enhancer of UV-initiated tumorigenesis. Supplementation with vitamin E or selenomethionine had no effect on hexavalent chromium-mediated enhancement of skin carcinogenesis suggesting that co-carcinogenic effects were not oxidant-mediated. It is noteworthy that the level of chromium in skin directly exposed to UV had significantly higher levels of chromium than underbelly skin that was not directly exposed to UV in mice exposed to UV and 5 ppm K2CrO4 ($P < 0.05$) (Davidson *et al.*, 2004). This raises an interesting question, does inflammation, whatever the source, facilitate chromium accumulation or delay chromium clearance? This is an important and understudied area. The identified co-carcinogenic effects of hexavalent chromium raise an intriguing possibility that much lower doses of chromium could be hazardous under certain circumstances when exposure to chromium in drinking water is combined with other harmful exposures. Another important area of research is an understanding of the role of Inflammation/colitis in hexavalent chromium carcinogenesis. It is well know that at least 20% of all cancers arise in association with infection and chronic inflammation and even those cancers that do not develop as a consequence of chronic inflammation, exhibit extensive inflammatory infiltrates with high levels of cytokine expression in the tumor microenvironment. Aberrant activation of NF-κB and/or STAT3 is found in over 50% of all cancers and renders premalignant and fully transformed cells resistant to apoptosis and speeds up their rate of proliferation, thereby increasing tumor growth. It is extremely important to test whether hexavalent chromium will be more carcinogenic and at lower doses in animals in which colitis was induced, for example by sodium dextran sulfate. **MOA. Interference with iron metabolism.** In rats neoplastic changes were found at sites of tissue contacts with the highest concentrations of hexavalent chromium, i.e. oral cavity. This may be explained by the

	immediate damaging effects of chromium on DNA and other cellular components. At the same time frequent nonneoplastic changes were observed in duodenum of male and female rats and neoplastic changes were observed in duodenum of male and female mice. These data cannot be explained by the direct effect of hexavalent chromium, which should be mostly reduced by the time it reaches small intestine. Considering that in other organs such as liver and kidney which accumulate significant amount of chromium no tumors were observed, it is important to do more research on the mechanism of tumor development in small intestine. Specifically, duodenum is a place where iron absorption takes place. An analysis of ferroreductase expression and iron metabolism will help to shed light on whether an alteration in iron metabolism in this tissue may be responsible for chromium carcinogenic effects.
Wise	This document is supposed to be limited to an oral drinking water exposure. It is inappropriate to extend any finding to "all routes of exposure" in this document and such evidence has not been considered or presented for dermal or inhalation routes. In its defense of a mutagenic mode of action, the Toxicological Review states on page 213 that: *"In addition to the evidence supporting a mutagenic mode of action in test animals, alternative or additional hypothesized modes of action for hexavalent chromium carcinogenicity have not been demonstrated."* There are three concerns with this statement. First, it seems to imply that other modes of action need to be "demonstrated" not simply supported. The frank reality is that no mode of action for Cr(VI) has been demonstrated, even the mutagenic mode of action is only supported and not demonstrated. There should not be a double standard here where the mutagenic mode of action needs to only be supported, while other modes must be demonstrated. The word "demonstrated" should be changed to "supported" to be consistent with the beginning of the passage. The second concern is that the statement is inaccurate. We supported an alternative mode of action to the induction of mutations in our paper that is cited in the Toxicological Review. Specifically, in Holmes, A.; Wise, SS; Wise, Sr., JP (2008) Carcinogenicity of hexavalent chromium. Indian J Med Res 128:353 – 372, we argue that the mechanism for Cr(VI) does not involve mutations in the primary sequence of the DNA as it is a weak mutagen. Instead, we argue for a genotoxic mechanism leading not to mutations but changes in chromosome number and structure. This point of view is not considered much in the Toxicological Review, but is well-supported in the review and does offer an alternative mode of action that should have been discussed. It is as well-supported as the mutagenic mode of action and so it is inaccurate to state other views have not been demonstrated to the extent a mutagenic mode of action has been. The third concern with the statement is that it seems to imply that the approach taken in determining the mode of action was to consider those possibilities suggested in reviews of the literature. Therefore, because there is no review article synthesizing the literature to suggest a mode of action, there are no other modes of actions to consider. A better approach would have been to consider the primary literature and consider some possible modes of action that emerge from the data, but that have not yet emerged as a review article.

	There are other modes of action that emerge and two possibilities are presented below. There are data to support these modes that should be synthesized, evaluated and considered. The mutagenic mode of action as the primary mode of action is not sufficiently scientifically supported or described in the Toxicological Review. Many concerns in the presentation with respect to proper citation of results, bias against cell culture studies, and an incomplete consideration of the primary literature are discussed above. The only approach taken was to consider all of these lesions in bulk as simply all representative of mutagenic events and not consider the possible confounding factors for each that may indicate they are not mutagenic events. A more careful consideration of the primary literature, considering each endpoint on its own merit could argue against a mutagenic mode of action that involves changes to the primary sequence of the DNA strand resulting in mutations. Cr(VI)-induced human tumors rarely contain such mutations and Cr(VI)-induced mutations are most often generated in experimental systems when one artificially forces them to occur by using extraordinarily high doses or systems with compromised repair and cell death pathways or by non-physiological exposure routes. It is unlikely that Cr(VI) is a mutagen at low doses. The most consistent outcome in the primary literature appears to be impacts on metaphase chromosomes. These outcomes occur at relatively low doses, in intact healthy human cells and across species in cell culture, whole animal and human worker studies. The question remains and this document does not address the underlying mechanism for this outcome. Induction of aneuploidy is another promising mode of action. One mode of action could involve direct damage to the DNA strand resulting in an alteration in chromosome structure or number. This mode would have key events that include: 1) Uptake of Cr(VI), 2) intracellular reduction of Cr(VI), 3) interaction of reductant products with DNA strands, 4) production of chromosomal changes, 5) escape of DNA repair and apoptosis and 6) expansion of damaged cells. Alternatively, the mode of action might not involve direct damage to the DNA. Instead, it could involve direct interactions with the mitotic spindle apparatus and be more of an epigenetic event. This mode would have key events that include: 1) Uptake of Cr(VI), 2) intracellular reduction of Cr(VI), 3) accumulation of intracellular reductant products, 4) interaction of reductant products with mitotic spindle apparatus, perhaps binding to the centrosomes, 5) production of chromosomal changes, 6) bypass of the spindle assembly checkpoint, 7) escape of apoptosis and 8) expansion of damaged cells.
Zhitkovich	Mutagenic mode of action: Hexavalent chromium was overwhelmingly positive for genotoxicity in a large variety of cells and organisms. It was also consistently mutagenic in bacterial and mammalian test systems. The mutagenicity and genotoxicity of Cr-6 result from a direct DNA-damaging mechanism, as evidenced by the induction of mutagenic chromium-DNA adducts and other forms of DNA damage. Formation of chromium-specific DNA lesions at environmentally relevant Cr-6 concentrations and sensitivity of genotoxic responses to manipulations of cellular DNA repair further support the role of direct DNA damage as a primary cause of genotoxicity. Since Cr-6 is taken up via ubiquitously expressed sulfate transporters and its metabolism in cells occur via ubiquitously present ascorbate, glutathione and cysteine, there is no reason to believe that

	the formation of DNA damage in the small intestinal cells and in more extensively studied cell types would be significantly different. Thus, diverse lines of evidence are fully consistent with a mutagenic mode of carcinogenic action for hexavalent chromium. The Draft clearly presented the main arguments for this designation. However, as pointed out above, Tables 4-22 and 4-23 need to be supplemented with information on Cr-DNA adducts. Supporting the importance of Cr-DNA adducts in chromate tumorigenicity are findings from the MOA study by the ACC in which levels of adducts were dramatically higher in the duodenum and jejunum of mice vs. rats. This result mirrors species differences in the intestinal carcinogenesis by chromate and it could not be explained by differences in tissue accumulation of chromium. In contrast to clear positive mutagenicity and genotoxicity data from *in vivo* studies and ascorbate-restored mammalian cell cultures, aneuploidy and epigenetic responses have not yet been tested in animal models and so far have been observed only in ascorbate-deficient cells. In fact, Sun et el. (2009) have found that the induction of epigenetic changes by chromate in human cultured cells occurs only under ascorbate-depleted conditions. The measurements of mutations in KRAS and p53 genes as part of the MOA study sponsored by the American Chemistry Council would not necessarily provide a clear answer about the mutagenic mode of action. A short 3-months duration of this study vs. 2 years for the NTP bioassay certainly diminishes its ability to detect mutations. Among the proposed mutation readouts, three KRAS codons represent a very small and consequently, insensitive mutagenic target. This gene was only rarely mutated in chromate-associated human lung cancers (Ewis et al. 2001). The p53 gene is also uncommonly mutated in cancers among chromate workers (Kondo et al. 1997). The presence or absence of KRAS or p53 mutations do not serve as a strong test for the validity of the mutagenic mode of carcinogenic action, as the frequency of cells with mutated KRAS or p53 can increase through selection of the pre-existing mutant clones whereas transformation process can result from mutagenic events in other components of KRAS and p53 pathways. For example, a large wave of early thyroid cancers among Chernobyl radioiodine-exposed children was caused by translocations in growth factor receptors with almost no RAS and p53 mutations (Nikiforov et al. 1996, Suchy et al. 1998, Williams 2002). <u>Potential alternative modes of action</u>: Two lines of in vivo evidence have been presented to point to a potentially nonmutagenic mode of carcinogenic action. One is based on the drinking water study by DeFlora et al. 2008, which found negative results for DNA damage in the duodenum of mice. However, as discussed in detail above, this study used assays that were insensitive for detection of DNA damage by the employed doses of Cr(VI)in drinking water. Therefore, the negative results of this work were expected and therefore, uninformative. The other observation leading to the discussion of nonmutagenic or indirectly mutagenic mechanisms of carcinogenicity was the presence of diffuse epithelial hyperplasia in the NTP bioassay. Although the NTP study has not found significant necrosis in the small intestine of exposed mice, it is quite possible that the observed hyperplasia was a typical manifestation of regenerative responses. A combination of increased proliferation and

	inflammation could be presented as an alternative mechanism for indirect induction of mutations due to higher rates of cell division and by reactive oxygen species released by the recruited inflammatory cells. This carcinogenic pathway would exhibit a strongly sublinear, threshold-type dose dependence, as it relies on the induction of cell death and small doses would not kill cells. Inflammatory events could also be linked to cell death of chromium-damaged cells, which release pro-inflammatory molecules. The extent of hyperproliferation in chromium-exposed groups was modest, and considering the overall very high rate of cell division in the small intestine, it is hard to see how somewhat faster replication would provide dramatically more spontaneous mutations required for cancer development. At best, the cytotoxicity-induced compensatory proliferation mechanism and the mutagenic mode should co-exist at high tumorigenic doses. The results from the MOA study sponsored by the ACC argue against significant inflammatory responses in the duodenum of chromate-exposed mice, as no increases in the levels of 8-oxodG and in the panel of 22 cytokines have been observed. A statistically significant drop in the ratio of GSH/GSSG was small in its magnitude, further demonstrating that tumorigenic doses were not associated with the state of strongly elevated oxidative stress and inflammation. The presence of chromium-induced hyperplasia could also be viewed as a manifestation of cancer-protective responses by the small intestine. Elimination of genetically damaged cells by apoptosis or another form of cell death is a firmly established protective mechanism against cancer. Thus, there are two opposing interpretations for the toxicological significance of the observed hyperplasia: one is pro-tumorigenic and another is anti-tumorigenic. The supralinear shape of dose-tumor incidence responses in the NTP-2008 studies for female mice is consistent with the engagement of cancer-protective mechanisms. Tumor incidence vs. dose in male mice visually displayed a linear dose-dependence (as shown in Stern 2010). Thus, a hypothetical cytotoxicity-based mechanism with the expected dose-response sublinearity is contradicted by the available evidence.
Zhu	A mutagenic mode of action was proposed as the primary mode of action. On the one hand, EPA discussed data gap and uncertainties about the mutagenic MOA and other possible MOAs. On the other hand, EPA defended the mutagenic MOA despite the lack of data evidence. For example, the only animal study that investigated target tissue genotoxicity (De Flora et al. 2008) reported negative results for DNA-protein crosslinks and DNA adducts in forestomach, glandular stomach, and duodenum of mice exposed to hexavalent chromium in drinking water for 9 month. EPA dismisses the negative finding on the basis of a shorter duration of the study compared with the 2-year NTP study.

B3. A two-year drinking water study in rats and mice (NTP, 2008) was selected for the derivation of an oral slope factor. Please comment on whether the selection of this study for quantification is scientifically supported and clearly described. Please identify and provide the rationale for any other studies that should be considered.

Reviewer	Comments
Byczkowski	Apparently, the two-year drinking water study by NTP (2008) is the only, up to date, appropriate cancer bioassay of Cr^{+6} by oral route (this document states - C.f. P. 224, L# 24: "*...No other adequate studies of hexavalent chromium carcinogenicity by ingestion are available...*")
Hamilton	There are no significant concerns about selection of this study, it is clearly the best available study for this type of risk assessment, with the caveats about how these data are interpreted and modeled as discussed elsewhere.
Nordberg	In this document it is not explained to the reader what decided the selection of chromium species and selection of doses and how the chosen doses relate to exposure in drinking water in the general population. Lethal doses should also be given for the chromium species that have been used in the quoted studies. To evaluate and compare the outcome of studies and concentration levels in tissues, a ratio of concentration in dry weight to concentration in wet weight would make it possible and easier to compare reported results and also of possible intake of hexavalent chromium in drinking water. It is told that the animals by increasing exposure to hexavalent chromium in drinking water showed a decrease in intake of drinking water. Influence on different tissues will be found in doing this. On page 112 NTP 2008 decreased body weights could be explained by reduced drinking water consumption.
Patierno	See previous comments above. The NTP study is the best study available but the interpretation of the data and conclusions drawn from it are incorrect. Important supplementary data is preliminarily discussed in the Public Comments.
Rossman	This seems to be the only choice.
Salnikow	Outside of my area of expertise.
Wise	This study is the proper study based on the available data. The study is flawed because only very high doses were considered in the study, thus, there is concern that it may not reflect events at lower doses. The EPA is in the unique position that a study that repeats the one above and extends it to lower doses is almost completed. The EPA should wait for the final results of that study to make the most informed analysis.
Zhitkovich	The selection of a two-year drinking water study in rats and mice by the NTP (2008) for the calculation of an oral slope factor is appropriate. The NTP study was well designed and well executed. No other multiple-dose chronic oral carcinogenicity study in animals is

	available and the dose-dependence from the single ecological study linking chromium-6 in drinking water to human stomach cancers cannot be reliably estimated.
Zhu	The selection of the NTP's 2-year drinking water study in rats and mice (NTP, 2008) is justified. Reasons for why existing epidemiological data were not used for estimating cancer slope factor are acceptable.

B4. The incidence of adenomas and carcinomas combined in the small intestine of male mice from the NTP (2008) two-year drinking water study were selected to serve as the basis for the quantitative cancer assessment. Please comment on whether this selection is scientifically supported and clearly described. Please identify and provide the rationale for any other endpoints that should be selected to serve as the basis for the quantitative cancer assessment.

Reviewer	Comments
Byczkowski	Even though in the NTP (2008) bioassays, only the exposures to the two highest doses of Cr^{+6} in mice and to the highest one dose in rats produced statistically significant carcinogenicity, the selection of this study for derivation of cancer slopes seems to be justified by the description provided in the document. Regarding the (common) practice of combining adenomas and carcinomas in modeling cancer risk, an explanation should be provided that the adenoma is a benign tumor of epithelial tissue with the tendency to become malignant, thus it may - or may not - lead to cancer. Also, the results of NTP (2008) cancer bioassay could be interpreted with the assumption of a threshold.
Hamilton	There is concern about selection of these endpoints to represent cancer risk in these animals as the basis for a human risk assessment. The high doses required to induce these lesions are well above a threshold level that would be of concern in humans under normal exposure scenarios, and these almost certainly represent a scenario where natural reductive defense mechanisms were overwhelmed by the doses of chromium used. Taken together with the more recent 90-day ACC sponsored studies, the MOA for chromium(VI) is most likely a non-mutagenic one involving tissue damage and reproliferation, and would only be seen at doses that are unlikely to ever occur in a human exposure setting, particularly via drinking water. Thus, a threshold based risk assessment is most appropriate, similar to the treatment used for the non-cancer endpoints which are likely to be directly related to the cancer MOA.
Nordberg	See comments above. It should be noted that pH is different in different parts of the gastrointestinal system.

Patierno	See previous comments above. The NTP study is the best study available but the interpretation of the data and conclusions drawn from it are incorrect. Important supplementary data preliminarily discussed in the Public Comments.
Rossman	This seems to be the only choice.
Salnikow	It seems that combining the incidence of adenomas and carcinomas in small intestine was the proper choice for modeling cancer risk. This is supported by the available data and clearly described. The fact that only highest doses produced a carcinogenic effect may indicate high reducing capacity in tested model systems (wild type mice and rats). As suggested in A2 exposing Gulo-/- mice or rats to hexavalent chromium may result in tumor appearance at lower doses. If this will be the case the extrapolation to environmentally relevant doses of chromium exposure will be more feasible.
Wise	If one is going to rely on this NTP study, the selection of the incidence of adenomas and carcinomas in the small intestine of male mice from the NTP (2008) two-year drinking water study to serve as the basis for the quantitative cancer assessment is appropriate. However, scientifically these lesions are not the same and are not necessarily linked. Thus, one or the other should be used.
Zhitkovich	The choice of the combined incidence of adenomas and carcinomas in the small intestine of male mice from the NTP-2008 study for the quantitative cancer assessment was based on a better fit of the multistage model for the male mouse data than for the female mouse data. However, it was unclear why a combination of male and female mouse data sets was not used.
Zhu	For dose-response assessment EPA considered the incidences of adenoma and carcinoma combined in the small intestine of male and female B6C3F mice (Tables 5.3 and 5.4), in the oral cavity (mucosa and tongue) (Tables 5.3 and 5.4) in rats (NTP 2008). (Note the denominators that determine the tumor incidence in small intestine are not consistent with those in Table 4.19). EPA did not consider the incidence of other neoplasm because the incidence is not dose-dependent. The incidence of adenoma or carcinoma in the oral cavity in both male and female rats elevated only at the highest dose, but not at the three lower doses (up to 2.1 and 2.4 mg/kg-d for male and female respectively). To fit a dose-response model to these incidence data that exhibited hockey-sticker shape of dose-response requires a nonlinear (curve-linear) functional form or even a threshold model. Such curve-linear pattern seems inconsistent with the hypothesized genotoxic MOA. The lack of dose-response in these two endpoints was cited as reason for not advancing these two endpoints for final dose-response analysis. Better justification is needed.

B5. The oral slope factor was calculated by linear extrapolation from the POD (i.e., the lower 95% confidence limit on the dose associated with 10% extra risk of tumors of the small intestine in male mice). Has the modeling been appropriately conducted and clearly described?

Reviewer	Comments
Byczkowski	The oral cancer slope factor was estimated in accordance with the U.S. EPA (2005) guidelines, but to this reviewer the linear cancer extrapolation seems inconsistent with the prior noncancer RfD modeling. While the early preneoplastic epithelial hyperplastic lesions have been modeled as a threshold phenomenon in derivation of RfD, the resultant neoplastic lesions were modeled as a no-threshold linear phenomenon in derivation of cancer slope factors. There seems to be some contradiction, as a threshold-bearing precursor cannot result in the no-threshold adverse effect. In addition, the allometric scaling of cancer slopes, extrapolated from animals to humans based on body weight (to the 3/4 power), seems to be inconsistent with the portal of entry site of action in the GI tract. Typically, this is the local concentration of a chemical at the target site/target tissue that drives an adverse response. Therefore, more appropriate could be a physiologically based pharmacokinetic (PBPK) scaling based on animal-to-human ratio of differences between the rate of absorption and the rate of reduction of Cr^{+6} to Cr^{+3} in gastrointestinal tract contents, as well as the rate of loss of Cr^{+6} from intestinal tract contents to the feces. It seems that appropriately developed PBPK model which recognizes physiological specificity of the different segments of gastrointestinal tract in animals and humans, as presented at the workshop by Summit Toxicology, LLP., may be critical in understanding the mode of action of orally administered Cr^{+6} and in quantitative evaluation of its effects.
Hamilton	As discussed in detail under G1. and G2. responses, this reviewer has considerable concerns about the use of a linear low-dose extrapolation model for assessing chromium cancer risk. The evidence, when objectively assessed, strongly argues for threshold mechanisms both in the gut and systemically, and there is little or no evidence that chromium reaches the systemic circulation as chromium(VI) under exposure scenarios of relevance to human exposures. There is no better candidate for departure from these default EPA assumptions than chromium if EPA is serious about evidence-based risk assessment. Many of the public comments that were available to the reviewers just before and after our May 2011 meeting also raise these issues, and the EPA should, in particular, wait until the recently reported 90-day MOA and PK studies are published and available to them, at which point they should give serious consideration to how these new data inform the likely MOA. Given that most toxicology profiles are only revised every 10-15 years, it is worth waiting for these studies, and taking them as well as the external reviewer comments in mind toward a revised document that will be more accurate and will better stand the test of time. The EPA might also consider asking for a National Research Council Special Emphasis Panel to review all these materials and make a recommendation to EPA regarding chromium(VI) as has been done for other several other key toxicants of concern. In any event the current draft's risk assessment treatment of chromium(VI) is highly flawed and grossly mischaracterizes the likely risk of human

	health effects of chromium(VI) in drinking water based on a careful and thorough assessment of all the available evidence.
Nordberg	The model is clearly described. However I feel very uneasy about extrapolation to lower exposure levels because of chosen exposure doses where LOAEL is identical to the lowest exposure dose administered. Other organizations like IARC do not perform any quantitative evaluation of carcinogenic agents/substances. Threshold concentrations are problematic because of lack of knowledge of how the carcinogenicity develops. Once an organism has been exposed to a substance that can give rise to cancer there is a possibility to such an effect to occur.
Patierno	See previous comments above. The linear extrapolation from the POD is not appropriate. CrVI toxicity and carcinogenicity demonstrates distinct non-linearity and there is little or no relation between what is observed at the highest doses in the NTP study and any physiologically-appropriate or environmentally-relevant exposure.
Rossman	This is outside my field of expertise.
Salnikow	Outside of my area of expertise.
Wise	The modeling has been appropriately conducted and clearly described. More methods are needed to make it clearer. It is possible that Cr(VI) acts via a threshold. The EPA is in the unique position that a study that repeats the NTP study and extends it to lower doses is almost completed. This study will help clarify if there is a threshold. The EPA should wait for the final results of that study to make the most informed analysis.
Zhitkovich	The calculation of the oral slope factor from the POD was appropriately performed. As per US-EPA 2005 Guidelines for Carcinogen Risk Assessment, a linear extrapolation to low doses was used based on the selection of the mutagenic mode of carcinogenic action for chromium-6. The ability of ingested chromium-6 to cause adverse effects at both environmentally relevant and much higher doses has been questioned, given the reported high chromate reducing capacity of the gastric juice and a limited systemic penetration of chromium after oral exposure (as extensively reviewed by the Draft). These considerations led to the formulation of the threshold model of chromium-6 carcinogenesis, which postulates that only doses that exceed the reducing capacity of the tissue (stomach for ingestion exposures) would be carcinogenic (DeFlora 2000). This model would argue that despite the selection of a mutagenic mode of action with the resulting recommendation for default linear extrapolation, the complete detoxification of low-to-moderate chromium-6 doses in the stomach makes it inappropriate to perform linear extrapolation from the POD. The ability of gastric juices to reduce/detoxify chromium-6 is generally accepted in the field; however, studies with human volunteers and other considerations argue against the completeness of the detoxification process. For example, the bioavailability for Cr(VI) was ~10-times higher than for Cr(VI) reduced with orange juice prior to ingestion

	(Kuykendall et al. 1996). The extent of chromium-6 reduction in the stomach is influenced by three factors: its reduction capacity, reduction rate and stomach emptying time. Based on the reported high reduction capacity of the stomach (>80 mg/day, DeFlora et al. 1997), the rate of reduction by gastric juice under fasting conditions could exhibit pseudo-first order kinetics for a broad range of chromium-6 concentrations. This means that the percentage of reduced chromium-6 could be the same for both very small amounts and much larger amounts. Reduction of chromium-6 by artificial gastric juice has been found to follow first order reaction kinetics (Gammelgaard et al. 1999). Consistent with the first-order reaction kinetics, Donaldson and Barreras (1966) found that human subjects excreted in the 24-hr urine 2.1% of ingested 20 ng radioactive chromium-6 whereas Kerger et al. (1997) found that ingestion of 5 mg chromium-6 by human volunteers led to about 1.43% excretion during the first 24 hr (1.43% excretion is a conservative $1/4^{th}$ estimate from the average 4-day excretion value of 5.7%). Thus, the bioavailability of 20 ng and 5 mg chromium-6 (250,000-fold range) looks quite similar. Donaldson and Barreras (1966) also performed a very important experiment on the bioavailability of 20 ng radioactive chromium-6 that was directly delivered into the duodenum of human subjects. In this case, they found that 10.6% of chromium was excreted in the urine. Since the duodenal delivery represent 100% nonreduced chromium-6, then the amount of nonreduced chromium-6 in their oral route experiment can be estimated from the urinary excretion of 2.1% divided by 0.106 = 19.8%. For the study by Kerger et al (2007), the same type of calculations gives an estimate of 14.3% nonreduced chromium-6 reaching the duodenum. Gammelgaard et al. (1999) calculated a half-life of 23 min for reduction of 0.1mg/L chromium-6 (current MCL for total chromium) by artificial gastric juice. After 1 hr, this reduction rate would leave 16.5% chromium-6.
Zhu	EPA carried out dose-response modeling and BMD estimation for the incidences of adenoma and carcinoma of small intestine in male and female mice separately. EPA stated that it relied on the multi-stage model because the model is preferred by the agency, but gave no justification or explanation. It went on to report an estimated slope of 0.09 (mg/kg-day) and 0.10 (mg/kg-day) for male and female mice respectively. In section 5.3.4 of the Review, EPA reported the CSFs derived on the basis of the cancer incidence of small intestine in male and female mice, and chooses that of male mice because of "the poor fit of the multistage model to the female mouse data". EPA did not provided adequate detail on the modeling efforts, or a discussion and justifications for its final selection (section 5.3.3 and 5.3.4). EPA did provide some detail in Appendix B2, which is essentially the direct output from running the BMDS software, but again no discussion or explanation of the output. It would be helpful and necessary that EPA substantially expand sections 5.3.3 and 5.3.4 to report in greater detail the modeling process, the issues encountered, and justify the decision and choice therein. On a more technical side, examination of Appendix B2 reveals that (1) no standard error is reported for the estimate of model coefficient, and (2) the coefficient of the second order in the polynomial was set to be zero, not estimated. The model did not fit the data at all despite a non-significant p-value for goodness-of-fit test. EPA did not give any explanation or discussion in this regard. The significance of a goodness-fit-test depends on sample size, and a non-significant result does not imply a correct model, especially within a range where there are no data. EPA should explore different options: trying

	different models, considering omitting the highest dose, or considering combine male and female mice. Inclusion of multiple studies, multiple endpoints, multiple model choices, and various BMR levels for deriving a POD is increasingly desirable towards a more systematic and quantitative risk assessment paradigm. It will afford an opportunity to quantify the underlying uncertainties and variations associated with the choices and options made each many stages of the risk assessment process. Within the context of CSF for hexavalent chromium, EPA is in a position to conduct a more thorough and comprehensive assessment by including multiple endpoints, different model forms that allow for nonlinear dose-response, various BMR levels. The outcome will then demonstrate a range for POD and CSF to permit a better quantification and better understanding of uncertainties and variations.

Additional Reviewer Comments

Additional Comments Submitted by Dr. Janusz Z. Byczkowski

Typos and errors *that should be corrected in the revised version*:

P. 24, L# 4: "...direct measurement of residual Cr(VI) in the *calorimetric* reaction..."
Please change to: "... colorimetric..."

P.26, L# 26: "...Michaelis-Menten uptake kinetics..."
Please change to: "...Michaelis-Menten-type uptake kinetics..."

P. 51, Figure 3-6, it would help the reader if non-standard abbreviations were explained in the figure legend - e.g., *DSB*, *SSB*, etc.

P. 52, L# 38: "...to describe kinetics *of* humans..."
Please change to: "...kinetics in humans..."

P. 84, L# 36: "...*colestasis*, as other markers of *colestasis*..."
Please change to: "...cholestasis..."

P. 229, L# 33: "...(see Section *4.6.3.4*)..."
Such a section does not exist in the reviewed document.

P. 244, L# 7: "...*Bukowksi*, JA..."
Please change to: "...Bukowski..."

Additional Comments Submitted by Dr. Konstantin Salnikow

Errata:

Regarding the form of the draft it should be noted that, although this is not a manuscript, it is necessary to correct errors and mistakes in the draft content.

Below are several examples:

1. **$K_2Cr_2O_4$** – does not exist (page 30, table 3-7; page 45), **should it be K_2CrO_4**.
2. Table 2-1, page 6 – **Cr_2O_3** is chromium (III), not hexavalent chromium. **$BaCrO_4$** is barium chromate, not barium oxide.
3. Table 2-5, page 18 – "accumulates Cr(V)", the intent of this statement is not clear because this form is short living and unlikely that is can accumulate.
4. Page 35 and Table 3-9, **K_2CrO_7** - does not exist.
5. None existing or wrong citations:

   Kumulainen, 1991 (page 7), **should be Kumpulainen**, **1992** (page 251).

   Salnikov and Zhitkovich, 2009 (page 50); Salnikov and Zhitkovich, 2008 (page 257), **should be Salnikow and Zhitkovich, 2008**.

   Costa and Klein, 2004 (page 66); Costa, M.; Klein, C.B. 2008 (page 245), **should be Costa and Klein, 2006**.

   LeVina et al., 2003, 2007, (page 50) **should be Levina et al., 2003**, **2007**.

   Kasprzak, 1996 (page 189), **no reference provided.**

   Campbell J.L.; Tan,Y.; Clewell, H.J. (2009) Development of a PBPK model for hexavalent chromium in rats and mice to estimate exposure to oral mucosa and small intestine. Toxicologist 108(1):98 (Abstract) Poster ID # 108. This is a **questionable citation**.

   Sun et al. (2009), (page 188), **no reference provided.**

   Davies, JM. (1979) Lung cancer mortality of workers in chromate pigment manufacture: An epidemiological survey. J Oil Chem Assoc 62:157-163, (page 245), **should be: J Oil Colour Chem Assoc 62:157-163**.

References:

Arita, A., and Costa, M. (2009). Epigenetics in metal carcinogenesis: nickel, arsenic, chromium and cadmium. *Metallomics* **1**, 222-228.

Chung, T. L., Brena, R. M., Kolle, G., Grimmond, S. M., Berman, B. P., Laird, P. W., Pera, M. F., and Wolvetang, E. J. (2010). Vitamin C promotes widespread yet specific DNA demethylation of the epigenome in human embryonic stem cells. *Stem Cells* **28**, 1848-1855.

Dai, H., Liu, J., Malkas, L. H., Catalano, J., Alagharu, S., and Hickey, R. J. (2009). Chromium reduces the in vitro activity and fidelity of DNA replication mediated by the human cell DNA synthesome. *Toxicol Appl Pharmacol* **236**, 154-165.

Davidson, T., Kluz, T., Burns, F., Rossman, T., Zhang, Q., Uddin, A., Nadas, A., and Costa, M. (2004). Exposure to chromium (VI) in the drinking water increases susceptibility to UV-induced skin tumors in hairless mice. *Toxicol Appl Pharmacol* **196**, 431-437.

De Flora, S., D'Agostini, F., Balansky, R., Micale, R., Baluce, B., and Izzotti, A. (2008). Lack of genotoxic effects in hematopoietic and gastrointestinal cells of mice receiving chromium(VI) with the drinking water. *Mutat Res* **659**, 60-67.

De Flora, S., Iltcheva, M., and Balansky, R. M. (2006). Oral chromium(VI) does not affect the frequency of micronuclei in hematopoietic cells of adult mice and of transplacentally exposed fetuses. *Mutat Res* **610**, 38-47.

Epstein, A. C., Gleadle, J. M., McNeill, L. A., Hewitson, K. S., O'Rourke, J., Mole, D. R., Mukherji, M., Metzen, E., Wilson, M. I., Dhanda, A., Tian, Y. M., Masson, N., Hamilton, D. L., Jaakkola, P., Barstead, R., Hodgkin, J., Maxwell, P. H., Pugh, C. W., Schofield, C. J., and Ratcliffe, P. J. (2001). C. elegans EGL-9 and mammalian homologs define a family of dioxygenases that regulate HIF by prolyl hydroxylation. *Cell* **107**, 43-54.

Hewitson, K. S., McNeill, L. A., Riordan, M. V., Tian, Y. M., Bullock, A. N., Welford, R. W., Elkins, J. M., Oldham, N. J., Bhattacharya, S., Gleadle, J. M., Ratcliffe, P. J., Pugh, C. W., and Schofield, C. J. (2002). Hypoxia-inducible factor (HIF) asparagine hydroxylase is identical to factor inhibiting HIF (FIH) and is related to the cupin structural family. *J Biol Chem* **277**, 26351-26355.

Kasprzak, K. S., Diwan, B. A., Kaczmarek, M., Logsdon, D., Fivash, M. J., Jr., and Salnikow, K. (2011). Effects of Ascorbic Acid on Carcinogenicity and Acute Toxicity of Nickel Subsulfide, and on Tumor Transplants Growth in Gulonolactone Oxidase Knock-Out Mice and Wild-type C57BL Mice *Toxicol Appl Pharmacol* **submitted**.

Kondo, K., Hino, N., Sasa, M., Kamamura, Y., Sakiyama, S., Tsuyuguchi, M., Hashimoto, M., Uyama, T., and Monden, Y. (1997). Mutations of the p53 gene in human lung cancer from chromate-exposed workers. *Biochem Biophys Res Commun* **239**, 95-100.

Kondo, K., Takahashi, Y., Hirose, Y., Nagao, T., Tsuyuguchi, M., Hashimoto, M., Ochiai, A., Monden, Y., and Tangoku, A. (2006). The reduced expression and aberrant methylation of p16(INK4a) in chromate workers with lung cancer. *Lung Cancer* **53**, 295-302.

Lando, D., Peet, D. J., Gorman, J. J., Whelan, D. A., Whitelaw, M. L., and Bruick, R. K. (2002). FIH-1 is an asparaginyl hydroxylase enzyme that regulates the transcriptional activity of hypoxia-inducible factor. *Genes Dev* **16**, 1466-1471.

Maeda, N., Hagihara, H., Nakata, Y., Hiller, S., Wilder, J., and Reddick, R. (2000). Aortic wall damage in mice unable to synthesize ascorbic acid. *Proc. Natl. Acad. Sci. U. S. A.* **97**, 841-846.

Mahon, P. C., Hirota, K., and Semenza, G. L. (2001). FIH-1: a novel protein that interacts with HIF-1alpha and VHL to mediate repression of HIF-1 transcriptional activity. *Genes Dev* **15**, 2675-2686.

Mirsalis, J. C., Hamilton, C. M., O'Loughlin, K. G., Paustenbach, D. J., Kerger, B. D., and Patierno, S. (1996). Chromium (VI) at plausible drinking water concentrations is not genotoxic in the in vivo bone marrow micronucleus or liver unscheduled DNA synthesis assays. *Environ. Mol. Mutagen.* **28**, 60-63.

Mizushima, Y., Harauchi, T., Yoshizaki, T., and Makino, S. (1984). A rat mutant unable to synthesize vitamin C. *Experientia* **40**, 359-361.

Myllyharju, J. (2003). Prolyl 4-hydroxylases, the key enzymes of collagen biosynthesis. *Matrix Biol.* **22**, 15-24.

O'Flaherty, E. J. (1996). A physiologically based model of chromium kinetics in the rat. *Toxicol Appl Pharmacol* **138**, 54-64.

O'Flaherty, E. J., Kerger, B. D., Hays, S. M., and Paustenbach, D. J. (2001). A physiologically based model for the ingestion of chromium(III) and chromium(VI) by humans. *Toxicol. Sci.* **60**, 196-213.

Onkelinx, C. (1977). Compartment analysis of metabolism of chromium(III) in rats of various ages. *Am J Physiol* **232**, E478-484.

Salnikow, K., and Kasprzak, K. S. (2005). Ascorbate depletion: a critical step in nickel carcinogenesis? *Environ Health Perspect* **113**, 577-584.

Salnikow, K., and Zhitkovich, A. (2008). Genetic and epigenetic mechanisms in metal carcinogenesis and cocarcinogenesis: nickel, arsenic, and chromium. *Chem. Res. Toxicol.* **21**, 28-44.

Sharma, S., Kelly, T. K., and Jones, P. A. (2010). Epigenetics in cancer. *Carcinogenesis* **31**, 27-36.

Shi, Y. (2007). Histone lysine demethylases: emerging roles in development, physiology and disease. *Nat Rev Genet* **8**, 829-833.

Snow, E. T. (1991). A possible role for chromium(III) in genotoxicity. Environ Health Perspect 92, 75-81.

Sun, H., Zhou, X., Chen, H., Li, Q., and Costa, M. (2009). Modulation of histone methylation and MLH1 gene silencing by hexavalent chromium. *Toxicol Appl Pharmacol* **237**, 258-266.

Takahashi, Y., Kondo, K., Hirose, T., Nakagawa, H., Tsuyuguchi, M., Hashimoto, M., Sano, T., Ochiai, A., and Monden, Y. (2005). Microsatellite instability and protein expression of the DNA mismatch repair gene, hMLH1, of lung cancer in chromate-exposed workers. *Mol. Carcinog.* **42**, 150-158.

Uddin, A. N., Burns, F. J., Rossman, T. G., Chen, H., Kluz, T., and Costa, M. (2007). Dietary chromium and nickel enhance UV-carcinogenesis in skin of hairless mice. *Toxicol Appl Pharmacol* **221**, 329-338.

Zhitkovich, A. (2005). Importance of chromium-DNA adducts in mutagenicity and toxicity of chromium(VI). *Chem. Res. Toxicol.* **18**, 3-11.

Additional Comments Submitted by Dr. Anatoly Zhitkovich

REFERENCES

Cheng L, Sonntag DM, de Boer J, Dixon K. Chromium(VI)-induced mutagenesis in the lungs of big blue transgenic mice. J Environ Pathol Toxicol Oncol. 19(3): 239-249 (2000).

De Flora S. Threshold mechanisms and site specificity in chromium(VI) carcinogenesis. Carcinogenesis 21(4): 533-541 (2000).

De Flora S, Camoirano A, Bagnasco M, Bennicelli C, Corbett GE, Kerger BD. Estimates of the chromium(VI) reducing capacity in human body compartments as a mechanism for attenuating its potential toxicity and carcinogenicity. Carcinogenesis 18(3): 531-537 (1997).

De Flora S, D'Agostini F, Balansky R, Micale R, Baluce B, Izzotti A. Lack of genotoxic effects in hematopoietic and gastrointestinal cells of mice receiving chromium(VI) with the drinking water. Mutat Res. 659(1-2): 60-67 (2008).

Donaldson RM Jr, Barreras RF. Intestinal absorption of trace quantities of chromium. J Lab Clin Med. 68(3): 484-493 (1966).

Ewis AA, Kondo K, Lee J, Tsuyuguchi M, Hashimoto M, Yokose T, Mukai K, Kodama T, Shinka T, Monden Y, Nakahori Y. Occupational cancer genetics: infrequent ras oncogenes point mutations in lung cancer samples from chromate workers. Am J Ind Med. 40: 92-97 (2001).

Gammelgaard B, Jensen K, Steffansen B. In vitro metabolism and permeation studies in rat jejunum: organic chromium compared to inorganic chromium. J Trace Elem Med Biol.13(1-2): 82-88 (1999).

Gibb H.J., Lees P.S.J., Pinsky P.F. and Rooney B.C. Lung cancer among workers in chromium chemical production. Am. J. Ind. Med., **38**, 115-126 (2000).

Glaviano A, Nayak V, Cabuy E, Baird DM, Yin Z, Newson R, et al. Effects on hTERT on metal ion-induced genomic instability. Oncogene 25: 3424-3435 (2006).

Itoh S, Shimada H. Clastogenicity and mutagenicity of hexavalent chromium in lacZ transgenic mice. Toxicol. Lett. 91(3): 229-233 (1997).

Itoh S, Shimada H. Bone marrow and liver mutagenesis in lacZ transgenic mice treated with hexavalent chromium. Mutat. Res. 412(1): 63-67 (1998).

Kerger BD, Finley BL, Corbett GE, Dodge DG, Paustenbach DJ. Ingestion of chromium(VI) in drinking water by human volunteers: absorption, distribution, and excretion of single and repeated doses. J Toxicol Environ Health 50(1): 67-95 (1997).

Kondo, K., Hino, N., Sasa, M., Kamamura, Y., Sakiyama, S., Tsuyuguchi, M., Hashimoto, M., Uyama, T., and Monden, Y. Mutations of the p53 gene in human lung cancer from chromate-exposed workers. Biochem. Biophys. Res. Commun. 239: 95-100 (1997).

Kuykendall JR, Kerger BD, Jarvi EJ, Corbett GE, Paustenbach DJ. Measurement of DNA-protein cross-links in human leukocytes following acute ingestion of chromium in drinking water. Carcinogenesis 17: 1971-1977 (1996).

Lan L, Nakajima S, Oohata Y, Takao M, Okano S, Masutani M, Wilson SH, Yasui A. In situ analysis of repair processes for oxidative DNA damage in mammalian cells. Proc Natl Acad Sci USA 101(38): 13738-13743 (2004).

Macfie A., Hagan E. and Zhitkovich A. Mechanism of DNA-protein cross-linking by chromium. Chem. Res. Toxicol. 23: 341-347 (2010).

Montel-Hagen A, Kinet S, Manel N, Mongellaz C, Prohaska R, Battini JL, Delaunay J, Sitbon M, Taylor N. Erythrocyte Glut1 triggers dehydroascorbic acid uptake in mammals unable to synthesize vitamin C. Cell 132: 1039-1048 (2008).

Nikiforov YE, Nikiforova MN, Gnepp DR, Fagin JA. Prevalence of mutations of ras and p53 in benign and malignant thyroid tumors from children exposed to radiation after the Chernobyl nuclear accident. Oncogene 13: 687-693 (1996).

Nualart FJ, Rivas CI, Montecinos VP, Godoy AS, Guaiquil VH, Golde DW, Vera JC. Recycling of vitamin C by a bystander effect. J Biol Chem. 278(12): 10128-10133 (2003).

Pillay K, von Blottnitz H, Petersen J. Ageing of chromium(III)-bearing slag and its relation to the atmospheric oxidation of solid chromium(III)-oxide in the presence of calcium oxide. Chemosphere 52(10): 1771-1779 (2003).

Quievryn G. and Zhitkovich A. Loss of DNA-protein crosslinks from formaldehyde-exposed cells occurs through spontaneous hydrolysis and an active repair process linked to proteosome function. Carcinogenesis 21: 1573-1580 (2000).

Quievryn G., Peterson E., Messer J. and Zhitkovich A. Genotoxicity and mutagenicity of chromium(VI)/ascorbate-generated DNA adducts in human and bacterial cells. Biochemistry 42: 1062-1070 (2003).

Reynolds MF, Peterson-Roth EC, Bespalov IA, Johnston T, Gurel VM, Menard HL, Zhitkovich A. Rapid DNA double-strand breaks resulting from processing of Cr-DNA cross-links by both MutS dimers. Cancer Res. 69(3): 1071-1079 (2009).

Slade R, Crissman K, Norwood J, Hatch G. Comparison of antioxidant substances in bronchoalveolar lavage cells and fluid from humans, guinea pigs, and rats. Exp Lung Res. 19(4): 469-484 (1993).

Standeven A.M. and Wetterhahn K.E. Ascorbate is the principal reductant of chromium (VI) in rat liver and kidney ultrafiltrates. Carcinogenesis 12: 1733-1737 (1991).

Standeven A.M. and Wetterhahn K.E. Ascorbate is the principal reductant of chromium(VI) in rat lung ultrafiltrates and cytosols, and mediates chromium-DNA binding in vitro. Carcinogenesis 13: 1319-1324 (1992).

Stern A.H. A quantitative assessment of the carcinogenicity of hexavalent chromium by the oral route and its relevance to human exposure. Env. Res. 110: 798-807 (2010).

Suchy B, Waldmann V, Klugbauer S, Rabes HM. Absence of RAS and p53 mutations in thyroid carcinomas of children after Chernobyl in contrast to adult thyroid tumours. Br J Cancer. 77(6): 952-955 (1998).

Sugiyama M., Patierno S. R., Cantoni O. and Costa M. Characterization of DNA lesions induced by CaCrO4 in synchronous and asynchronous cultured mammalian cells. Mol. Pharmacol. 29: 606–613 (1986).

Sun H, Zhou X, Chen H, Li Q, Costa M. Modulation of histone methylation and MLH1 gene silencing by hexavalent chromium. Toxicol. Appl. Pharmacol. 237(3): 258-266 (2009).

Tsapakos M. J., Hampton T. H. and Wetterhahn K. E. Chromium(VI)-induced DNA lesions and chromium distribution in rat kidney, liver, and lung. Cancer Res. 43: 5662–5667 (1983).

Williams D. Cancer after nuclear fallout: lessons from the Chernobyl accident. Nat Rev Cancer. 2(7): 543-549 (2002).

Zecevic A., Hagan E., Reynolds M., Poage G., Johnston T. and Zhitkovich A. XPA impacts formation but not proteasome-sensitive repair of DNA-protein crosslinks induced by chromate. Mutagenesis 25: 381-388 (2010).

Appendix A: Individual Reviewer Comments

POST-MEETING COMMENTS SUBMITTED BY

Janusz Z. Byczkowski, DABT, Ph.D.

Contract No. EP-C-07-024
Task Order No. 92
March 3, 2011

Toxicological Review of Hexavalent Chromium

Post-meeting

Answers to General Questions

G1.

Logical - yes; clear - mostly; concise - no, as there is a very large body of information covered by this Toxicological Review.

While the scientific evidence of both noncancer and cancer hazards of oral exposures to Cr^{+6} has been appropriately reviewed and synthesized, the conclusions and numerical derivation of toxicity values are mostly based on strict (default) interpretation and literal application of U.S EPA guidelines, rather than on the current scientific understanding of the mode of action of Cr^{+6}.

G2.

It seems that up to date (around the year 2009), all important studies (and some unimportant too) have been already covered by this Toxicological Review. However, I strongly suggest that the results of study presented at the workshop by ToxStrategies, Inc., and especially the physiologically based pharmacokinetic (PBPK) modeling by Summit Toxicology, LLP., should be included in the revised Toxicological Review of Hexavalent Chromium document.

Answers to Chemical-Specific Charge Questions:

(A) Oral Reference Dose (RfD) for Hexavalent Chromium

A1.

The two-year drinking water study by NTP (2008) seems to be the most comprehensive from all available chronic bioassays, and thus, it is suitable as the basis for derivation of the RfD.

However, there is still some concern regarding the selection of the NTP study for developing of RfD (this document states - C.f. P. 85, L# 1: "*...Urinalysis showed dose-related decreased volume and increased specific gravity, consistent with decreased water intake. NTP (2007) suggested that decreased water intake was due to decreased palatability...*" and then, P.89. L# 34: "*...Drinking water consumption was reduced...*")

Thus, the reduced drinking water consumption, and consequently at least partial dehydration, may have increased the osmolality of gastrointestinal fluid, which could be a significant confounder in the chronic

toxicity study, even though (according to the quotation on P. 120) the Technical Report by NTP (2008) attempted to dismiss such a concern.

A2.

Diffuse hyperplasia, in itself, is considered to be a physiological (normal) response to several stimuli, as the cells of a hyperplastic growth remain sensitive to normal regulatory control mechanisms. Such a physiological proliferation of cells may be secondary to several pathological factors (e.g., the increased osmolality, changed pH, infection by Helicobacter, etc). Still, the proliferation in "diffuse hyperplasia" is a normal process - although, it may be generated in response to abnormal condition (in contrast to neoplasia, where the proliferation in itself becomes abnormal). On the other hand, the hyperplasia is a common early preneoplastic response to potentially carcinogenic stimuli.

Considering its rather ubiquitous and nonspecific character, it is remarkable that even no single case of epithelial hyperplasia was found during the two-year study by NTP (2008) in duodena of as many as 100 male and female control mice. Taking this result at a face value, it may be assumed that the diffuse epithelial hyperplasia in duodena could be an early biomarker of oral exposure to Cr^{+6}, at least in the two-year NTP (2008) bioassay study.

However, another end point - the pathological changes in the liver were noted in the same study with significantly increased frequency at the lowest dose employed. Even though they may be considered somewhat less "specific" than the epithelial hyperplasia (because they were observed at low frequency also in controls), but perhaps they could be more relevant to the systemic toxicodynamics of Cr^{+6} - appearing further away from the portal of entry then the hyperplasia and apparently being less sensitive to the potentially confounding effects of the reduced drinking water consumption.

A3.

The benchmark dose modeling was applied in accordance with the *U.S. EPA Benchmark Dose Technical Guidance Document (2000)* in the prescribed manner. However, the modeled BMD, derived from the diffuse epithelial hyperplasia, was supported only by the three dose-points (including zero dose). In contrast, the pathological changes in the liver were consistently fitted, for both female rats and mice with Log-logistic model which included all the five data points (including controls), and thus, they could be considered to be robust dose-response adverse effects for derivation of BMD. So, the pathological changes in the liver may be used as alternative end points for derivation of BMD.

A4.

While the uncertainty factors (UFs) were applied in accordance with the U.S. EPA guidelines, the use of the UF = 10 for extrapolating the toxicity value based on effects in the portal of entry of Cr^{+6} in animals to

predict GI effects in humans, seems to be problematic. Thus, both the reductive capacity of human gastrointestinal fluids and the antioxidant protection of human tissues exceed those in mice. So, adequately nourished and hydrated humans should be less vulnerable than mice to the adverse GI effects of oral exposure to Cr^{+6}, rather then 10 times more sensitive, as the U_A=10 may suggest.

(B) Carcinogenicity of Hexavalent Chromium

B1.

Even though the U.S. EPA (2005) guidelines were applied appropriately, there is no direct evidence of dose-dependent carcinogenicity by orally administered Cr^{+6} in humans (C.f. P. 236, L# 33: "*...EPA concluded that the exposure-response analyses presented by Zhang and Li (1997), Beaumont et al. (2008), and Kerger et al. (2009) are not based on the quality of data that is needed to support a conclusion regarding the presence or absence of a dose-response among the observed cancer rates in these villages. The other epidemiologic studies did not find a significant correlation between hexavalent chromium concentrations in drinking water (or proximity to the source of hexavalent chromium soil contamination) and cancer...*")

The classification of hexavalent chromium as "*likely to be carcinogenic to humans by the oral route of exposure*" is based on carcinogeniesis observed only at the highest dose levels employed in animal studies. Therefore, it may, or may not be relevant to humans at environmentally relevant exposure concentrations.

B2.

The positive laboratory results of mutagenicity tests do not prove genotoxicity and are not necessarily biologically relevant to humans exposed *in vivo* to the environmentally relevant concentrations of Cr^{+6}.The mutagenicity results from humans occupationally exposed to Cr^{+6} (Table 4-25) were inconsistent (this document states - C.f. P. 178, L# 14: "*...In general, associations between hexavalent chromium exposure and mutagenicity in workers are uncertain...*") and actually, the results presented in the document did not prove the direct genotoxic mode of action of Cr^{+6} i*n vivo*.

An alternative, indirect mode of action seems plausible to this reviewer. As discussed in Section 4.5.2., the intracellular products of one-electron reduction of Cr^{+6} *in vivo*, causing oxidative stress and free-radical damage to cellular macromolecules, seems to be responsible for initiating carcinogenicity at relatively low dose levels. Erosion of GI tract mucosa with inflammation that followed at high Cr^{+6} dosage (discussed in section 4.2.1) apparently caused promotion in the lesions, which eventually progressed to benign and malignant tumors. Importantly, such a mode of action implies a threshold phenomenon, *i.a*. due to antioxidant protection of cells.

B3.

Apparently, the two-year drinking water study by NTP (2008) is the only, up to date, appropriate cancer bioassay of Cr^{+6} by oral route (this document states - C.f. P. 224, L# 24: "*...No other adequate studies of hexavalent chromium carcinogenicity by ingestion are available...*")

B4.

Even though in the NTP (2008) bioassays, only the exposures to the two highest doses of Cr^{+6} in mice and to the highest one dose in rats produced statistically significant carcinogenicity, the selection of this study for derivation of cancer slopes seems to be justified by the description provided in the document.
Regarding the (common) practice of combining adenomas and carcinomas in modeling cancer risk, an explanation should be provided that the adenoma is a benign tumor of epithelial tissue with the tendency to become malignant, thus it may - or may not - lead to cancer.
Also, the results of NTP (2008) cancer bioassay could be interpreted with the assumption of a threshold.

B5.

The oral cancer slope factor was estimated in accordance with the U.S. EPA (2005) guidelines, but to this reviewer the linear cancer extrapolation seems inconsistent with the prior noncancer RfD modeling. While the early preneoplastic epithelial hyperplastic lesions have been modeled as a threshold phenomenon in derivation of RfD, the resultant neoplastic lesions were modeled as a no-threshold linear phenomenon in derivation of cancer slope factors. There seems to be some contradiction, as a threshold-bearing precursor cannot result in the no-threshold adverse effect.
In addition, the allometric scaling of cancer slopes, extrapolated from animals to humans based on body weight (to the 3/4 power), seems to be inconsistent with the portal of entry site of action in the GI tract. Typically, this is the local concentration of a chemical at the target site/target tissue that drives an adverse response. Therefore, more appropriate could be a physiologically based pharmacokinetic (PBPK) scaling based on animal-to-human ratio of differences between the rate of absorption and the rate of reduction of Cr^{+6} to Cr^{+3} in gastrointestinal tract contents, as well as the rate of loss of Cr^{+6} from intestinal tract contents to the feces.
It seems that appropriately developed PBPK model which recognizes physiological specificity of the different segments of gastrointestinal tract in animals and humans, as presented at the workshop by Summit Toxicology, LLP., may be critical in understanding the mode of action of orally administered Cr^{+6} and in quantitative evaluation of its effects.

Additional Comments:

Typos and errors *that should be corrected in the revised version*:

P. 24, L# 4: "...direct measurement of residual Cr(VI) in the *calorimetric* reaction..."
Please change to: "... colorimetric..."
P.26, L# 26: "...Michaelis-Menten uptake kinetics..."
Please change to: "...Michaelis-Menten-type uptake kinetics..."

P. 51, Figure 3-6, it would help the reader if non-standard abbreviations were explained in the figure legend - e.g., *DSB*, *SSB*, etc.

P. 52, L# 38: "...to describe kinetics *of* humans..."
Please change to: "...kinetics in humans..."

P. 84, L# 36: "...*colestasis*, as other markers of *colestasis*..."
Please change to: "...cholestasis..."

P. 229, L# 33: "...(see Section *4.6.3.4*)..."
Such a section does not exist in the reviewed document.

P. 244, L# 7: "...*Bukowksi*, JA..."
Please change to: "...Bukowski..."

POST-MEETING COMMENTS SUBMITTED BY

Joshua W. Hamilton, Ph.D.

Post-Meeting Reviewer Comments on
EPA Draft Toxicological Review of Hexavalent Chromium
(dated September 2010)

Joshua W. Hamilton Ph.D. 6/12/2011
Senior Scientist, Bay Paul Center, Marine Biological Laboratory
Professor (MBL), Pathology and Laboratory Medicine, Brown University

General Charge Questions:

G1. Is the Toxicological Review logical, clear and concise? Has EPA clearly presented and synthesized the scientific evidence for non-cancer and cancer hazard?

G2. Please identify any additional studies that would make a significant impact on the conclusions of the Toxicological Review.

Reviewer Response:

In general, the report is a concise presentation of the vast primary literature for chromium toxicology and carcinogenicity and the writing is generally clear. However, there are specific sections with deficiencies as noted below in detail, and many other sections and specific comments that are not logical. In many cases a statement in one section is either not a logical extension of the data presented, or is in opposition to a statement elsewhere. Overall, the greatest concern is with the logic regarding the choice of a mode of action (MOA), which is the basis for many of the subsequent assumptions that are made, the default values that are chosen, and risk assessment modeling that follows from these choices. In this reviewer's strong opinion – and in the consensus opinion of the external reviewers who are experts in this area and who discussed this at the May 12, 2011 meeting – Cr(VI) is highly unlikely to act via a mutagenic mode of action in vivo. Rather, a careful review of existing information, as well as emerging studies all strongly indicate that the likely MOA involves a threshold mechanism that supports both the physiological uptake-reduction model of DeFlora and the cellular uptake-reduction model of Wetterhahn that were previously proposed. The current EPA draft document concludes that chromium(VI) acts via a mutagenic MOA by all routes of exposure, a conclusion that is illogical given the current state of knowledge of chromium biology and toxicology as already presented in this draft report, and also based on the recently emerging data from a series of 90-day rodent MOA studies sponsored by the American Chemistry Council (ACC). This is the most important and central point since the choice of a mutagenic MOA then drives all other considerations in this document. Specific areas of concern are outlined by chapter and section below in detail in a combined response to G1. and G2.

Chapter 1

EPA should include more definitive information about the literature it reviewed that contributed to this draft report. It currently states that the relevant literature was reviewed through September 2010 but the external reviewers all noted at the May 12 meeting a number of gaps in the literature being cited in the current draft. In addition, it would be helpful to know how many studies the EPA identified as being part of the chromium literature, how many it reviewed, how many it set aside or did not review, what criteria were used to include or exclude a study, etc. For example, a statement such as: *"The EPA identified 26,839 peer-reviewed scientific publications in PubMed from 1950 through September 2010 using the keyword 'chromium.' Of these, 9,456 were determined to be relevant to the current draft based on the criteria of covering aspects of chromium biology, toxicology, environmental chemistry and epidemiology."*

Chapter 2

On page 14 the draft states that,*"Natural occurrence of hexavalent chromium is rare …"* This statement should be qualified since geochemical surveys by the U.S. Geological Survey and others have indicated that there are background levels of naturally occurring Cr(VI) in most groundwater, and that these levels are typically in the range of 2-5 ppb but can be up to 3-5 times higher than that in certain areas. Likewise recent reports of Cr(VI) levels in soil and house dust indicate that there are natural sources, or at least sources that cannot be identified as being specifically anthropogenic, that contribute to the background levels and background exposures of people throughout the U.S. A more detailed literature review and discussion regarding background levels of Cr(VI) in soil, air and water, including more up-to-date information on such levels, should be included in this chapter to provide context for subsequent discussions regarding human exposure through drinking water. Keep in mind that, until recently, analytical methods to detect total chromium and to speciate chromium(VI) accurately were difficult, with higher detection levels than many natural sources contain. Further, speciation of chromium(VI) at very low levels has been very problematic until recently, so much of the older environmental data are inaccurate or had non-detect values where reanalysis has revealed widespread chromium(VI) background levels in the environment. This is of importance not only for understanding the potential contribution of these background levels to total chromium exposure but also in setting practical regulatory levels. Clearly it is of little value for the EPA to calculate a maximum contaminant level (MCL) or public health goal (PHG) that is lower that typical background levels since this would be virtually impossible to achieve by most public drinking water systems with limited resources, and because such background levels are unlikely to represent a significant health risk (see discussion below).

Chapter 3

This is an important chapter that would greatly benefit from reorganization and from synthesis of information. A number of studies are described here sequentially, and for several of these the draft document speculates on possible mechanisms. However, the order of presentation is not logical, and there is little in the way of more global synthesis of results and the conclusions that can be drawn – or those areas that remain controversial or poorly understood – while there are several areas where a statement in one section is contradicted by a statement elsewhere. Because the toxicokinetics of chromium is so central to its biology and toxicology, this is a critical chapter for its overall evaluation and therefore this chapter should be revised.

Regarding organization, Section 3.3 is extremely important for understanding the studies described throughout the remainder of the chapter, providing context for extracellular versus intracellular reductive metabolism. It is suggested that this section should be moved to just before or immediately after the current Section 3.1. Likewise, Section 3.6 on Cr(III) and its nutritional benefit and essentiality is extremely important for understanding how the body normally absorbs, distributes and excretes chromium. From the standpoint of human environmental exposures, Cr(VI) is primarily, but not exclusively an anthropogenically derived form of chromium that is principally encountered and used in occupational settings, but humans and all other life forms have been dealing, both physiologically and biochemically, with Cr(III) and reduction of background levels of Cr(VI) for the entire history of life on Earth. It is recommended that Section 3.6 should be moved to the beginning of this chapter and greatly expanded. The current section on this important topic is extremely short, superficial, and inaccurately presents this subject as an area of controversy in the field.

Cr(III) nutritional biochemistry has been extensively studied over the past fifty-plus years with a large and robust literature. Only two toxicologists are cited as the sources of the "current debate" about this, whose individual views have been well aired in their review articles but which represent views that are generally considered to be well outside the mainstream of toxicology and nutritional biochemistry. Citing their views so prominently in this short section does not balance well with the wealth of studies and numerous other investigators over decades who have concluded that there is a beneficial role of Cr(III) in human and animal nutrition and have demonstrated an underlying biochemistry and physiology that supports this role. This section should be greatly expanded and treated in a more balanced fashion since it sets the stage for understanding how Cr(III) is treated by the body in all aspects of toxicokinetics, and therefore also provides valuable insight into Cr(VI) toxicokinetics that largely explains much of the experimental literature on chromium disposition in intact animals and humans as discussed in more detail below. See for example reviews by W Mertz (J Nutr 1993, 123:626-633; Nutr Rev 1995, 53:179-185), RA Anderson (Reg Tox Pharm 1997, 26:S35-41), HC Lukaski (Ann Rev Nutr 1999, 19:279-302) and JB Vincent (J Am College Nutr 1999, 18:6-12) for summaries of this earlier literature.

Some of this information is alluded to – for example, the end of the primary paragraph on page 44 beginning with *"Aitio et al. (1988) developed …"*, which represents an extremely important body of literature

on Cr(III) toxicokinetics and which should be moved forward with section 3.6 and greatly expanded by citing other relevant literature. There are dozens of studies of Cr(III) uptake and kinetics. The current chapter leaves the impression that Cr(III) gastrointestinal (GI) uptake is uniformly low, and that chromium is not normally found above background levels in urine, therefore if one administers Cr(VI) and sees a dose-dependent increase in blood, urinary or tissue levels it is evidence of Cr(VI) uptake as Cr(VI). But there are a number of uptake studies of chromium picolinate and other natural and man-made Cr(III) complexes, as well as Cr(III) uptake in chromium-sufficient versus chromium-deficient diets, that defy this simple interpretation. For example, one study described on page 45 by Kerger et al. (1997) reported that *"ingestion of chromium picolinate resulted in significantly elevated urine concentrations such that participants routinely exceeded background."* Similar elevations have been reported for chromium picolinate in the nutritional literature. Likewise, other studies have shown an inverse correlation between Cr(III) levels in diet and the uptake, distribution, tissue storage and excretion of Cr(III), indicating that the body tightly regulates Cr(III) kinetics to maintain a steady-state body burden and availability of the nutritionally active form of Cr(III), and can actively take up Cr(III) from the GI when the body senses that it is deficient, or decrease uptake and increase excretion when internal stores are sufficient or exceeded.

A related and key concept that is alluded to in this section but never addressed directly is the behavior of chromium in serum and red blood cells (RBC). It has been well established, beginning with RBC labeling studies going back to the 1950's in which RBC are incubated with Cr(VI) ex vivo, that Cr(VI) is readily taken up by RBC, rapidly reduced, and in the process forms highly stable chromium adducts on hemoglobin and other macromolecules which are very long lived, essentially remaining intact for the lifetime of the cell. In this way the half-life of RBC in humans and experimental animals was established (with human RBCs having a half-life of ca. 110-120 days) and this tool has also been used to look at RBC turnover. This is alluded to on page 27, where it states that *"The partitioning of hexavalent chromium from plasma into erythrocytes is significant. It has been used as a biomonitoring endpoint … and is responsible for the observed residence time of chromium in whole blood …"* Conversely, in the human and animal studies that were described in Chapter 3, it has been consistently shown that when Cr(VI) is administered orally or by gavage, there is a transient increase in both serum and RBC levels which rapidly return to baseline. Thus, it is highly unlikely that the chromium in the blood of these animals and humans was Cr(VI), or else the RBC would have been stably labeled. It was noted on page 45, for example, in describing the human studies of Paustenbach et al. (1996) where there was oral exposure to Cr(VI) that *"both plasma and RBC chromium concentrations returned rapidly to background levels within a few days, again suggesting that concentrations of 10 mg Cr(VI)/L or less in drinking water of humans appears to be completely reduced to Cr(III) prior to systemic distribution."* This is an extremely important experimental observation in humans – and one of the most important statements in this draft document -- that directly addresses the issue of whether Cr(VI) is taken up by the human GI as Cr(VI) and whether it survives as Cr(VI) in the circulation.

Similar results were seen in the Sutherland et al. (2000) rat study in which the blood kinetics, and lack of chromium increases in brain or other distal tissues argued strongly that it was Cr(III) rather than Cr(VI) that was taken up by the gut. Were this not the case, they should have observed stable chromium labeling of the RBC and elevated chromium levels in distal tissues. But they reported the opposite, and this also strongly indicates in this key rodent study that Cr(VI) failed to survive as Cr(VI) in crossing into the bloodstream from a GI exposure. And elsewhere in Chapter 3, similar results are reported that would lead to the same conclusion, yet the text alludes to transient RBC uptake as possible evidence that Cr(VI) is being taken up from the GI tract as Cr(VI). This is a highly flawed, illogical argument that appears throughout this document. Likewise it is now well known that there are specific uptake, transport and storage mechanisms for nutritionally active Cr(III) that must be taken into account in any measurements of chromium in the blood or other tissues. In fact, none of the studies presented in Chapter 3 provide any direct evidence that any Cr(VI escapes the GI tract as Cr(VI) except perhaps where normal gastric reduction is bypassed or the bolus doses are so large as to completely overwhelm the reductive capacity of the gut, which is likely what occurred in the NTP (2008) study. This should be more clearly described in this chapter, perhaps with a new section prior to current section 3.5 that presents the theoretical PBPK model since this model also critically relies on how one interprets the in vivo data.

Specific edits suggested below are based on these concerns (changes underlined):

- Page 24, Line 24 – *"…for the chromium administered as hexavalent …"*
- Page 25, Line 7 – *"…of the chromium administered as hexavalent …"*
- Page 26, Line 3 – *"…. Generally, absorbed chromium is …*"
- Page 26, Line 9 – *"… toxicokinetics of chromium, …."*
- Page 26, Line 16 – *"… comparing administration of Cr(III) ..."*
- Page 30, Line 2 – *"… bioavailability of chromium administered as …"*
- Page 35, Lines 7-11 – two sentences beginning with *"The reason for the higher …"* This is highly speculative and should be deleted. This could all be Cr(III) rather than Cr(VI) using the arguments above.
- Page 36, Lines 25-28 – end of sentence beginning with *"… indicating that a portion of the Cr(VI) escaped extracellular reduction …"* This is also highly speculative and is actually counter to the data presented, which clearly show transient blood levels that are indicative of Cr(III) distribution, not Cr(VI), as more appropriately alluded to in Lines 32-34 where is says *"Brain, ovarian, and whole-blood concentrations were below detection limits in all exposed groups. The lack of concentrations in whole-blood was attributed to rapid delivery of Cr to tissues and clearance of plasma Cr."* The lack of stable blood chromium clearly indicates that it could not have been absorbed as Cr(VI) or else the RBC would have shown significant and stable elevations.
- Page 38, Line 13 – *"… did not alter GI uptake appreciably at these concentrations."*

- Page 41, Line 1 – *"Chromium is capable of crossing the placenta."*
- Page 41, Line 15-17 – *"Absorption and elimination of chromium was evaluated …. Following ingestion by human volunteers of either trivalent or hexavalent chromium in single or multiple drinking water doses."*
- Page 42, Lines 23-26 – This statement beginning with *"Because the Cr(VI) increases …"* is incorrect. Increases in RBC chromium that are stable over time, with the same half-life as the RBC, would be indicative of uptake of chromium as Cr(VI) by the RBC, but if the increase in RBC chromium is transient, as in this case, then it cannot be due to Cr(VI) in the blood but is most likely a Cr(III) complex that is carried by the RBC but not covalently bound. Evidence for this is cited elsewhere in this chapter (see middle of page 44 and top of page 46, for example) and in the broader Cr(III) literature. The draft report is illogical since it currently argues this issue both ways depending on the study being discussed.
- Page 42, Lines 30-35 – These two sentences beginning with *"The higher bioavailability …"* are highly speculative and well outside the boundaries of the actual data presented and discussed above. If the report is going to speculate, it should pull all this into a separate section and synthesize it across all the studies that were cited. Otherwise delete these speculations since they do not have a factual foundation.
- Page 49, last line – *" … chromium administered as Cr(VI) distributes …"*
- Page 58, Lines 2-3 – *" … greater percentage of chromium administered as Cr(VI) than Cr(III) is absorbed."* Delete sentence reading *"This implies that some Cr(VI) escaped reduction …"* since this is not implied by the actual experimental data based on the above arguments.

Taken together, these studies, as argued above, lead one to only one logical conclusion: Cr(VI) is not taken up by the gut as Cr(VI), nor does it survive as Cr(VI) in the systemic circulation in these studies. It is only by greatly exceeding the normal doses and reductive capacity of the gut that one can see any signs of Cr(VI) surviving to reach other tissues. Therefore there is a clear biochemical barrier, a threshold, to Cr(VI) uptake and systemic exposure under normal physiological conditions. This must clearly be taken into account in any analysis of MOA and resulting risk assessment for this toxicant.

Chapter 4

Liaoning Province, China studies – At the beginning of this chapter (Pages 68-76) the EPA presents an extensive review and discussion of the various reports related to the study of populations in China near a site of chromium-contaminated drinking water from a nearby industry. Depending on the authors and methods used – particularly certain assumptions regarding age adjustment, use of an urban area as a control population, and exposure estimates -- the reports of this data set either find no statistical association between stomach cancer incidence or a modest elevation. The other epidemiology studies cited in this chapter report

no statistical correlation between drinking water chromium exposure and cancer incidence. It is of considerable concern to this reviewer that the EPA has chosen in some places to highlight this one positive report and elevate it to the level of their major recommendations, such as on Page 239 of Chapter 6 (Major Conclusions) where they state that there is *"evidence of an association between oral exposure to hexavalent chromium and stomach cancer in humans."* Yet elsewhere, they state, appropriately, that *"this risk has not been established in other populations exposed to drinking water contaminated with hexavalent chromium. The epidemiology data are not sufficient to establish a causal association between exposure to hexavalent chromium by ingestion and cancer."* (Page 201, Lines 20-23; and similar language on Page 205, Lines 33-36)

It is inappropriate to "cherry-pick" a single study – indeed, a single treatment among three treatments of the same study – if this is going to influence their major recommendations regarding the human carcinogenicity of Cr(VI) via ingestion. Many of my other comments and edits relate to this concern, as well as previous concerns regarding the toxicokinetics of Cr(VI) via ingestion and the misinterpretation of those results with respect to mode of action and carcinogenic potential in animals and humans as detailed below. This reviewer feels strongly that the Liaoning Province study and its various treatments should be set aside, since the initial study (first reported in 1987) is highly flawed by today's epidemiology standards, there is a lack of information that would allow others to reevaluate this study in the manner that would most directly address the potential correlation, and the three subsequent treatments by original authors Zhang and Li (1997), by Beaumont et al. (2008) and by Kerger et al. (2009) -- each of which makes slightly different assumptions in order to fill critical data gaps -- reached different conclusions. It is also important to point out that this population was exposed not just to extremely high levels of Cr(VI) but also to industrial effluent which contained high levels of a number of other chemicals of concern including sulfates, acids and other toxicants. Thus, even if an association was found, it is not possible to attribute this to Cr(VI) per se and could be the result of either other contaminants or a statistical anomaly based on the high rates of stomach cancer in China which are diet- and province-related. For these reasons this study and its series of treatments cannot be the basis for risk characterization or risk assessment, and should be set aside.

Section 4.4 also requires extensive revision and expansion. The first paragraph reports only briefly on the extensive literature examining occupational exposures and cancer, principally lung cancer. This section, and this literature, should receive a much more extensive treatment since these studies are our best data regarding human exposures to Cr(VI). It should be noted that in addition to inhaling extremely high levels of Cr(VI) prior to the advent of industrial hygiene practices in the 1960's and 1970's (levels as high as several mg chromium(VI) per cubic meter) these workers had extensive dermal exposures, and also had extensive ingestion exposure since it is well known that individuals exposed to high levels of dust swallow a large fraction of what they inhale either through direct deposition in the mouth, nose and throat, and via mucocilliary clearance of the pulmonary system. Thus, these occupational epidemiology studies are

extremely important in understanding the toxicology of chromium(VI). Several key points will be made below citing a few critical studies, but the EPA should more thoroughly review this entire literature.

The U.S EPA's current risk assessment for chromium(VI) via inhalation is based principally on the studies by Mancuso of older worker populations through the 1960's prior to the advent of modern industrial hygiene practices. Similar risks were observed in other occupational studies of workers from the 1940's through 1970's who were exposed to much higher levels of chromium than are encountered in occupational settings today, such as those by RB Hayes et al. (Intl J Epi 1979, 8:365-374; Am J Indust Med 1989, 16:127-133), JM Davies (Br J Indust Med 1984, 41:158-168), T Sorahan et al. (Br J Indust Med 1987, 44:250-258), and R Kishi et al. (Am J Indust Med 1987, 11:67-74). Subsequent epidemiology studies of workers exposed to lower levels of chromium since the 1960's have not only provided estimates of risk at these high doses, but have also provided important information as to lower occupational levels at which no increases in lung cancer or other health effects were observed. These more modern studies have also taken into account other factors, such as accounting for cigarette smoking and other confounding variables, whereas many of the older studies did not control for these important confounders (see for example, KD Rosenman, Am J Indust Med 1996, 29:491-500). It should be noted that, where it was reported, virtually all the lung cancer cases in these occupational studies occurred in smokers (see for example Pastides et al., Am J Indust Med 1994, 25:663-675; T-C Aw, Reg Tox Pharm 1997, 26:S8-12). These studies not only provide better estimates of the actual health risks attributable to occupational chromium exposure, but also an estimate of a practical threshold below which we would predict either no effects, or risk of effects that are so low that it cannot readily be detected even in large populations of exposed people.

Gibb et al. performed a follow-up study (Am J Indust Med 2000, 38:115-126) of a worker population in Baltimore MD that had previously been studied by Hayes et al. (Int J Epi 1979, 8:365-374) in examining the relationship between chromium(VI) exposure and cancer incidence. In the Gibb study, the workers were stratified according to different levels of cumulative exposure to chromium, allowing a more detailed examination of the potential dose-response. Cumulative exposure was expressed as $\mu g/m^3$-years (1,000 ng/m^3-years), integrating both the chromium level and the total time of exposure at that level. This is similar to smoking data that express cumulative dose as "pack-years" and is based on the observation that the risk of a 40 pack-year smoker who used 1 pack per day for 40 years is similar to that of a 40 pack-year smoker who used 2 packs per day for 20 years. In the Gibb study, the lowest quartile of workers had exposure to chromium between 0 and 1.5 $\mu g/m^3$-years (1,500 ng/m^3-years). This group had an observed/expected lung cancer ratio of 0.96, i.e., it was slightly less than expected from the comparison population (the general population of Maryland) that had no occupational chromium exposure.

Pastides et al. examined a group of chromate production company workers in North Carolina (cited above), focusing on the possible differences in risk between cohorts of workers who were exposed to chromium under the older conditions and processes of the 1940's through 1960's and those who began work

after 1971 in a modernized factory in which both the chemical process and the exposure levels to chromium had been modified. They found a slightly increased risk of lung cancer, proportional to exposure, in the older cohort working under the higher dose exposure conditions, as had been reported previously in other studies. However, the workers in the modernized factory had no excess of lung cancer, all cancers, heart disease, or all causes of death over an 18 year period. Personal monitors for the workers indicated that the chromium(VI) levels were all below 50,000 ng/m^3, and most were below 25,000 ng/m^3, with the majority in the range of 500-10,000 ng/m^3. Average duration of employment was 9.5 years, such that cumulative dose would have averaged 4,750-95,000 ng/m^3-years. Dividing the workers into two groups of exposure, i.e., those working less than 10 years versus those working more than 10 years, indicated no difference in mortality, further suggesting that these workers had no significant increase in cancer or other health risks from either the higher or lower chromium exposures. Similarly, Aw reported (cited above) that workers in the more modernized plants who were occupationally exposed to chromium since the 1960's showed no increase in disease risk, as was also noted by S Langard et al. (Br J Indust Med 1990, 47:14-19).

WJ Blot et al. performed a large and comprehensive study (J Occup Environ Med 2000, 42:194-199) of a group of 51,899 workers of the Pacific Gas & Electric (PG&E) Company. A sub-set of 3,796 these workers had been exposed occupationally to chromium(VI), either as gas generator workers or trainees at the Kettleman CA station which used chromium as a rust inhibitor in cooling tower water at PG&E natural gas transfer stations from the 1950's through the 1980's. Examination of these workers for specific cancers, all cancers, specific non-cancer diseases, and all diseases indicated no increased incidence in any adverse health outcomes in relation to chromium exposure. In fact, the total cancer and lung cancer standardized mortality ratios (SMRs, observed/expected ratios) were 0.64 and 0.81, and 0.55 and 0.57, respectively, for these two groups of chromium-exposed workers, which was less than those of the overall PG&E worker group and substantially less than those of the general California population against which they were compared. SMRs for all causes of death were also low (0.79 and 0.68, respectively). Likewise, JD Boice et al. performed a large and comprehensive study (Occup Environ Med 1999, 56:581-597) of a group of 77,965 workers at an aircraft manufacturing plant in California. A sub-set of 3,634 of these workers were exposed to chromates and other chemicals as part of airplane production for a total of 88,224 person-years of exposure and a mean of 24 working years per person of exposure. The SMR for total cancers was 0.93, and the SMR for lung cancer was 1.02. As with the Blot study, there was no association of any adverse cancer or non-cancer health outcome with chromium exposure in this group, nor did the overall worker population have an increase in overall or specific mortalities as compared to the general population despite exposure to a number of occupationally related chemicals.

Taken together, these occupational studies indicate that, although previous historical exposure conditions were associated with a modest risk of lung and respiratory cancer (average of 2- to 4- fold increased lifetime risk, as compared, for example, to a 10- to 20-fold increased risk for cigarette smokers), more recent

occupational exposures at or below the current regulatory limits indicate that these represent levels that do not elevate cancer risk even for lifetime occupational exposures. Moreover, the previous exposures of concern in workers from the 1930's through the 1960's were at levels that typically exceeded 1,000,000 ng/m^3 and also involved exposure to the most carcinogenic forms of chromates, i.e., the insoluble or slightly soluble forms such as lead chromate, zinc chromate and calcium chromate. The newer lifetime occupational exposure limits -- at which no increase in cancer risk or other health effects has been observed – represent daily exposures that are hundreds to thousands of times higher than would occur in an environmental setting or via U.S. drinking water. There are two other major conclusions that can be drawn from these occupational exposure studies. First, although dermal exposure to chromium(VI) was extensive – particularly prior to the advent of industrial hygiene practices in the 1970's – there is no evidence for increased risk of skin cancer, even in workers where the chromate levels were high enough to burn "chrome holes" in their skin or nasal septum. These chrome holes healed and were not associated with increased skin cancer risk in these workers. This is relevant to the very high doses of chromium(VI) used in the NTP studies and a possible MOA. Second, taken together these occupational studies do not demonstrate an increased risk of GI cancers or other internal cancers, despite the fact that these workers swallowed a significant fraction of the dusts they were exposed to in the air. These data were recently summarized in a meta-analysis published in 2010 (NM Gatto et al., Cancer Epi 2010, 34:388-399).

Other specific edits and comments (changes underlined):

- Page 200, Lines 1-2 – Delete the phrase *"… and evidence of an association between oral exposure to hexavalent chromium and stomach cancer in humans"* which is based on a single study and EPA's selective treatment of this result as discussed above.
- Page 200, Lines 18-20 – *"This study found evidence <u>of a modestly increased incidence of stomach cancer mortality (OR 1.69, CI 1.12-2.44)</u> from 1970 to 1978 …."*
- Page 201, Lines 20-23 – *"… was <u>reported in a re-examination of a single study</u> in JinZhou .."*
- Page 209, last section, Bioavailability – This section is highly flawed in logic and presentation as discussed under Chapter 3. Specific edits are as follows:
    - Page, 209, last two lines – *"Quantitative studies of GI absorption <u>of chromium administered as hexavalent chromium</u> have estimated …"*
    - Page 210, first line – Please note that hexavalent chromium was not measured, and without exception has never been measured systemically as <u>Cr(VI)</u> following GI absorption. It is an assumption that increased chromium uptake to the blood represents Cr(VI) uptake, but it could also represent other forms as discussed in Chapter 3 above. We know that certain forms of Cr(III) are much more readily taken up than others, and it is therefore possible, and perhaps quite likely, that reduction of Cr(VI) in the gut in the presence of organic molecules in the GI lumen leads to formation of complexes that are much more bioavailable than inorganic Cr(III) that is

typically found in food, water and soil. You could also modify this phrase as a separate sentence to read *"This may indicate that not all hexavalent chromium is reduced by the gastric juices of the stomach, or that reduction of Cr(VI) in this environment leads to formation of organic chromium complexes that are more readily absorbed than inorganic Cr(III)."*

- Page 210, Lines 7-12 - Delete the sentence beginning *"Thus, at oral doses within human exposure ranges …*" as per the argument above or modify to include alternative interpretations as suggested above.
- Page 210, Lines 28-30 – End of sentence beginning with *"… and uptake of hexavalent chromium into the tissues … "* delete this phrase or modify it as per the argument above.
- Page 210, last line – Add a sentence after the last sentence on this page reading *"However, none of those studies speciated the chromium that was absorbed systemically, and so the form(s) of chromium in the blood and other tissues is unknown following increased absorption of chromium following ingestion of hexavalent chromium. Therefore, it is not known whether chromium reaches the blood or distal tissues as chromium(VI) at doses relevant to human exposures. The lack of long-term labeling of RBCs by chromium in the animal and human studies argues that little, if any, chromium is absorbed as chromium(VI) under these exposure scenarios."*
- Page 213, Section 4.7.3.5. Lines 3-5 – This statement is completely incorrect; there is no evidence for it as argued above. Delete this, or modify as follows *"Chromium absorbed following ingestion exposure to chromium(VI) may be in forms that can reach the systemic circulation and distal tissues, thereby potentially affecting tissues beyond those at or near the site of entry. However, the form(s) of chromium following such uptake is not known."*

Chapter 5

This reviewer strongly objects to use of a linear low-dose approach for Cr(VI) risk assessment given the clear evidence for a threshold mechanism due to extracellular reduction of chromium at doses of relevance to human exposure via drinking water. The conclusion by EPA of a mutagenic action of chromium – most of which is based on cell culture data where chromium exposures and other parameters were extreme and where metabolism and intracellular exposure are far different than in vivo exposures – should not be the sole basis for use of this standard model which ignores the compelling toxicokinetic data summarized in Chapter 3 of this draft. More importantly, as discussed above for Chapter 4, Cr(VI) is unlikely to act via a mutagenic MOA in vivo, and requires extraordinary experimental manipulation to be positive in cell culture and in vivo mutagenicity studies. While it is clear that Cr(VI) can cause certain forms of DNA and chromosomal damage or other changes, it is not clear whether any of these is pre-mutagenic, and the in vivo data argue strongly against a mutagenic MOA under physiological conditions and normal routes of exposure. The document must more clearly differentiate between genotoxicity – i.e., damage to DNA or chromatin – and mutagenicity

– or frank mutations that may result from DNA damage. Chromium(VI) can induce DNA damage but is a very weak mutagen at best, particularly in vivo. It is far more likely, and most consistent with all available data, that chromium(VI) acts via a non-mutagenic mechanism that involves a clear threshold – two threshold actually, one of which is extracellular and chemical involving reduction of chromium(VI) to chromium(III) and the other of which is biochemical and involves a threshold for cellular effects that lead to cell damage and cell death, resulting in turn in tissue proliferative responses that ultimately increase tumor risk via well known mechanisms of repeated tissue injury, compensatory cell proliferation and re-population.

Given this most likely MOA based on a synthesis of several decades of chromium research, it is therefore inappropriate to use a linear low-dose extrapolation model for assessing risk via the ingestion route of exposure. It is clear from the animal and human studies that there is a threshold for in vivo effects that is based on the strong reducing capacity of the GI tract following oral exposures, and that at normal drinking water concentrations this will effectively protect from any in vivo exposure to Cr(VI) as Cr(VI). Thus, a more appropriate risk assessment method would be to do dose-response modeling from the 2008 NTP study and the more recent ACC-sponsored 90-day MOA studies, and then use an approach similar to that for the RfD to calculate, with appropriate safety factors, a drinking water MCL that is protective based on threshold mechanisms. This should be done for Cr(VI) rather than the current MCL that is for total chromium, but it is likely that an MCL in the range of 50-100 ppb is going to be fully protective, including several uncertainty factors that separate it from the departure point of any likely human health effects for even the most sensitive individuals.

Chromium is an excellent example of an opportunity to apply the concept of evidence-based risk assessment – which the EPA has claimed to be promoting for many years but has not, to date, actually applied in any meaningful risk assessment -- since there is a strong and compelling argument for use of a non-linear, threshold-based mechanism for chromium that logically leads to a real-world risk assessment that is based on that mechanism. Setting aside the 1987 Zhang and Li study and subsequent re-analyses as per the arguments above, there is not a single credible epidemiology study linking exposure to chromium via ingestion with cancer risk or any other long-term health effects, including in the extensive occupational epidemiology literature which includes several decades of extremely high-dose exposure cohorts. And even taking into account the Zhang and Li study, there is not a single peer-reviewed report linking any health effects to chromium(VI) at levels within a hundred-fold of likely environmental exposures via drinking water. The NTP 2008 animal data showed evidence of a cancer increase only at the highest doses, and the more recent ACC studies demonstrated hyperplasia consistent with a non-mutagenic MOA, which is also consistent with a threshold mechanism and which argues against developing a linear cancer slope factor from those data.

Chapter 6

There is considerable disconnect between the conclusions provided in Chapter 6 and the more considered and detailed discussions of the primary data in the previous chapters. The language should be modified to reflect this understanding such that the conclusions and their application to risk assessment of chromium(VI) follow logically from the scientific evidence. Specific edits:

- Page 235, Lines 18-19 – *" … resulting in substantial, and in some cases complete reduction of hexavalent chromium to trivalent chromium depending on the concentration, dose, and precise route and method of exposure."*
- Page 235, Lines 21-26 – *"The extent of absorption of chromium from ingesting hexavalent chromium appears to be determined by both the solubility….. in the GI tract, but ingestion of both trivalent and hexavalent chromium results in systemic uptake of chromium. Trivalent chromium does not readily cross cell membranes except as part of certain organic complexes. Hexavalent chromium, if absorbed systemically, can easily …."*
- Page 235, Lines 27-29 – *"Chromium absorbed systemically from the gut following ingestion of hexavalent chromium is distributed throughout the body. …. If hexavalent chromium is absorbed without extracellular reduction, it can cross cell membranes and, once inside the cell, …."*
- Page 236, Line 3 – *"Chromium absorbed systemically following hexavalent chromium ingestion is eliminated primarily in the urine as trivalent chromium."*
- Page 239, Lines 11-12 – delete the phrase *".. and evidence of an association between oral exposure to hexavalent chromium and stomach cancer in humans."* This is a significant statement to make, and as discussed above in reference to Chapter 4, the Zhang and Li studies (1987, 1997) should not be used to assess human cancer risk for oral exposure to chromium.
- Page 240, Lines 12-14 – As noted for Chapter 5 above, there is considerable concern with the default choice of a linear low-dose extrapolation model for cancer risk.

Chemical-Specific Charge Questions:

(A) Oral Reference Dose (RfD) for Hexavalent Chromium

A1. A two-year drinking water study of sodium dichromate dihydrate in rats and mice (NTP, 2008) was selected as the basis for the derivation of the RfD. Please comment on whether the selection of this study as the principal study is scientifically supported and clearly described. Please identify and provide the rationale for any other studies that should be selected as the principal study.

A2. Diffuse epithelial hyperplasia in the duodenum of female mice was selected as the critical effect for the RfD. Please comment on whether the selection of this critical effect is scientifically supported and clearly described. Please identify and provide the rationale for any other endpoints that should be selected as the critical effect.

A3. Benchmark dose (BMD) modeling was applied to the incidence of diffuse epithelial hyperplasia in the duodenum of female mice to derive the point of departure (POD) for the RfD. Has the BMD modeling been appropriately conducted and clearly described? Is the benchmark response (BMR) selected for use in deriving the POD (i.e., a 10% increase in the incidence of diffuse epithelial hyperplasia) scientifically supported and clearly described?

A4. Please comment on the rationale for the selection of the uncertainty factors (UFs) applied to the POD for the derivation of the RfD. Are the UFs scientifically supported and clearly described? If changes to the selected UFs are proposed, please identify and provide a rationale.

Reviewer Response:

No concerns regarding A1. And A2. This reviewer would point out that the hyperplasia that was chosen as this endpoint, while appropriate for this RfD, is also appropriate for considering the carcinogenic MOA as well, as argued above and below and taking into considering the recently reported ACC studies that should be considered in this regard. This reviewer is not an expert on modeling and cannot comment on A3 in detail. No concerns regarding A4. Uncertainty factors are policy decisions, not scientific ones, and we can neither prove nor disprove any of the assumptions on which they are based nor can we accurately determine when and how such factors might be applied. Based on previous EPA doctrine, these seem to be consistent with previous applications.

(B) Carcinogenicity of Hexavalent Chromium

B1. Under EPA's 2005*Guidelines for Carcinogen Risk Assessment* (www.epa.gov/iris/backgrd.html), hexavalent chromium is *likely to be carcinogenic to humans* by the oral route of exposure. Is the cancer weight of evidence characterization scientifically supported and clearly described?

B2. A mutagenic mode of carcinogenic action by all routes of exposure is proposed as the primary mode of action for hexavalent chromium. Please comment on whether this determination is scientifically supported and clearly described. Please comment on data available for hexavalent chromium that may support an alternative primary mode of action.

B3. A two-year drinking water study in rats and mice (NTP, 2008) was selected for the derivation of an oral slope factor. Please comment on whether the selection of this study for quantification is scientifically supported and clearly described. Please identify and provide the rationale for any other studies that should be considered.

B4. The incidence of adenomas and carcinomas combined in the small intestine of male mice from the NTP (2008) two-year drinking water study were selected to serve as the basis for the quantitative cancer assessment. Please comment on whether this selection is scientifically supported and clearly described. Please identify and provide the rationale for any other endpoints that should be selected to serve as the basis for the quantitative cancer assessment.

B5. The oral slope factor was calculated by linear extrapolation from the POD (i.e., the lower 95% confidence limit on the dose associated with 10% extra risk of tumors of the small intestine in male mice). Has the modeling been appropriately conducted and clearly described?

Reviewer Response:

Regarding B1. and as outlined in the detailed comments under G1. and G2., this reviewer is concerned that the evidence for carcinogenicity is not strong in animals and is not supported by human epidemiology. As also noted in comments under G1. and G2., there is considerable concern with statements by EPA in sections of this draft, particularly under Major Conclusions in Chapter 6, regarding evidence for human carcinogenesis based on a single human epidemiology study that in turn is a re-analysis of another study that lacks critical information that would be useful in fully assessing relative cancer risk. However, based on the current criteria for selection of this designation, it appears to be consistent with EPA doctrine since there is evidence of increased tumors in animals under certain exposure conditions.

B2. There is considerable evidence that Cr(VI) is genotoxic in cell culture and in vitro, and under certain extreme conditions it can also be shown to be mutagenic. However, there is far less support that Cr(VI) is genotoxic or mutagenic in vivo by the oral route of exposure at doses of relevance to humans; conversely, there is considerable evidence that there are protective threshold mechanisms that significantly impact the ability of Cr(VI) to reach target tissues and cause DNA damage under physiological conditions. In addition, while alternative mechanisms are briefly discussed, these are essentially dismissed without extensive treatment. As noted in the document, there is not a large literature on alternative mechanisms, but this is largely because, since the discovery of the genotoxic potential of Cr(VI) some forty years ago, most of the field has only focused on this one aspect, and I suspect it would be very difficult to get peer-reviewed funding to study non-genotoxic mechanisms for this toxicant. It is important to note, however, that there are no reports of increased skin cancer under occupational exposure settings, despite the fact that workers until the past few decades were directly exposed to Cr(VI) on the skin to the extent that they formed “chrome holes” that eventually healed. Yet this direct application and clear signs of chromium reduction and toxicity directly on the skin produced no increased skin cancer risk. Likewise, the occupational exposure literature only recently investigated the role of smoking status in chromium-related lung cancer risk. The increased risk of lung cancer associated with Cr(VI) exposure is modest considering the exposure levels and duration of exposures that span decades, and virtually all of the cancer cases in the epidemiology studies were seen in smokers, suggesting an interaction but one that is very modest. This in turn suggests alternative mechanisms such as inflammation, oxidative damage, damage-induced proliferation, and other mechanisms not directly tied to the ability of Cr(VI) to enter cells and damage DNA. These should considered and explored in more detail, since they are the basis for many of the assumptions regarding the risk of cancer from oral exposure to Cr(VI).

B3. There are no significant concerns about selection of this study, it is clearly the best available study for this type of risk assessment, with the caveats about how these data are interpreted and modeled as discussed elsewhere.

B4. There is concern about selection of these endpoints to represent cancer risk in these animals as the basis for a human risk assessment. The high doses required to induce these lesions are well above a threshold level that would be of concern in humans under normal exposure scenarios, and these almost certainly represent a scenario where natural reductive defense mechanisms were overwhelmed by the doses of chromium used. Taken together with the more recent 90-day ACC sponsored studies, the MOA for chromium(VI) is most likely a non-mutagenic one involving tissue damage and reproliferation, and would only be seen at doses that are unlikely to ever occur in a human exposure setting, particularly via drinking water. Thus, a threshold based risk assessment is most appropriate, similar to the treatment used for the non-cancer endpoints which are likely to be directly related to the cancer MOA.

B5. As discussed in detail under G1. and G2. responses, this reviewer has considerable concerns about the use of a linear low-dose extrapolation model for assessing chromium cancer risk. The evidence, when objectively assessed, strongly argues for threshold mechanisms both in the gut and systemically, and there is little or no evidence that chromium reaches the systemic circulation as chromium(VI) under exposure scenarios of relevance to human exposures. There is no better candidate for departure from these default EPA assumptions than chromium if EPA is serious about evidence-based risk assessment. Many of the public comments that were available to the reviewers just before and after our May 2011 meeting also raise these issues, and the EPA should, in particular, wait until the recently reported 90-day MOA and PK studies are published and available to them, at which point they should give serious consideration to how these new data inform the likely MOA. Given that most toxicology profiles are only revised every 10-15 years, it is worth waiting for these studies, and taking them as well as the external reviewer comments in mind toward a revised document that will be more accurate and will better stand the test of time. The EPA might also consider asking for a National Research Council Special Emphasis Panel to review all these materials and make a recommendation to EPA regarding chromium(VI) as has been done for other several other key toxicants of concern. In any event the current draft's risk assessment treatment of chromium(VI) is highly flawed and grossly mischaracterizes the likely risk of human health effects of chromium(VI) in drinking water based on a careful and thorough assessment of all the available evidence.

POST-MEETING COMMENTS SUBMITTED BY

Monica Nordberg, Ph.D.

Charge Questions

General Charge Questions:

G1. Is the Toxicological Review logical, clear and concise? Has EPA clearly presented and synthesized the scientific evidence for noncancer and cancer hazard?

EPA has presented and synthesized present knowledge about non-cancer and cancer hazards for hexavalent chromium. However, some further literature could be included in the document and paid attention to i.e, Langård and Costa Chapter 24 Chromium In: Nordberg GF, Fowler BA, Nordberg M and Friberg LT (Eds.) (2007) Handbook on the Toxicology of Metals, 3rd edition, Elsevier 487-510.

Other chapters of interest e.g.,Chapter 10 Carcinogenicity by Ke, Costa and Kazantzis page 177-196. One chapter (14 by G. Nordberg and B A Fowler) deals with Risk assessment pages 281-301.

A comparison between criteria for classification of carcinogenicity should be done between IARC, EU and USA. Hexavalent chromium is classified as a human carcinogen. This evaluation is also taken by USEPA for inhaled hexavalent chromium and related lung cancer. It should be highlighted that for some metals e.g., arsenic it has been reported in the scientific literature that oral intake i.e., via drinking water also can give rise to lung cancer though oral intake mostly is referred to cancer in the oral cavity or gastrointestinal system.

My question is if the lung was studied in the NTP studies or any of the animal studies reported in the given report? Same question goes for the epidemiological studies that are cited?

Table 3-7 page 30 reports levels of chromium in female controls both in kidney and in bone. It is not easy to find any comments on this in the document. Is there any analytical problem in this study?

G2. Please identify any additional studies that would make a significant impact on the conclusions of the Toxicological Review.

To my understanding there are new experimental studies with more for this purpose fitted doses. It could be worthwhile to await the outcome of these studies to find out whether more appropriate values for both NOAEL and LOAEL will be reported and include these in an appendix if feasible.

Any further data obtained in the cited NTP studies should be included and presented to the reader. It would be of interest to know if there are any data on lung cancer or other effects from the NTP studies.

During the workshop a number of ongoing studies were presented and it was suggested that they be paid attention to. It is always an advantage to get more and more information and research is always going on.

In my opinion it is however important to set recommendations for exposure to toxic agents in order to protect humans from developing adverse health effects. It is a human right to be protected from unwanted exposure which also will cause unnecessary worry during the time from alert to protection. People expect regulatory agencies to make evaluations and set exposure limits. Studies underway even if published in peer review scientific journals should be carefully evaluated and scrutinized by EPS´s working group to determine if presented data is reliable e.g., based on a number of factors such as, just to mention a few, how large are the studies and what is the power o the study, analytical procedures that include quality control so data is validated and to be trusted. Based on experience it takes time before data will be available even for ongoing studies. I recommend that IRIS, EPA sets a recommendation based on information presented in the draft document. In case important information which can change any evaluation shows up in time, such data can be included in the final document as an appendix or addendum. It is important in Risk Assessment to keep in mind that any recommendation set for exposure levels values needs to be reevaluated over time because by new techniques e.g., rapid development of usage of "omics" has to be considered. In view of said it is important to draw conclusions now and on data available now and not to wait.

Chemical-Specific Charge Questions:

(A) Oral Reference Dose (RfD) for Hexavalent Chromium

A1. A two-year drinking water study of sodium dichromate dihydrate in rats and mice (NTP, 2008) was selected as the basis for the derivation of the RfD. Please comment on whether the selection of this study as the principal study is scientifically supported and clearly described. Please identify and provide the rationale for any other studies that should be selected as the principal study.

EPA suggests an oral RfD of $9x^{10-4}$ mg/kg-day.

The epidemiology studies in Liaoning province, China (p 68-76 in draft report) reported increased incidence of cancer after intake of drinking water contaminated with hexavalent chromium. It is stated in the document to be the only reported human data. That study supports the statement of hexavalent chromium in drinking water to be carcinogenic.

It should be explained to the reader why sodium dichromate dehydrate was chosen for oral exposure study. Data in the literature indicates that bioavailability and bioaccesibility depends on species of the compound and also exposure media. Are there any data on hexavalent chromium species in the drinking water in the general environment? The NTP studies that are reported have used doses that are much higher than reported present concentration in drinking water in the general environment. Thus values for LOAEL is identical to lowest administered dose. It has not been possible because of applied doses to set a NOAEL.

A2. Diffuse epithelial hyperplasia in the duodenum of female mice was selected as the critical effect for the RfD. Please comment on whether the selection of this critical effect is scientifically supported and clearly described. Please identify and provide the rationale for any other endpoints that should be selected as the critical effect.

Perhaps some information on possible effects on the lung should be comment on. The document should give information about solubility of different chromium (VI) species should be given and specifically for the chromium species that have been used in the quoted studies. Soluble salts are mentioned on page 54 under 7 but soluble in what media is not mentioned. The reference WHO/IPCS (2006) Environmental Health Criteria 234, Elemental speciation in Human Health Risk Assessment, WHO, Geneva is recommended to be included.

A3. Benchmark dose (BMD) modeling was applied to the incidence of diffuse epithelial hyperplasia in the duodenum of female mice to derive the point of departure (POD) for the RfD. Has the BMD modeling been appropriately conducted and clearly described? Is the benchmark response (BMR) selected for use in deriving the POD (i.e., a 10% increase in the incidence of diffuse epithelial hyperplasia) scientifically supported and clearly described?

Yes

A4. Please comment on the rationale for the selection of the uncertainty factors (UFs) applied to the POD for the derivation of the RfD. Are the UFs scientifically supported and clearly described? If changes to the selected UFs are proposed, please identify and provide a rationale.

The reason for chosen UFs is clearly and properly described.

It is known that the reduction of hexavalent chromium to chromium three is influenced by vitamin C. This can perhaps be used in setting UFs and thus not only choose the standard UFs of 10 between species and 10 for interindividual differences. Humans can not synthesize vitamin C and are thus depending on vitamin C supplementation. The tested animals i.e., the mouse and the rat both produce vitamin C themselves. In this context a laboratory animal that resembles the human by being depended on vitamin C supplementation might be used in future studies. The concentration of vitamin C in tissues and organs are very important in

evaluation of carcinogenic metals. It is likely to be involved in the mechanism in causing cancer and plays a role in the MOA.

(B) Carcinogenicity of Hexavalent Chromium

B1. Under EPA's 2005 *Guidelines for Carcinogen Risk Assessment* (www.epa.gov/iris/backgrd.html), hexavalent chromium is *likely to be carcinogenic to humans* by the oral route of exposure. Is the cancer weight of evidence characterization scientifically supported and clearly described?

Though it is an American document prepared for US I would recommend also to consult and cite documents published by the United Nations Organizations such as International Agency for Research on Cancer (IARC) and World Health Organization (WHO). Recommended literature is IARC Monographs on the Evaluation of Carcinogenic Risks to Humans Volume 49 (1990), Lyon, France and Chromium, Nickel and Welding 1-677 pages and IARC Monographs on the Evaluation of Carcinogenic Risks to Humans Volume 100 which is in preparation and further information on www.iarc.fr

Hexavalent chromium is classified as a human carcinogen. It is not clear to the reader why classification can be different upon different route of exposure.

B2. A mutagenic mode of carcinogenic action by all routes of exposure is proposed as the primary mode of action for hexavalent chromium. Please comment on whether this determination is scientifically supported and clearly described. Please comment on data available for hexavalent chromium that may support an alternative primary mode of action.

Again this might be linked to differences among hexavalent chromium species regarding for example solubility in body fluids.

Somewhere in the document it should be pointed that iron can reduce hexavalent chromium to chromium three. This has been done in some products. This is touched by on page 48 line 18.

The document describes in detail the possible MOA. IARC 1990 stated "… chromium (VI) compounds on the basis of the combined results of epidemiological studies, carcinogenicity studies in experimental animals, and several types of other relevant data which support the underlying concept that chromium(VI) ions generated at critical sites in the target cells are responsible for the carcinogenic action observed" However, also alternatives like DNA- methylation and other epigenetic mechanisms should be considered, because for many metals DNA-methylation is recognized as a possible mode of action also addressed as mechanism for carcinogenicity. Effects on cell signalling and gene expression may also serve as mechanisms

involved in carcinogenesis of metallic compounds. See further discussion in review by Davidsson et al, Chapter 5 in Nordberg et al (eds) Handbook on the Toxicology of Metals, p79-100

B3. A two-year drinking water study in rats and mice (NTP, 2008) was selected for the derivation of an oral slope factor. Please comment on whether the selection of this study for quantification is scientifically supported and clearly described. Please identify and provide the rationale for any other studies that should be considered.

In this document it is not explained to the reader what decided the selection of chromium species and selection of doses and how the chosen doses relate to exposure in drinking water in the general population. Lethal doses should also be given for the chromium species that have been used in the quoted studies. To evaluate and compare the outcome of studies and concentration levels in tissues, a ratio of concentration in dry weight to concentration in wet weight would make it possible and easier to compare reported results and also of possible intake of hexavalent chromium in drinking water. It is told that the animals by increasing exposure to hexavalent chromium in drinking water showed a decrease in intake of drinking water. Influence on different tissues will be found in doing this. On page 112 NTP 2008 decreased body weights could be explained by reduced drinking water consumption.

B4. The incidence of adenomas and carcinomas combined in the small intestine of male mice from the NTP (2008) two-year drinking water study were selected to serve as the basis for the quantitative cancer assessment. Please comment on whether this selection is scientifically supported and clearly described. Please identify and provide the rationale for any other endpoints that should be selected to serve as the basis for the quantitative cancer assessment.

See comments above. It should be noted that pH is different in different parts of the gastrointestinal system.

B5. The oral slope factor was calculated by linear extrapolation from the POD (i.e., the lower 95% confidence limit on the dose associated with 10% extra risk of tumors of the small intestine in male mice). Has the modeling been appropriately conducted and clearly described?

The model is clearly described. However I feel very uneasy about extrapolation to lower exposure levels because of chosen exposure doses where LOAEL is identical to the lowest exposure dose administered. Other organizations like IARC do not perform any quantitative evaluation of carcinogenic agents/substances. Threshold concentrations are problematic because of lack of knowledge of how the carcinogenicity develops. Once an organism has been exposed to a substance that can give rise to cancer there is a possibility to such an effect to occur.

POST-MEETING COMMENTS SUBMITTED BY

Steven R. Patierno, Ph.D.

Toxicological Review of Chromium

Charge Questions

General Charge Questions

1. Is the Toxicological Review logical, clear and concise? Has EPA clearly presented and synthesized the scientific evidence for non-cancer and cancer hazard?

I will offer a response to this question in the form of general comments regarding specific sections of the Review in order of appearance in the text. In taking this approach my comments will also directly address questions (A)1-4 and (B)1-5.

Page 7: The Environmental Protection Agency (EPA) should not be referencing a 2006 review article by Costa and Klein to site background on environmental chemistry. This review article was not a critical review of the environmental chemistry of chromium. Even if the general background in that review article is accurate, The Toxicological Review of Hexavalent Chromium (TRHC) should cite primary references from chemical or environmental journals or compendiums. Also, this paper is mis-labeled in the reference list as a 2008 paper. [Correct reference is "Toxicity and Carcinogenicity of Chromium Compounds in Humans" Crit. Rev. Tox.: 36(2):155-163, 2006].

Moreover, the premise of this review article [in essence that even very low exposures to any form of CrVI, including in drinking water, can cause virtually any type of cancer in virtually every organ, as well as plethora of assorted other diseases], and the preceding review article on which it was largely based ["Toxicity and Carcinogenicity of Cr(VI) in Animal Models and Humans" Crit. Rev. Tox.: 27:431-442, 1997], should not be universally accepted by the EPA without critical evaluation. Much of the epidemiological methodology applied in these papers is flawed. In these papers, the author(s) repeatedly and selectively tabulated whatever instances could be found in any of the many epidemiologic studies of chromium, of an elevated Standard Mortality Ratio (SMR). These were presented with no mention of the fact that most of these instances were small, non-statistically significant elevations (likely to be random fluctuations due to the large breadth of the studies), which were either ignored or discounted by the original authors because of confounding factors. The paper also failed to take into account that many of the small, non-statistically significant elevations in some cancers in one selected study, were counter-balanced by either no elevation or decreased SMRs in other studies. This "tabulation" approach does not constitute a true meta-analysis and is also statistically incorrect.

There are also additional reasons that the EPA should be circumspect about citing either of these articles. The 2006 article, and its preceding counterpart published in 1997, were written and published at a time when the senior author was actively engaged as an expert witness for the plaintiffs in high-profile hexavalent chromium lawsuits. This involvement was not disclosed in the 1997 article, which was focused on attempting to implicate low dose exposure to CrVI in a broad array of human cancers. In the Acknowledgements section of the 2006 article there is partial disclosure that production of the paper was paid for in part by Baron and Budd. In fact, Baron and Budd is one the law firms with whom the senior author was under contract with as an expert witness for the plaintiffs in an active lawsuit. This article specifically tried to implicate CrVI as a human drinking water carcinogen even at very low doses, as well as suggesting that exposure to CrVI causes a broad array of other diseases, including neuropsychiatric problems, for which there is no support. If the EPA is going to site these review articles it is critical that EPA conduct an independent critical review of every paper sited in these review articles. In the latter scenario it is certain that EPA will reach a different conclusion.

I am not of the opinion that a scientist who serves as an expert witness should have to disclose all litigation-related work in all scientific publications, particularly not in reports of original laboratory research into mechanisms of action, or even in review articles that give an unbiased evaluation of the existing literature, especially as it relates to basic mechanisms of action. Indeed, in the world of chromium toxicology it is hard to find experts who have not participated in some sort of chromium-related litigation. However, these two articles do not merely describe original laboratory research or present an unbiased review of the literature (note that part of the 2006 review article is a recapitulation of an already published journal article on UV light and chromium exposure). These two particular articles are essentially position/opinion papers, with speculative declarations that even very low dose exposures to soluble CrVI can cause virtually any kind of cancer (and other disease) in virtually any organ, a theory of obvious benefit to any plaintiff's case in chromium-related litigation, but one that is not supported by either epidemiological studies or in vivo animal studies.

Page 20-21: Although the draft TRHC frequently describes each specific study in this section and offers a conclusion/interpretation, the TRHC discussion of the Donaldson and Barrera paper ends with a reference to Table 3-1 with no summary. There is important information in Table 3-1 that strongly supports the capacity of gastric juice to rapidly reduce CrVI to CrIII. Note that the uptake of Cr in intestinal rings was virtually identical, whether the starting material was CrIII without gastric juice or CrVI plus gastric juice at pH 1.4.

The text at the bottom of page 21 and the top of page 24 seems to be nuanced to cast doubt on the body of work of DeFlora, by using the word "suggested" (second line from the bottom of pg 21), and then suggesting

that the values of reducing capacity given by DeFlora "should be considered with some caution". This "caution" is based on speculation found in the paper cited (Zhitkovich, 2005), and reiterated in the 2006 Costa and Klein article (from where the draft TRHC apparently drew its language). This speculation is addressed in the supplementary materials under "Public Comments". The TRHC and the EPA should not cast doubt on the body of work by DeFlora, based on unsubstantiated speculation.

Page 24-5: The TRHC should recognize and illustrate the main point of the absorption studies cited: no matter whether the original starting material is CrVI or CrIII there is limited absorption and little retention of either: fecal recovery in rats was 98% for CrIII and 97.7% for CrVI (pg 24) and in humans was 99.6% for CrIII and 89.4% for CrVI. Pretreatment of CrVI with gastric juice completely inhibited absorption of CrVI after direct perfusion into the small intestine. On pg 27 another study indicates that 99% of CrVI is recovered in feces using rats gavaged with CrVI. On pg 28 another study indicates that maximal uptake after gavage of rats with CrVI occurred in liver and was only 1%. Absorption in other organs was in the range of 0.1 to 0.2%. It is important to note that in all of these absorption studies, including drinking water studies, the increased tissue distribution was only observed after chronic administration of more than 5 ppm. Many of the studies used greater than 100ppm. The main point that there is very little absorption and retention of Cr even after administration of CrVI.

Page 26: It is incorrect to state that absorbed "hexavalent" chromium is distributed throughout the body. Few studies actually speciated the Cr found in organs distal to the route of administration and even extremely large doses of CrVI, large enough to saturate reduction in the stomach and GI tract, do not deliver much more than trace amounts of CrVI to most distal organs because of the vast reducing capacity of blood components. The vast majority of Cr reaching distal organs arrives as CrIII. The TRHC should make this absolutely clear.

Page 34: The TRHC should provide an accurate summation of Table 3-8 which compares tissue chromium after ingestion of a very large, gastric reduction-saturating dose (12.9 mg/L) of either CrIII or CrVI. The only "organ" that showed consequential increased levels of Cr after CrVI compared to CrIII was blood. Most of the other organs exhibited only trace amounts of Cr, even after this huge dose of CrVI, except for the intestine which showed significant and nearly identical increased Cr concentrations after both CrIII and CrVI. This supports the conclusions of the supplementary data in the Public Comments showing that some sections of the intestine (jejunum for example) are sites of Cr accumulation, regardless of whether the source material is CrIII or CrVI.

Page 35: Note that there is no increased accumulation after 8 weeks of exposure compared to 4 weeks of exposure, even at the enormous dose of 130 ppm (mg/L).

Page 36: The NTRC should not interject the commentary statement: "indicating that a portion of the CrVI escaped intracellular reduction in the GI tract and became bioavailable for systemic distribution". Like almost every other study this study measured total tissue Cr and did not speciate tissue Cr, and the NTRC therefore cannot speculate on what the form of Cr was that reached the tissues. Note in Table 3-8 the accumulation of Cr in the intestine and blood after CrIII.

Page 37: Note the obvious threshold of increased bone and kidney concentrations after 10 ppm compared to all lower doses, as well as estimated body Cr burdens. Note that in liver there is a significant Cr burden even after ZERO CrVI exposure. Note the strangely compressed scale of the Y axis: even the increase in females at 10 ppm is only an increase from 0.3 to less than 0.5. These data demonstrate the exact opposite of the conjecture on page 36: there is a clear threshold of accumulation indicating saturation of reductive capacity.

Pages 38-39: Virtually every study shows the same thing. The NTP study used a "low dose" that is already higher than the 10 ppm in the Sutherland study. What is being referred to here as a "dose-dependent" increase is already supra-saturation of gastric reductive capacity. What these studies really show is how little Cr is absorbed, even in tissues that are directly exposed (glandular stomach and forestomach), even after massive doses are administered.

Page 41: It is inappropriate to make such an unqualified statement as found at the top of this page: "Hexavalent chromium is capable of crossing the placenta". This is only true in the highly contrived circumstances referenced below the statement wherein pregnant mice were given an IV injection of a massive dose of CrVI (10mg/kg).

Page 42: The TRHC does a good job describing the bioavailability and kinetics of Cr absorption in humans after CrIII, CrVI in OJ and CrVI. It correctly acknowledges that the CrVI-OJ was completely reduced and that even the full dose of CrVI was insufficient to overwhelm the reducing capacity of blood. The potential explanations offered are correct but need to add another possibility. Often overlooked is the fact that not all CrIII is alike. Anyone who works with CrIII in the laboratory knows that it undergoes aging in aqueous solution, even visibly changing color with time after solubilization. It is possible that CrIII generated from newly reduced CrVI (as in the CrVI-OJ) may have some different biological parameters than straight CrIII made up in water and allowed sit for a couple of days. In fact overall absorption of newly formed CrIII may be higher than aged CrIII, possibly as a function of its ability to form complexes with biological ligands that may alter its absorption potential.

Page 49 bottom: It is inaccurate to state that "CrVI distributes to other tissues, notably the blood, kidney, and liver." Except for the cases of treatment with extreme doses, or use of pathways like intra-intestinal instillation or IP injection, the vast majority of Cr that arrives at distal tissues is CrIII. Once again, it is critical that TRHC make that fact clear, otherwise it gives the appearance of non-objectivity.

Page 50, last paragraph: The TRHC should not simply reiterate speculation that is found in the papers it cited (Zhitkovich 2005, Costa and Klein 2006), in suggesting that the mutagenicity of Cr may be underestimated in cultured cells because of lower levels of intracellular ascorbate when cells are cultured in absence of added ascorbate. Indeed, it is just as likely that the mutagenicity of CrVI in cultured cells is grossly overestimated, because the lack of ascorbate in the extracellular medium allows CrVI to persist in the extracellular medium thereby maximizing its uptake as the hexavalent oxyanion. At the very least the TRHC should discuss both possibilities and not give the appearance of bias.

Page 58: It is inaccurate to state that model simulations "imply" that some CrVI escaped reduction in the stomach. This is circular reasoning. The "input" data that went into formulating these models was based on experiments wherein massive doses of CrVI were administered, doses that would clearly exceed reductive capacity. It is not appropriate to then state that the model simulation "implies" that some CrVI escaped reduction, as though the model now supports a novel biological observation. It would be completely expected for the model to predict that scenario since it would logically emanate from the very data that was used to formulate the model. It is critical that the TRHC indicate that these models do not apply to, or accurately predict, the toxicokinetics of low, environmentally relevant doses of CrVI. The discussion in the THRC does become more balanced on page 64 where the non-linear aspects of CrVI uptake, reduction and bio-distribution are given some weight.

Page 66: This section (3.6) needs to be completely rewritten as it lends undue weight to an opinion expressed in only one or two papers, at least one of which was written under financial inducement by a law firm with a vested interest in characterizing all Cr, including CrIII, as a potential hazard (see preceding comments). The TRHC needs to not indiscriminately cite speculation found in review articles without more rigorous analysis. Except for those few biased citations it is almost universally accepted that CrIII is an essential element.

Page 68: Section 4.1.2, last sentence: This is nearly the ultimate example of how critically important it is for the TRHC to do its own critical analysis of the literature. The paper cited, (Bick et al, 1996) should not be cited under any circumstances and in fact it should be retracted from the scientific literature. Two of the authors, Walter Lack and Thomas Girardi, were two of the lead lawyers for the plaintiffs in several high-profile chromium lawsuits, now immortalized by the Hollywood movie "Erin Brockovich". They listed their

"academic" credentials as the Department of Hematology at the University of Tasmania in Australia. The other three authors (Costa, Bick and Teitlebaum) were paid expert witnesses for the plaintiffs in the same case, which was active at the time. None of this was disclosed in the paper. The two cases of Non-Hodgkin's lymphoma discussed in this case report were plaintiffs in the active lawsuit and the information was supplied by the lawyers. Moreover, at best this report is merely a case-report (not even a case-control study), merely reporting that two people in Hinckley CA, at that time, had been diagnosed with Non-Hodgkin's lymphoma.

In contrast, the draft TRHC does not yet, but should reference the recent work of Dr. John Morgan, an epidemiologist for the California Cancer Registry. He has been tracking cancer incidence in the town of Hinckley CA (the "Brockovich" town) from 1996 to present. He recently published data showing that from 1996 to 2008, not only is there no excess of total cancer or any specific cancer in Hinckley, there are actually fewer cancers than expected.

Pages 71-80: The draft TRHC conducts a very thorough depiction of the different interpretations of the Liaoning Province situation. What seems to get lost in the details is the larger picture. This is a Province of a country wherein the background rates of both stomach and lung cancer are high even in non-chromium exposed comparison groups, indicating the presence of other contributing risk factors. This is a situation where exposure is characterized in terms of high dose, long term "yellow water", yet despite this potential significant exposure, the question of whether there is an additional modest increase in risk for stomach cancer hangs on whether a particular industrial area is included in the comparison group or not. There is much controversy surrounding the reports of cancer risk in this Province, but after discussing the controversy the draft THRC aligns itself with the method of re-analysis of Beamont et al. The THRC should then also cite the commentary by Allan Smith [Epidemiology 19:24, 2008] which accompanied the Beamont article: although Smith is sympathetic to Beamont's attempt to re-analyze the data, he also describes the extensive weaknesses of the approach. This is not the kind of data that a regulatory decision should be based on.

Page 81: The NTP toxicology studies on subchronic oral exposure (Section 4.2.1) are technically well done. The principle issue that needs to not be lost in the detail is that the lowest dose was 62.5 ppm, an enormous concentration of little or no environmental relevance. This is a "yellow water" situation to the extreme. Despite these enormous doses most of the observations did not exhibit a consistent pattern of dose or duration dependence. It is also important to recognize that these enormous doses of CrVI actually serve to deliver an enormous amount of CrIII to the organs and cells in question. Remembering that CrIII is not without biological activity (acting as a co-factor in insulin action), it is entirely possible the some of the observed

effects are due to the physiological effects of massive CrIII overload. The extensive new data provided by ToxStrategies, described in the Public Comments, needs to be incorporated into the TRHC.

Page 84: Again, a consistent relationship between severity and dose was not observed. This implies the presence of effects caused by indirect mechanisms, likely chronic inflammation and/or tissue damage only observed at the highest doses (see below). Urinalysis shows effects due to decreased water intake due to poor palatability of the yellow water. This dehydration alone is capable of rendering epithelial tissues more fragile. Changes in organ weights were only observed at doses above 500ppm (pg 86).

Page 87-108: The results are described repeatedly as "without clear dose-response relationship". Indeed, minimal to mild histiocytic cellular infiltration was observed in all groups including the control animals. Even less toxicity was observed in mice compared to rats; in fact even at 1000 ppm for 3 months there was no evidence of any hepatotoxicity, only mild changes in some hematological indices that were attributed to changes in body weight (probably caused by massive CrIII overloading and its potential effects on insulin and glucose metabolism). What needs to be emphasized here is that the lowest dose used in any of these studies is at or above saturation of gastric reductive capacity and yet still very little toxicity was observed except at the two highest doses (and often only at the one highest dose) (Tables 4-12, 4-13, 4-14). At the lower end of these very high doses, only inconsistent observations were made and when "toxicity" was reported it was generally ranked minimal to mild. Only the index of Liver (fatty change) was ranked as moderate, but that was identical to the ranking of that same index in the Controls. The main point here should be that these are massive doses and they are eliciting minimal effects. This important concept should not be lost in the mass of detailed results.

Pages 109-120: The NTP carcinogenesis studies in rats and mice show that there is no carcinogenic response except at the two highest doses that also produce chronic tissue damage at the sites of carcinogenicity. The dose-response is definitively non-linear, as is the absorption data described above. Given that the lowest dose is already above the reductive capacity of the oral cavity and stomach, these data provide strong evidence of the protective effects of the reductive capacity of blood components.

It should be noted that the NTP's published report by Stout et al [Hexavalent Chromium is Carcinogenic to F344/N Rats and B6C3F1 Mice after Chronic Oral Exposure, Environmental Health Perspectives 117: 716, 2009] presents an inaccurate Discussion of potential mechanism of action, drawn heavily from the 2006 Costa and Klein article, especially in criticizing the work of DeFlora. In point of fact, the results of the NTP assay, and the extensive additional data found in the Public Comments generated by a group of investigators around the country funded by ToxStrategies, give nearly definitive proof that the work of DeFlora is correct.

Even the lowest dose of the NTP assay exceeds the reductive capacity of the oral cavity and upper digestive tract. Yet little toxicity and no carcinogenicity is observed except at the two highest doses.

The argument by Stout et al that the NTP doses were below gastric reduction-saturation, based on a supra-linear (decreasing response with dose) rather than sub-linear (increasing response with dose) dose response is incorrect. If the doses were below saturation of reductive capacity, as the dose increased the ratio of unreduced CrVI to reduced CrVI (CrIII) in the stomach would increase (due to depletion of reductive capacity), and absorption would show an increasing rate of response (opposite of what was observed) because of an increased percentage of the total Cr that would be in the unreduced hexavalent state. Yet both absorption and toxicity exhibit a decreasing rate of response with dose in the NTP assay. This would actually be expected at supra-saturation doses: once the reductive capacity of the oral, digestive and blood components is exceeded, the organs receiving the highest amount of CrVI will sustain inflammatory tissue damage provoking tissue regeneration. It is unlikely that such tissue damage would display dose dependence since it only occurred at the two highest doses of the assay and it is a complex, disseminated biological response. It is likely then that a combination of three factors contribute to the high dose carcinogenic response: (i) tissue damage with regenerative cell profieration, (ii) regenerative cell proliferation in the presence of macromolecular damage, and (iii) regenerative cell proliferation occurring in the presence of massive CrIII loading, which may affect insulin-dependent proliferative signaling.

Pages 122-149: For these studies on the potential reproductive toxicities caused by CrVI one can only hope that the TRHC and EPA will remember the 16th century adage of Paracelcus "all substances are poisons, the right dose differentiates a poison from a remedy". These studies show reproductive toxicity at huge doses of CrVI, often given using invasive administration procedure (IP or IV injection), with little relevance to environmental exposure levels.

Pages 176-178: The in vivo studies showing DNA damage or mutagenicity in cells peripheral to the point of administration of CrVI were only positive when massive doses of CrVI were administered by gavage, direct instillation, or intravenous injection. Although some studies claim to find mutations in the absence of cytotoxicity these are highly contrived systems: for example eye spots in offspring of pregnant female mice given huge doses (62.5 ppm) of CrVI in drinking water. All studies of mutagenesis in cultured mammalian cells, including human cells, demonstrate that mutagenesis is only observed at doses that produce some degree of cytotoxicity and replication arrest. It should also be clarified in the TRHC that DNA damage and mutagenicity should not be equated: while mutagenicity may result from DNA damage, the relationship is not simple or linear and is further complicated by DNA repair. Also, it is unclear whether all forms of DNA or chromatin alterations (collectively termed DNA damage) are pre-mutagenic. For example, in silico studies

on DNA-protein crosslinks suggest that under certain circumstances CrIII can serve as binary crosslinking agent between small peptides and DNA. However, in in vitro studies in cultured cells and in in vivo studies, it is not clear what is actually being measured by assays for DNA-protein crosslinks. This phenomenon may in fact only indicate that chromatin isolated from certain cells exhibits a higher degree of condensation during isolation, rendering chromatin proteins more difficult to extract. What appear to be DNA-protein crosslinks can be actually be observed in cells treated with agents that do not participate in or catalyze formation of an actual binary crosslink.

Pages 202-214 (section 4.7.3.2): Many of the preceding comments directly address and provide major qualifications to the MOA discussed in this section, including the interpretation of reductive capacity found in the Stout et al report of the NTP assay.

It is clear that the EPA is faced with a unique situation in assessing the MOA of Cr(VI) at it relates to low-dose risk assessment. It is abundantly clear from all the science that the effects of Cr(VI) at the massive doses necessary to produce tissue toxicity and carcinogenesis in rodents, have no bearing on the effects of low-dose, environmentally-relevent exposures. This is consistently borne out by epidemiological, animal and cell experimentation.

This is especially pertinent in relation to whether or not Cr(VI) should be considered with a mutagenic MOA. I have spent more than 25 years studying the genotoxic properties of Cr(VI) and I have frequently contributed to the plethora of studies showing DNA damage and what we thought was associated mutagenesis. There is no doubt that Cr(VI) can be forced to be genotoxic and "mutagenic" under experimentally contrived systems and at high doses that evoke major amounts of cell death. However, in hindsight many of us "DNA damage and repair" scientists have come to appreciate several important factors: (i) DNA damage is only observed at very high dose that kill a lot of cells, (ii) Cr(VI) is at best a very weak "mutagen", requiring very high doses that kill most cells and experimental "backflips" to select for survivors, and (iii) what we thought was "mutagenesis" is actually selection for stochastic cell survivors of massive toxic insult. Dr. Rossman's group has shown that the base sequence of the genes used for mutation detection and selection is intact and that the changes in gene expression enabling selection are epigenetic, not mutagenic. Our group has shown that what we really selected for at toxic exposures are cells that are resistant to apoptosis, and Dr. Zhitkovich's group has shown that the "mutant" cells were actually surviving cells that were selected for changes in specific forms of DNA repair. Again, this only occurs at doses that kill a lot of cells, not dis-similar to the high-dose rodent assays wherein tumors were only observed at doses that produced chronic and fairly severe tissue damage. This harkens to what is sometimes viewed as a landmark study of lung cancer and occupational exposure to high doses of Cr(VI) by Gibb et al. Occupational exposure to in the chromate production

industry was categorized into 4 quartiles. The lowest two quartiles are huge levels of exposure by "environmental" standards, orders of magnitude beyond the even the highest known environmental exposures. The lowest quartile of exposure was essentially a No Effect Level (no elevated risk) and the slightly elevated risk ratio in the second quartile was not statistically significant. Interestingly, of the total of 120 lung cancer cases found in chromium-exposed workers, 116 were also smokers.

The EPA may be under certain historical regulatory precedents and pressures to deem Cr(VI) with a mutagenic mode of action simply because there are published studies that have "Cr(VI)" and "mutation" equated in the title (some of these papers are my own), but this decision would not be based on science. At high, tissue damaging doses one can get tumors to form and those tumors will have mutations in specific genes because that is the molecular etiology of how that particular cancer develops. It will have no relation to any chemically-specific mutations caused by Cr(VI) because Cr(VI) is an exceedingly poor mutagen. Even at the low end of very high doses there is NO MOA because there is NO toxicity, no mutagenecity, and no carcinogenesis. Extrapolating linearly from events observed at the two highest doses of the NTP assay, to anything close to reality for environmental exposure, is simply not scientific. If ever there was a textbook case to be made for a "threshold carcinogen", it is Cr(VI).

General Charge Questions:

G2. The TRHC should absolutely consider the extensive new data being provided by ToxStrategies and presented in part in the supplementary section under Public Comments.

Chemical-Specific Charge Questions:

A1. The two-year drinking water study of sodium dichromate dyhydrate in rats and mice (NTP, 2008) is the most thorough and technically well-conducted study available. It is likely the best study available for selection. However, the interpretation of and conclusions drawn from that study need to be re-evaluated in light of the issues raised in my preceding comments and the additional data shown in the Pubic Comments and coming available from a multi-institutional study sponsored by ToxStrategies.

A2. The selection of Diffuse epithelial hyperplasia in the duodenum of female mice as the critical effect for the RfD should be re-evaluated in light of the issues raised in my preceding comments. It must be considered in the context of the non-linear, dose-related issues discussed above regarding saturation of reductive capacity and definitive threshold data for toxicity and carcinogenicity.

A3. See answer to A2 above.

A4. The Uncertainty Factors must be re-evaluated in the context of the non-linear dose-response data, the clear evidence of thresholds for toxicity and carcinogenicity and the fact that these high-dose, supra-saturation experiments cannot be extrapolated linearly to low or vanishingly small doses.

(B) Carcinogenicity of Hexavalent Chromium

B1. The cancer weight of evidence characterization is not scientifically supported. The conclusion that hexavalent chromium is "likely to be carcinogenic to humans by the oral route of exposure" is not scientifically supportable given the issues raised in my comments above. The non-linear dose data in both the NTP studies and the data preliminarily discussed in the Public Comments clearly demonstrate that the toxicities and carcinogenesis observed at these extremely high, obviously supra-saturating doses, cannot and should not be extrapolated to lower doses. See detailed comments above.

B2. The determination of a mutagenic mode of action by all routes of exposure should be re-evaluated before proposing it as the primary mode of action. Nearly all indices (NTP studies, inhalation studies, mammalian cell mutagenesis studies etc) indicate that carcinogenicity of CrVI is only observed under exposure conditions that evoke cellular toxicity, inflammatory tissue damage, and tissue regeneration. The DNA damage and presumed mutagenicity (actually epigenetic or stochastic selection of cells that survived toxicity) of CrVI is only observed at doses that also cause cell death and tissue damage. In vivo, these effects are only achieved at very large doses that clearly overwhelm the reductive capacity of the oral cavity, stomach and blood components resulting in a sharp threshold of carcinogenesis only at the two highest NTP doses. I have spent more than 25 years studying the molecular mechanisms of CrVI genotoxicity and mutagenesis and I have a deep appreciation for its capacity to interact with cells and alter DNA and DNA replication and transcription. However, just because CrVI is capable of causing DNA damage and what we thought was "mutagenicity" (see above) in carefully contrived experimental systems, does not mean that it does so under physiologically and environmentally relevant conditions. It is much more likely that the chronic tissue damage, with accompanying inflammation and subsequent proliferative regeneration, possibly in the presence of unrepaired DNA damage, all of which are only observed at the highest doses, is the principle mode of action.

B3. See previous comments above. The NTP study is the best study available but the interpretation of the data and conclusions drawn from it are incorrect. Important supplementary data is preliminarily discussed in the Public Comments.

B4. See previous comments above. The NTP study is the best study available but the interpretation of the data and conclusions drawn from it are incorrect. Important supplementary data preliminarily discussed in the Public Comments.

B5. See previous comments above. The linear extrapolation from the POD is not appropriate. CrVI toxicity and carcinogenicity demonstrates distinct non-linearity and there is little or no relation between what is observed at the highest doses in the NTP study and any physiologically-appropriate or environmentally-relevant exposure.

POST-MEETING COMMENTS SUBMITTED BY

Toby G. Rossman, Ph.D.

Toxicological Review of Hexavalent Chromate

General Charge Questions

G1. Is the Toxicological Review logical, clear and precise? Has EPA clearly presented and synthesized the scientific evidence for noncancer and cancer hazard?

In general, this was a clearly written document. However, my area of expertise is in the mutagenic and epigenetic mechanisms of action of carcinogens, and I found many more problems in those areas than in the rest of the document. I will discuss these in section B2.

Here I will mention some errors in the rest of the text.

p. 38, end of 1st paragraph reads, "Uptake in guinea pigs did not appear to generally differ from that of rodents". The guinea pig (*Cavia porcellus*) is a rodent.

p. 46, section 3.3, refers to transport of the hexavalent chromium oxyanion (for clarity, should this read chromate/dichromate?) by sulfate and phosphate transport systems (should be pleural). It is claimed that this allows accumulation in cells at higher concentrations than the extracellaular concentration. Neither the transport systems nor the evidence for higher intracellular accumulation are referenced. Actually, what allows higher accumulations is the fact that Cr(6) is reduced in the cell to Cr(3), which cannot get out, so what accumulates is Cr(3).

p. 201, 1st full paragraph: It is claimed that the key precursor events leading to chromium-induced mutagenicity have been identified in animals. This is not so. It is not even true for mammalian cells in culture. Some ideas have been derived from cell culture studies (but with Cr-damaged shuttle vectors). Almost nothing is known about mutagenicity in animals, and nothing at all is known about the genetic changes occurring in animal tumors or in the target tissues.

p. 202. Section 4.7.3. For reasons that will become clearer in my response to B2, this section is very flawed. I will mention just one point here: The confusion between "mutagenic" and "genotoxic" must be cleared up throughout this section (as well as throughout Section 4.5.). The thinking on this issue is very sloppy. A mutagenic mode of action is just that: it requires mutagenesis.

G2. Additional studies that would make a significant impact on the conclusions of the Toxicological Review.

Some of these will be presented in section B2, as they pertain to mode of action.

It is extremely important that the new information supported by American Chemical Council (performed by ToxStudies and others) should be considered before the final document is completed. They address a number of missing data sets. It is already clear that proliferative increases occur in the mouse duodenum at doses of Cr(6) lower than those that cause tumors. Also, there is evidence for cytotoxicity at these lower concentrations that may be driving the proloferative responses.

The fact that Cr is an essential element needs to be addressed. What are the implications for a threshold?

It is possible that dietary Cr(6) is significant and should be evaluated. All parts of grain contain Cr(6) and 10% of the Cr in bread is Cr(6) (Mishra et al., Food Chem. Toxicol. 33:393-397, 1995; Soares et al., J. Agric. Food Chem. 58:1366-1370).

River waters have a median Cr value [which is probably Cr(6)] of 10 ppb (range <1-30), and even rainwater has a range from 0.14-0.9 (ATSDR, Chromium, Draft for Public Comment, online).

A recent meta-analysis of cancers of the G.I. tract among those occupationally exposed to Cr(6), concludes that these workers are not at greater risk than the general population (Gatto et al., Cancer Epidemiol. 34:388-399, 2010). Inhalation exposure usually leads to G.I. exposure, suggesting a possible threshold if the ingested dose can be estimated.

(A) Oral Reference Dose (RfD) for Hexavalent Chromium

A1. A two-year drinking water study of sodium dichromate dihydrate in rats and mice (NTP, 2008) was selected as the basis for the derivation of the RfD. Please comment on whether the selection of this study as the principal study is scientifically supported and clearly described. Please identify and provide the rationale for any other studies that should be selected as the principal study.

This does seem like the best and most complete study to use.

A2. Diffuse epithelial hyperplasia in the duodenum of female mice was selected as the critical effect for the RfD. Please comment on whether the selection of this critical effect is scientifically supported and clearly described. Please identify and provide the rationale for any other endpoints that should be selected as the critical effect.

This seems like an appropriate choice, but it's outside my area of expertise.

A3. Benchmark dose (BMD) modeling was applied to the incidence of diffuse epithelial hyperplasia in the duodenum of female mice to derive the point of departure (POD) for the RfD. Has the BMD modeling been appropriately conducted and clearly described? Is the benchmark response (BMR) selected for use in deriving the POD (i.e., a 10% increase in the incidence of diffuse epithelial hyperplasia) scientifically supported and clearly described?

This does seem like the best and most complete study to use (but it's a bit outside my area of expertise).

A4. Please comment on the rationale for the selection of the uncertainty factors (UFs) applied to the POD for the derivation of the RfD. Are the UFs scientifically supported and clearly described? If changes to the selected UFs are proposed, please identify and provide a rationale.

This is outside my area of expertise.

(B) Carcinogenicity of Hexavalent Chromium

B1. Under EPA's 2005*Guidelines for Carcinogen Risk Assessment* (www.epa.gov/iris/backgrd.html), hexavalent chromium is *likely to be carcinogenic to humans* by the oral route of exposure. Is the cancer weight of evidence characterization scientifically supported and clearly described?

Given the fact that there is not enough human data to firmly establish carcinogenicity, but there is animal data, "likely to be" is reasonable but "possibly carcinogenic at high dose" would be more accurate.

B2. A mutagenic mode of carcinogenic action by all routes of exposure is proposed as the primary mode of action for hexavalent chromium. Please comment on whether this determination is scientifically supported and clearly described. Please comment on data available for hexavalent chromium that may support an alternative primary mode of action.

By definition, the "mode of action" (MOA) of a carcinogen is "a sequence of key events and processes, starting with interaction of the agent with a cell, proceeding through operational and anatomical changes, and resulting in cancer formation" (USEPA, 2005). For mutagenesis to be a carcinogenic MOA, the agent must at the very least cause heritable mutations in mammalian cells. The mutations should be induced in a concentration range with low toxicity (preferably similar to concentrations seen in human exposures), and the mutations should be induced in the target tissues in animal experiments and in humans. Human and animal tumors should also show genetic alterations consistent with the types of mutations induced by the agents, and these should be early events.

The information about Cr(6) is lacking for much of these criteria. In fact, the human tumor data support an epigenetic mechanism more than a mutagenic one.

Genotoxic is not the same as mutagenic, and sections 4.5 and 4.73 must be completely rewritten, as they consistently confuse these terms. Standard genotoxicity assays were not designed to inform specific modes of tumor induction. With the exception of mutagenesis, these other assays (non-mutagenic assays) do not measure heritable events, but rather measure evidence of DNA damage or its repair. Non-mutagenic assays include chromosome aberrations, micronuclei, comet assays, DNA lesion measurements, and DNA repair assays. These assays are useful for hazard identification or as biomarkers of exposure. They provide only supportive evidence that mutagenesis might be a MOA. DNA damage *per se* does not inform us about eventual heritable change, which is the true issue. Most (but not all) mutagens cause heritable changes in DNA sequences by causing damage to DNA (pre-mutagenic lesions) that is converted to mutation after cell division.

Table 4-21 should be deleted, as results in bacteria are not relevant to tumorigenic MOA. A simple statement that Cr(6) is mutagenic in bacteria should suffice (referencing a review such as Klein CB. "Carcinogenicity and genotoxicity of chromium" In: Toxicology of Metals (Chang LW, ed). Boca Raton, FL:CRC Press, 1996, pp.205–219.

Table 4-22 represents positive results in a group of assays that measure both mutagenic and non-mutagenic endpoints (including a paper on epigenesis, Klein et al., 2002, which should be removed, as this is not a genotoxic event; neither is disruption of mitosis, which can have many causes). Table 4.22 has neither information on the concentrations inducing positive results, nor on the toxicity of those treatments.

This table is also deficient in the most important results in mammalian cells, i.e. mutagenesis. All of the studies reported are mouse lymphoma cell studies, yet later in the document (page 203), reference is made to mutations at the HGPRT locus in "Chinese Hamster ovary cells (V79 and AT3-2)" V79 is not a Chinese hamster ovary cell, it is a Chinese hamster lung fibroblast. In fact, one such study using V79 cells (Sugiyama et al., Mutat. Res. 260:19-23, 1991) shows a modest positive effect only in a narrow concentration range. A CHO line (AA8) showed a small increase (3.4 fold) at a dose giving 75% survival (Brooks et al., 2008). There is a fuller discussion of chromate mutagenicity (with references) in: Klein CB. "Carcinogenicity and genotoxicity of chromium" In: Toxicology of Metals (Chang LW, ed). Boca Raton, FL:CRC Press, 1996, pp.205–219. This article points out that chromium compounds are mutagenic in a narrow dose range,

possible because of persistent toxicity after treatment (e.g. residual toxicity was seen a week after treatment of V79-derived G12 cells).

Chromosome aberrations and DNA strand breaks can occur as a result of cytotoxicity. Dead cells do not become tumors. Unless assays for cytotoxicity are performed, it is not possible to know whether DNA damage occurs in cells that can replicate to form clones. Traditional cytogenetic assays rely on short-term cell survival to generate the mitotic figures necessary for analyses; the long-term viability of these treated cells cannot be determined. Thus, the relevance of this kind of data for carcinogenic MOA is questionable. To measure cytotoxicty, the gold standard is clonal survival, a method that is common in gene mutation assays, but not in other genotoxicity assays. Short-term survival assays, such as MTT, neutral red, and trypan blue, as well as measurements of mitotic index that are commonly used in cytogenetic assays, fail to detect early or delayed apoptotic events. Trypan blue detects only necrosis. MTT and neutral red assays can be delayed to allow time for apoptosis to develop, at which point the results approach clonal survival (Komissarova et al., Toxicol. Appl. Pharmacol. 202:99–107, 2005). As mentioned above, Cr(6) causes delayed residual toxicity (Klein et al. Environ. Health Perspect. 102 (suppl 3):63-67, 1994), and thus clonal survival or at least apoptosis at later times after exposure, are essential in establishing cytotoxicity levels. Normal human fibroblasts show ~80% loss of clonality after a 25h exposure to 2 μM sodium chromate [Vilcheck et al., Environ. Health Perspect. 110 (Sup5):773-777, 2002].

Micronuclei can result from DNA damage or from malsegregation of chromosomes. It has been recommended that this assay should be performed under conditions of high survival (an increase of >90% in number of viable cells) and that markers for apoptosis and necrosis be included [Kirsch-Volders, et al. (2003) Report from the in vitro micronucleus assay working group. Mutat. Res. 540:153-163]. In the case of Cr(6), at lower concentrations, most of the micronuclei are kinetecore-positive, meaning that they arise from malsegregation and not DNA strand breaks (Seoane and Delout, Mutat. Res. 490:99-106, 2001; Figgitt et al., Mutat. Res. 688:53-61, 2010). Those that are kinetecore-negative (arising from chromosome breaks) occurred only at the highest concentrations. Thus, Cr(6) induces aneuploidy rather than DNA damage at lower concentrations (Holmes et al., 2010; Figgitt et al., Mutat. Res. 688(1-2):53-61, 2010). Aneugenesis is caused by alterations in proteins, not DNA, and has thresholds.

It should also be noted that the mouse lymphoma assay (MLA), chromosome aberration assay (CA) and micronucleus assay (MN) give a large number of false positives, even compared with the Ames test. Chemicals that are non-carcinogenic after thorough testing in both male and female rats and mice are often positive in these assays (Kirkland et al., Mutation Research 584 (2005) 1–256).

The Comet assay detects single and double strand DNA breaks as well as alkali-labile sites. Nucleotide excision repair (NER) and base excision repair (BER) of adducts can create breaks as intermediates. Single strand breaks are quickly repaired and are not regarded as significant premutagenic lesions. During apoptosis, DNA fragmentation into segments of 180 base pairs occurs, whether or not the apoptosis was induced by a genotoxic event (Choucroun et al. 2001, Mutat. Res. 478:89-96; Henderson et al.,.1998, Mutagenesis 13:89-94.) Necrotic cells also display DNA damage (Fairbairn et al., 1996 Scanning 18:407-416.). In order to avoid false positive responses, Henderson et al. (1998) suggests that the concentration of test substance should produce >75% viability.

In summary, standard genotoxicity assays from hazard identification exercises cannot be used to establish a mutagenic MOA, because these assays do not measure heritable events and because the doses used in such assays are usually too high.

Other MOA's have not been adequately considered. These include, for example, selection for Cr-resistance, resistance to apoptosis, and aneuploidy. The evidence for a mutagenic MOI is weak. Mutations can result from DNA damage, but can also be a secondary effect of the loss of mismatch repair, aneuploidy, and other types of genomic instability (in other words, it is a later effect). With the exception of the mouse lymphoma system, Cr(6) is only weakly mutagenic in mammalian cells, rarely giving more than a 3-fold increase in mutant fraction over background levels (in endogenous genes), and in a very narrow (and toxic) dose-range with a strong threshold (reviewed in Nickens et al., 2010).

In some cases the "mutations" have been shown to be epimutations resulting from altered DNA methylation (Klein et al., 2002). Since none of the other studies on mammalian cells looked for epigenetic inactivation, this calls into question whether the "mutants" seen are really mutants. These are important considerations for the MOA of Cr(6), since cells grown in the presence of Cr(6) show selection for cells with inactivated mismatch repair (MMR) genes. These cells are Cr(6)-resistant and could be the result of either mutation or epigenetic inactivation (reviewed in Salnikow and Zhitkovich, 2008). Cells with epigenetically inactivated MLH1 (a MMR gene) were seen in human lung A549 cells exposed to Cr(6) (Sun et al., Toxicol. Appl. Pharmacol. 237:258-266, 2009). MMR-deficient cells are mutators (having a high spontaneous mutation rate) and show microsatellite instability. An important consideration for MOA is the fact that chromium-induced lung cancer cells also show epigenetically-inactivated MMR genes (Takahashi et al., 2005). Also against the idea of a mutagenic MOI is the fact, discussed in Salnikow and Zhitkovich (2008), that Cr-induced lung tumors in humans lack p53 mutations, in contrast to lung tumors associated with other agents such as tobacco smoke, and the fact that the few mutations found do not correspond to the types of mutations caused by Cr in *in vitro* systems. The fact that Cr is essential also implies that oral Cr(VI) could supply the necessary Cr, again implying a threshold at nontoxic doses. Also, experiments from the Costa laboratory (Davidson et al.,Toxicol. Appl. Pharmacol. 196:431-437, 2004; Uddin et al., Toxicol. Appl. Pharmacol. 221:329-338, 2007) showing that chromate in drinking water is a cocarcinogen with solar UV, and the implications of this finding, are not discussed.

Other problems (by page):

Page 176, top of the page, is a good example of the confusion between mutagenicity and other endpoints. The statement "Hexavalent chromium-induced mutagenicity has been demonstrated following oral exposure" is misleading. There is only one mutagenicity assay showing positive effects on eyespots (presumed deletions) in offspring of female rats given drinking water with 62.5 mg Cr(6)/L. The deletions were not confirmed, so the eyespots might be epigenetic events (as Klein found with so-called mutants). All of the other assays are for non-mutagenic endpoints, and tend to be negative for drinking water exposure, but positive for gavage (a more toxic type of exposure).

Page 178, 4.5.1.2, it is claimed that mutagenicity has been evaluated in humans experimentally exposed to hexavalent chromium. No such studies appear in Table 4-25. The paragraph mistakenly refers to mutagenicity many times.

Page 186: It is not ER (excision repair) that is responsible for removal of bulky lesions, but NER (nucleotide excision repair) that is. Reynolds et al. 2004 is missing in references.

Page 187, bottom. It doesn't make sense that mice given 0 mg/kg Cr(6) should have a significant level of apoptosis (compared to what?).

Page 188: The Dai et al 2009 paper does not measure mutation frequency in human cells.

Page 190: 3rd paragraph: The mutational spectrum of chromate is not clear. See the review by Klein referenced above.

Page 202: Key Events, #3: The authors skip from discussing mechanisms of DNA damage by Cr to "overall genomic instability which can lead to mutations if not adequately repaired". Genomic instability can occur as a result of other factors besides DNA damage, and genomic instability is not repaired (DNA damage can be, but the repair often leads to apoptosis). As discussed above, toxic exposure would play a role in the selection of Cr-resistant and/or apoptosis-resistant cells. There is no obvious tie-in here between DNA damage and mutagenesis as a key event, since cells resistant to Cr could have arisen by epigenetic silencing (and this may also be a mechanism in resistance to apoptosis). In a sense, this point is recognized in #4, but in postulating apoptosis as a key event, the authors do not seem to realize the implications, i.e. that a toxic dose is needed for carcinogenicity. They suggest that selection for resistance to apoptosis is due to mutations (either pre-existing or Cr-induced) but there is no evidence for this. Besides altered DNA methylation, other mechanisms for the appearance of Cr(VI)-resistance in exposed cells include epigenetic effects via altered histone modification (Sun et al., Toxicol. Appl. Pharmacol. 237:258-266, 2009) as well as reduction of Cr(VI) transport via down-regulation of sulfate ion transporter activity, and resistance to apoptosis via altered gene expression (upregulation of survival pathways and down-regulation of apoptotic pathways) (discussed in Nickens et al., 2010)

p. 204-210: What is described as "mutagenicity" is not in all cases.

p. 206, end of paragraph 1: It is claimed that there is evidence that Cr(6) induces mutagenicity in tissues at the site of entry and systemically at doses relevant to human exposure. Where is the evidence for this?

p. 206, bottom: Again, it is important to note that DNA damage can lead to apoptosis as well as mutation, so it does not necessarily support a mutagenic MOA.

p. 207: De Flora et al., 2008, did not look for mutagenesis, they looked for DNA damage.

p. 208: Neither O'Brien et al., 2005 nor Eastmond et al., 2008 is in references.

p. 209: Low (non-toxic) concentrations would not provide selective pressure for Cr-resistant, mismatch repair deficient cells. Especially in vivo, toxicity would be the driving force to stimulate the outgrowth of such cells.

p. 209, 2nd. paragraph: This is a lot of speculation (should be, may be).

p. 211, bottom: It is claimed that the study by NTP found no evidence of tissue damage or necrosis. Did they look for apoptosis? It is also claimed that most available studies found Cr-induced genetic damage at doses below those that inducing cytotoxicity. This has not been demonstrated, since clonal survival (the only assay that will detect delayed toxicity) was not performed in these studies.

p. 212, end: "…giving rise to mutagenicity (including DNA adduct formation, DNA damage, gene mutations, chromosomal aberrations and micronuclei formation" This is nonsense, as is the next sentence. There is no evidence for mutagenicity at the target tissue at all.

p. 213: More confusion between mutagenicity and other endpoints. It is not true that other hypothesized MOA's have not been demonstrated. There is actually more tumor evidence for an epigenetic MOA. Thus, the weight of evidence favors the alternative. This is not a numbers game. Epigenetic studies are relatively new, compared with DNA damage and other "genotoxic" studies, so there are fewer studies.

p. 214 top: Has EPA concluded this? Then what is the purpose of reviewing this document?

p. 238, bottom: More confounding of mutagenesis and other endpoints.

Additional papers showing epigenetic effects of Cr

Schnekenburger et al., (2007) Chromium cross-links histone deacetylase1-DNA methyltransferase1 complexes to chromatin, inhibiting histone remodeling marks critical for transcriptional activation. Mol. Cell Biol. 27(20): 7089-101.

Sun et al. (2011) Comparison of gene expression profiles in chromate-transformed BEAS-2B cells. PloS ONE 6(3): e17982. Doi:10.1371/journal.pone.0017982

Ali et al. (2011) Aberrant DNA methylation of some tumor suppressor genes in lung cancers from workers with chromate exposure. Mol. Carcinogenesis 50(2):88-99

B3. A two-year drinking water study in rats and mice (NTP, 2008) was selected for the derivation of an oral slope factor. Please comment on whether the selection of this study for quantification is scientifically supported and clearly described. Please identify and provide the rationale for any other studies that should be considered.

I do not think that a linear no threshold approach is valid.

B4. The incidence of adenomas and carcinomas combined in the small intestine of male mice from the NTP (2008) two-year drinking water study were selected to serve as the basis for the quantitative cancer assessment. Please comment on whether this selection is scientifically supported and clearly described. Please identify and provide the rationale for any other endpoints that should be selected to serve as the basis for the quantitative cancer assessment.

This seems to be the only choice.

B5. The oral slope factor was calculated by linear extrapolation from the POD (i.e., the lower 95% confidence limit on the dose associated with 10% extra risk of tumors of the small intestine in male mice). Has the modeling been appropriately conducted and clearly described?

This is outside my field of expertise.

POST-MEETING COMMENTS SUBMITTED BY

Konstantin Salnikow, Ph.D.

Revised comments on the Integrated Risk Information System (IRIS) assessment of hexavalent chromium carcinogenicity.

These comments are part of an external peer-review of the scientific basis supporting the human health assessment of hexavalent chromium that will appear on the U.S. Environmental Protection Agency online database IRIS.

Comments are prepared by Dr. Konstantin Salnikow, Ph.D. Program Director, Division of Cancer Biology, Cancer Cell Biology Branch, National Cancer Institute, NIH.

G1. Is the Toxicological Review logical, clear and concise? Has EPA clearly presented and synthesized the scientific evidence for noncancer and cancer hazard?

In September 2010, the U.S. Environmental Protection Agency prepared the "Toxicological Review of Hexavalent Chromium" to assess health risks associated with hexavalent chromium exposure. This document will appear on the Agency's online database, the Integrated Risk Information System (IRIS). The existing IRIS file for hexavalent chromium, prepared in 1998, does not consider hexavalent chromium to be carcinogenic by the oral route of exposure. The purpose of the new document is to update the IRIS regarding noncancer and cancer health effects associated with oral exposure to hexavalent chromium after considering the latest scientific evidences.

The prepared draft of the "Toxicological Review of Hexavalent Chromium" provides a detailed analysis of the data obtained in several studies carried out in 2008, 2009 by the National Toxicology Program along with other studies, formulates a mutagenic mode of carcinogenic action, and suggests that the reduction of orally administered hexavalent chromium in guts, even in low doses, is incomplete. The draft also calculates a cancer slope factor for humans. Recognizing the importance and relevance of this document, this reviewer needs to address some shortcomings of the document. In general this is a dense document cataloguing many diverse and sometimes controversial studies in the area of hexavalent chromium toxicology and carcinogenesis. Of course the limitations in available experimental data obtained from existing animal models, ongoing investigations regarding the mode of action (MOA) of hexavalent chromium, and uncertainties in epidemiological data make it difficult to prepare a comprehensive document that will fully address public health concerns.

Chapter 3 should be reorganized and more emphasis should be given to the role of chromium III in toxico- and pharmacokinetics as well as in biological effects produced by hexavalent chromium. Hexavalent chromium is generally considered a much more potent mutagen and carcinogen than trivalent chromium. Lack of carcinogenic effects observed with trivalent chromium compounds can be explained by poor permeability of cell membrane for this ion. However, considering that the end product of intracellular reduction of hexavalent chromium is trivalent chromium, which may accumulate in tissues, it is important to consider what role intracellularly deposited trivalent chromium may play in chromium toxicity and carcinogenesis. The majority of studies indicate that after intracellular reduction of hexavalent chromium to trivalent chromium, it can form various damaging DNA adducts. These adducts can inhibit the enzymatic activity of DNA polymerases, simultaneously increasing the rate of replication and the processivity of the DNA polymerase, and thereby decreasing its fidelity and causing more frequent errors. The frequency of errors increases with a dose-dependent increase in mutation frequency *in vitro* (Snow, 1991; Salnikow and

Zhitkovich, 2008). Unfortunately, it is not clear how applicable these studies are to understanding the effects of trivalent chromium on DNA synthesis and cellular metabolism in vivo because the experiments were done in either in test tubes or in artificial model systems with concentrations far exceeding those obtained through environmental exposure (Snow, 1991; Dai *et al.*, 2009). Numerous attempts have been done to study the distribution and retention of chromium (III) in vivo. Onkelinx studied tissue retention of $^{51}CrCl_3$ in groups of female Wistar rats of various ages (35, 60, and 120 days) after a single intravenous injection of trace amounts of $^{51}CrCl_3$ (Onkelinx, 1977). The study showed that total excretory clearance is the sum of three components: urinary clearance (fu), fecal clearance (fd), and a residual clearance (fs), corresponding to an apparently irreversible deposition of chromium into long term body reservoirs. Consistent with the model, ^{51}Cr was found to accumulate with time in several organs such as bone, kidney, spleen, and liver after a single intravenous injection of $^{51}CrCl_3$. These data are supported by those obtained by O'Flaherty (O'Flaherty, 1996) and others indicating that the retention of chromium III by bone, liver, kidney, and spleen is prolonged.

Also, to make the toxicological review concise, I suggest eliminating Table 4-21"In vitro genotoxicity studies of hexavalent chromium in nonmammalian cells" and Table 4-24 "In vivo genotoxicity studies of hexavalent chromium in *D. melanogaster"* because these are irrelevant to the MOA of hexavalent chromium in mammalian systems.

There are numerous errors throughout the text of the Draft. Some of them are shown at the end of these comments as Errata.

G2. Please identify any additional studies that would make a significant impact on the conclusions of the Toxicological Review.

Key issues that should have an impact on the conclusions of the draft of the Toxicological Review of Hexavalent Chromium are: 1) the use of appropriate animal models, 2) an understanding of the chromium carcinogenic MOA, including genotoxic (mutagenic) and non-genotoxic (epigenetic) mechanisms and their interrelations, 3) the co-carcinogenic effects of hexavalent chromium, and 4) the role of iron metabolism in chromium carcinogenesis. Unfortunately, studies to address these issues are either not done or are in the early stages of research. Thus, it is too early to draw any conclusive decisions on risk assessment of hexavalent chromium in drinking water.

Are used animal models appropriate for risk assessment?

Although the NTP studies provide evidence that oral exposure to hexavalent chromium induced tumors in rodents, the main argument against these studies is that the toxic and carcinogenic effects could be achieved/observed only at high chromium concentrations, which significantly exceed human exposure levels. Also, it is noted that biological effects were seen only at chromium concentrations that overwhelmed the cellular defense systems (reducing capabilities). Ascorbate is a major reducing agent of hexavalent chromium in biological fluids and tissues (see review (Zhitkovich, 2005; Salnikow and Zhitkovich, 2008)). Humans cannot synthesize ascorbate in the body because of a mutation in the L-gulono-γ-lactone oxidase gene coding for the final enzyme in ascorbate metabolism and thus ascorbate is supplemented through the diet. Unlike humans, laboratory mice and rats, used for carcinogenicity assays, are capable of synthesizing ascorbate endogenously. Because ascorbate regulates many cell and tissue functions that are critical for cancer development, the changes in the level of ascorbate should be considered in animal models of choice (Salnikow and Kasprzak, 2005). It is impossible to deplete tissue ascorbate levels by metal exposure in wild-type rodents because the enzyme producing ascorbate will be up-regulated when the level of ascorbate drops below a critical point. To avoid this problem for *in vivo* testing of the toxic and carcinogenic effects of heavy metals, which efficiently destroy ascorbate, an appropriate model is the use of mice or rats that like humans

cannot synthesize ascorbate (Kasprzak *et al.*, 2011). Two rodent model systems unable to synthesize ascorbate are available: Gulo-/- mice (Maeda *et al.*, 2000), and a similar rat strain (Mizushima *et al.*, 1984). Our preliminary data show that when Gulo-/- mice were supplemented with ascorbate in drinking water their blood and tissue ascorbate levels were undistinguishable from that in wild type mice. However, ascorbate levels were significantly decreased by metal exposure in Gulo-/- mice but not in wild-type mice, in which the enzyme responsible for ascorbate production was activated in response to metal exposure (ascorbate depletion) (Kasprzak *et al.*, 2011). In this model system we found that the reduction in ascorbate levels increased acute toxicity induced by Ni_3S_2 in Gulo-/- mice and that Gulo-/- mice were more susceptible than wild-type mice to nickel-induced carcinogenesis. Additionally, in tumor transplantation assays, Gulo-/- mice had shorter tumor latency than wild-type mice. After the lag period established tumor growth rates were comparable in Gulo-/- and wild-type mice. Although cancer initiation and development is a very complicated process our results indicate that ascorbate is a potentially important part of the molecular mechanisms of metal carcinogenesis and acute toxicity.

Ascorbate is involved in diverse biological activities. Ascorbate is essential for the function of numerous 2-oxoglutarate-dependent hydroxylases. This group of hydroxylases includes the asparaginyl and prolyl hydroxylases, FIH-1 and PHD1, PHD2, PHD3, which are responsible for HIFα hydroxylation (Epstein *et al.*, 2001; Mahon *et al.*, 2001; Hewitson *et al.*, 2002; Lando *et al.*, 2002); the collagen prolyl-4-hydroxylases (Myllyharju, 2003), which is critical for extracellular matrix formation; and a new class of histone and DNA demethylases that remove methyl group through hydroxylation (Shi, 2007). We already pointed out that the level of ascorbate is critical for metal carcinogenesis mainly by affecting epigenetic pathway (Salnikow and Zhitkovich, 2008). More recently a link between changes in ascorbate concentration and DNA demethylation of human embryonic stem cells (hESCs) has been identified (Chung *et al.*, 2010). Thus, ascorbate levels have the potential to directly impact the differentiation of hESCs and the reprogramming of somatic cells.

Given that ascorbate has diverse cellular functions and ascorbate levels are critical to interpreting carcinogenic effects of heavy metals, the animal models described in the Toxicological Review are not the most appropriate. The results obtained in NTP 2007 studies are consistent with the idea that ascorbate is an important factor to consider. In preliminary toxicokinetic studies in which animals were exposed to chromium in drinking water for 21 days chromium concentrations in the blood of the guinea pigs (which are unable to synthesis ascorbate) was greater than chromium concentrations in the blood of the rats or mice suggesting greater absorption (less reduction) of chromium in guinea pigs.
http://ntp.niehs.nih.gov/ntp/htdocs/ST_rpts/tox072.pdf

I suggest that new studies similar to the NTP studies with several dietary concentrations of ascorbate and lower chromium does (i.e., those more relevant to environmental exposures), be done in ascorbate-deficient rats or mice or both animal models. Additionally, because of more efficient depletion of ascorbate in tissues of Gulo-/- animals by chromium these animal models will show whether Cr(III) and Cr(VI) kinetic that were developed using the wild type animals (O'Flaherty, 1996; O'Flaherty *et al.*, 2001) will be applicable to Gulo-/- animals. This will allow for adjustment of kinetic models, if needed, and identification of new or confirming known compartments of chromium retention.

A1-A4 Oral Reference Dose (RfD) for Hexavalent Chromium

Outside of my area of expertise

Carcinogenicity of Hexavalent Chromium

B1.

Hexavalent chromium has been classified by IARC as carcinogenic to humans (group 1) via inhalation route of exposure based on results obtained in human and animal studies. However, when animals were exposed to hexavalent chromium in drinking water the carcinogenic effects were observed only at very high doses, which are irrelevant to human exposure. These results seems to cast doubt on carcinogenicity of hexavalent chromium via oral route of exposure and yet as I already pointed out in G2 the reason for only high chromium doses producing carcinogenic effect may be stemming from the inappropriate animal models which have higher protective ascorbate levels as compared to humans. Although, more human and animal studies are required to make an informed conclusion the ability of hexavalent chromium to produce tumors makes it likely to be carcinogenic by oral exposure.

Another important consideration is that hexavalent chromium is a co-carcinogen and consumption of water with other toxic or carcinogenic compounds will result in unraveling chromium carcinogenic effects at much lower doses. Additionally, people with chronic inflammation of digestive tract could be more susceptible to chromium-induced carcinogenesis.

B2.

MOA. Genotoxic effect of hexavalent chromium.

The draft of the Toxicological review provides a substantial body of information regarding the mutagenic potential of hexavalent chromium and concludes that hexavalent chromium is carcinogenic by a mutagenic MOA. Indeed this topic has been studied extensively. The results of in vitro and in vivo studies provide substantial evidence for the mutagenic activity of hexavalent chromium, which is mediated through the generation of the highly reactive chromium intermediates penta- and tetravalent chromium, reactive oxygen species, and trivalent chromium formed during the intracellular reduction of hexavalent chromium. These chromium and oxygen species can react with DNA, leading to oxidative DNA damage, chromium-DNA adducts, DNA strand breaks, and chromosomal aberrations. Despite these studies, the significance of chromium-induced DNA damage in the mechanisms of chromium carcinogenicity is not clear. If DNA damage/mutations are important and a causative factor in chromium-induced carcinogenesis, then these mutations should be frequent in chromium-induced tumors. However, very few publications exist in this respect. In animal studies, De Flora et al. (De Flora *et al.*, 2008) found no evidence of DNA-protein crosslinks and DNA adducts in the duodenum following drinking water chromium exposures. Other available hexavalent chromium drinking water exposure studies that measured mutagenicity in mice also could not detect evidence of micronucleus induction in the blood or bone marrow (Mirsalis *et al.*, 1996; De Flora *et al.*, 2006; De Flora *et al.*, 2008). Analysis of lung cancers from chromate-exposed workers revealed that p53 mutations are not very frequent, with only six missense mutations identified in 4 (20%) of the 20 chromate lung cancer samples (Kondo *et al.*, 1997). There were fewer mutations in the patients with lung cancers who had been exposed to chromate than in those who had not (20% vs about 50%). This study also revealed that there was no association between p53 mutations and the period spent working in chromate factories. It is conceivable that chromium causes genotoxic effects, damage DNA, but this damage is efficiently repaired and do not play any role in carcinogenic effects of chromium.

Thus, in order to confirm or refute a possible role of chromium genotoxic/mutagenic effects in chromium-induced carcinogenesis, comprehensive analyses of mutations in oncogenes and tumor suppressor genes in experimentally induced tumors in animals should be done first, followed by more detailed sequence analyses

of chromium-exposed human tumors. A feasible study that could be done in a short period of time (assuming that tumor samples collected in the NTP studies are stored and frozen) is **exon only global sequencing** of DNA from chromium-induced rat tumors versus spontaneous tumors (several spontaneous tumors of different origin were observed in NTP 2008 study). These studies will allow comparison of tumor driving mutations versus passenger mutations.

MOA, Epigenetic effects of hexavalent chromium.

Global changes in the epigenetic landscape are a hallmark of cancer. The initiation and progression of cancer, traditionally seen as a genetic disease, is now realized to involve epigenetic abnormalities along with genetic alterations. Recent advancements in the rapidly evolving field of cancer epigenetics have shown extensive reprogramming of every component of the epigenetic machinery in cancer including DNA methylation, histone modifications, nucleosome positioning and non-coding RNAs, specifically microRNA expression (Sharma *et al.*, 2010). Epigenetic effects have also been observed following hexavalent chromium exposure (Salnikow and Zhitkovich, 2008; Arita and Costa, 2009). Increased DNA methylation was observed in the promoter region of the tumor suppressor gene p16 and the MMR gene hMLH1, indicating that chromium can induce epigenetic effects (Takahashi *et al.*, 2005; Kondo *et al.*, 2006). Gene transcription has also been shown to be affected by exposure to hexavalent chromium in vitro via epigenetic mechanisms. Sun et al. (Sun *et al.*, 2009) found alterations in the levels of histone methylation in human lung A549 cells exposed to hexavalent chromium, indicating the capability of these exposures to lead directly to changes in gene expression. Taken together, these studies suggest that epigenetic mechanisms may contribute to the carcinogenicity of hexavalent chromium. However, it is not clear whether epigenetic changes produced by chromium exposure are acting alone or linked to chromium genotoxic effects and that both genetic and epigenetic changes are essential for tumor appearance and evolution. More research is needed in this area including the identification of changes in DNA methylation (analyses of frequency of inherited silencing of tumor suppressors in tumors and in miRNA expression patterns following chromium exposure in animal models and humans before any conclusions can be drawn regarding the role of epigenetics in the carcinogenic effects of hexavalent chromium.

MOA. Co-carcinogenic effects of chromium.

Co-carcinogenic effects of hexavalent chromium were reviewed recently (Salnikow and Zhitkovich, 2008). The majority of occupational and probably all environmental exposures to hexavalent chromium occur as co-exposures with other carcinogens. The most common examples of co-exposures occur among stainless steel welders, and among hexavalent chromium-exposed workers who are also smokers. Two reports from the Costa Lab (Davidson *et al.*, 2004; Uddin *et al.*, 2007) provided strong experimental data demonstrating that hexavalent chromium can act as a potent co-carcinogen for UV-induced skin tumors. In both studies, the presence of hexavalent chromium in drinking water caused dose-dependent increases in the frequency of skin tumors in UV-irradiated hairless mice. Hexavalent chromium alone produced no tumors, indicating that it acted a strong enhancer of UV-initiated tumorigenesis. Supplementation with vitamin E or selenomethionine had no effect on hexavalent chromium-mediated enhancement of skin carcinogenesis suggesting that co-carcinogenic effects were not oxidant-mediated. It is noteworthy that the level of chromium in skin directly exposed to UV had significantly higher levels of chromium than underbelly skin that was not directly exposed to UV in mice exposed to UV and 5 ppm K_2CrO_4 ($P < 0.05$) (Davidson *et al.*, 2004). This raises an interesting question, does inflammation, whatever the source, facilitate chromium accumulation or delay chromium clearance? This is an important and understudied area. The identified co-carcinogenic effects of hexavalent chromium raise an intriguing possibility that much lower doses of chromium could be hazardous

under certain circumstances when exposure to chromium in drinking water is combined with other harmful exposures.

Another important area of research is an understanding of the role of Inflammation/colitis in hexavalent chromium carcinogenesis. It is well know that at least 20% of all cancers arise in association with infection and chronic inflammation and even those cancers that do not develop as a consequence of chronic inflammation, exhibit extensive inflammatory infiltrates with high levels of cytokine expression in the tumor microenvironment. Aberrant activation of NF-κB and/or STAT3 is found in over 50% of all cancers and renders premalignant and fully transformed cells resistant to apoptosis and speeds up their rate of proliferation, thereby increasing tumor growth. It is extremely important to test whether hexavalent chromium will be more carcinogenic and at lower doses in animals in which colitis was induced, for example by sodium dextran sulfate.

MOA. Interference with iron metabolism.

In rats neoplastic changes were found at sites of tissue contacts with the highest concentrations of hexavalent chromium, i.e. oral cavity. This may be explained by the immediate damaging effects of chromium on DNA and other cellular components. At the same time frequent nonneoplastic changes were observed in duodenum of male and female rats and neoplastic changes were observed in duodenum of male and female mice. These data cannot be explained by the direct effect of hexavalent chromium, which should be mostly reduced by the time it reaches small intestine. Considering that in other organs such as liver and kidney which accumulate significant amount of chromium no tumors were observed, it is important to do more research on the mechanism of tumor development in small intestine. Specifically, duodenum is a place where iron absorption takes place. An analysis of ferroreductase expression and iron metabolism will help to shed light on whether an alteration in iron metabolism in this tissue may be responsible for chromium carcinogenic effects.

B3.

Outside of my area of expertise.

B4.

It seems that combining the incidence of adenomas and carcinomas in small intestine was the proper choice for modeling cancer risk. This is supported by the available data and clearly described. The fact that only highest doses produced a carcinogenic effect may indicate high reducing capacity in tested model systems (wild type mice and rats). As suggested in A2 exposing Gulo-/- mice or rats to hexavalent chromium may result in tumor appearance at lower doses. If this will be the case the extrapolation to environmentally relevant doses of chromium exposure will be more feasible.

B5.

Outside of my area of expertise.

Errata:

Regarding the form of the draft it should be noted that, although this is not a manuscript, it is necessary to correct errors and mistakes in the draft content.

Below are several examples:

1. **$K_2Cr_2O_4$** – does not exist (page 30, table 3-7; page 45), **should it be K_2CrO_4**
2. Table 2-1, page 6 – **Cr_2O_3** is chromium (III), not hexavalent chromium. **$BaCrO_4$** is barium chromate, not barium oxide.
3. Table 2-5, page 18 – "accumulates Cr(V)", the intent of this statement is not clear because this form is short living and unlikely that is can accumulate.
4. Page 35 and Table 3-9, **K_2CrO_7** - does not exist
5. None existing or wrong citations:
   Kumulainen, 1991 (page 7), **should be Kumpulainen**, **1992** (page 251).
   Salnikov and Zhitkovich, 2009 (page 50); Salnikov and Zhitkovich, 2008 (page 257), **should be Salnikow and Zhitkovich, 2008**.
   Costa and Klein, 2004 (page 66); Costa, M.; Klein, C.B. 2008 (page 245), **should be Costa and Klein, 2006**.
   LeVina et al., 2003, 2007, (page 50) **should be Levina et al., 2003**, **2007**.
   Kasprzak, 1996 (page 189), **no reference provided.**
   Campbell J.L.; Tan,Y.; Clewell, H.J. (2009) Development of a PBPK model for hexavalent chromium in rats and mice to estimate exposure to oral mucosa and small intestine. Toxicologist 108(1):98 (Abstract) Poster ID # 108. This is a **questionable citation**.
   Sun et al. (2009), (page 188), **no reference provided.**
   Davies, JM. (1979) Lung cancer mortality of workers in chromate pigment manufacture: An epidemiological survey. J Oil Chem Assoc 62:157-163, (page 245), **should be: J Oil Colour Chem Assoc 62:157-163**

References:

Arita, A., and Costa, M. (2009). Epigenetics in metal carcinogenesis: nickel, arsenic, chromium and cadmium. *Metallomics* **1**, 222-228.

Chung, T. L., Brena, R. M., Kolle, G., Grimmond, S. M., Berman, B. P., Laird, P. W., Pera, M. F., and Wolvetang, E. J. (2010). Vitamin C promotes widespread yet specific DNA demethylation of the epigenome in human embryonic stem cells. *Stem Cells* **28**, 1848-1855.

Dai, H., Liu, J., Malkas, L. H., Catalano, J., Alagharu, S., and Hickey, R. J. (2009). Chromium reduces the in vitro activity and fidelity of DNA replication mediated by the human cell DNA synthesome. *Toxicol Appl Pharmacol* **236**, 154-165.

Davidson, T., Kluz, T., Burns, F., Rossman, T., Zhang, Q., Uddin, A., Nadas, A., and Costa, M. (2004). Exposure to chromium (VI) in the drinking water increases susceptibility to UV-induced skin tumors in hairless mice. *Toxicol Appl Pharmacol* **196**, 431-437.

De Flora, S., D'Agostini, F., Balansky, R., Micale, R., Baluce, B., and Izzotti, A. (2008). Lack of genotoxic effects in hematopoietic and gastrointestinal cells of mice receiving chromium(VI) with the drinking water. *Mutat Res* **659**, 60-67.

De Flora, S., Iltcheva, M., and Balansky, R. M. (2006). Oral chromium(VI) does not affect the frequency of micronuclei in hematopoietic cells of adult mice and of transplacentally exposed fetuses. *Mutat Res* **610**, 38-47.

Epstein, A. C., Gleadle, J. M., McNeill, L. A., Hewitson, K. S., O'Rourke, J., Mole, D. R., Mukherji, M., Metzen, E., Wilson, M. I., Dhanda, A., Tian, Y. M., Masson, N., Hamilton, D. L., Jaakkola, P., Barstead, R., Hodgkin, J., Maxwell, P. H., Pugh, C. W., Schofield, C. J., and Ratcliffe, P. J. (2001). C. elegans EGL-9 and mammalian homologs define a family of dioxygenases that regulate HIF by prolyl hydroxylation. *Cell* **107**, 43-54.

Hewitson, K. S., McNeill, L. A., Riordan, M. V., Tian, Y. M., Bullock, A. N., Welford, R. W., Elkins, J. M., Oldham, N. J., Bhattacharya, S., Gleadle, J. M., Ratcliffe, P. J., Pugh, C. W., and Schofield, C. J. (2002). Hypoxia-inducible factor (HIF) asparagine hydroxylase is identical to factor inhibiting HIF (FIH) and is related to the cupin structural family. *J Biol Chem* **277**, 26351-26355.

Kasprzak, K. S., Diwan, B. A., Kaczmarek, M., Logsdon, D., Fivash, M. J., Jr., and Salnikow, K. (2011). Effects of Ascorbic Acid on Carcinogenicity and Acute Toxicity of Nickel Subsulfide, and on Tumor Transplants Growth in Gulonolactone Oxidase Knock-Out Mice and Wild-type C57BL Mice *Toxicol Appl Pharmacol* **submitted**.

Kondo, K., Hino, N., Sasa, M., Kamamura, Y., Sakiyama, S., Tsuyuguchi, M., Hashimoto, M., Uyama, T., and Monden, Y. (1997). Mutations of the p53 gene in human lung cancer from chromate-exposed workers. *Biochem Biophys Res Commun* **239**, 95-100.

Kondo, K., Takahashi, Y., Hirose, Y., Nagao, T., Tsuyuguchi, M., Hashimoto, M., Ochiai, A., Monden, Y., and Tangoku, A. (2006). The reduced expression and aberrant methylation of p16(INK4a) in chromate workers with lung cancer. *Lung Cancer* **53**, 295-302.

Lando, D., Peet, D. J., Gorman, J. J., Whelan, D. A., Whitelaw, M. L., and Bruick, R. K. (2002). FIH-1 is an asparaginyl hydroxylase enzyme that regulates the transcriptional activity of hypoxia-inducible factor. *Genes Dev* **16**, 1466-1471.

Maeda, N., Hagihara, H., Nakata, Y., Hiller, S., Wilder, J., and Reddick, R. (2000). Aortic wall damage in mice unable to synthesize ascorbic acid. *Proc. Natl. Acad. Sci. U. S. A.* **97**, 841-846.

Mahon, P. C., Hirota, K., and Semenza, G. L. (2001). FIH-1: a novel protein that interacts with HIF-1alpha and VHL to mediate repression of HIF-1 transcriptional activity. *Genes Dev* **15**, 2675-2686.

Mirsalis, J. C., Hamilton, C. M., O'Loughlin, K. G., Paustenbach, D. J., Kerger, B. D., and Patierno, S. (1996). Chromium (VI) at plausible drinking water concentrations is not genotoxic in the in vivo bone marrow micronucleus or liver unscheduled DNA synthesis assays. *Environ. Mol. Mutagen.* **28**, 60-63.

Mizushima, Y., Harauchi, T., Yoshizaki, T., and Makino, S. (1984). A rat mutant unable to synthesize vitamin C. *Experientia* **40**, 359-361.

Myllyharju, J. (2003). Prolyl 4-hydroxylases, the key enzymes of collagen biosynthesis. *Matrix Biol.* **22**, 15-24.

O'Flaherty, E. J. (1996). A physiologically based model of chromium kinetics in the rat. *Toxicol Appl Pharmacol* **138**, 54-64.

O'Flaherty, E. J., Kerger, B. D., Hays, S. M., and Paustenbach, D. J. (2001). A physiologically based model for the ingestion of chromium(III) and chromium(VI) by humans. *Toxicol. Sci.* **60**, 196-213.

Onkelinx, C. (1977). Compartment analysis of metabolism of chromium(III) in rats of various ages. *Am J Physiol* **232**, E478-484.

Salnikow, K., and Kasprzak, K. S. (2005). Ascorbate depletion: a critical step in nickel carcinogenesis? *Environ Health Perspect* **113**, 577-584.

Salnikow, K., and Zhitkovich, A. (2008). Genetic and epigenetic mechanisms in metal carcinogenesis and cocarcinogenesis: nickel, arsenic, and chromium. *Chem. Res. Toxicol.* **21**, 28-44.

Sharma, S., Kelly, T. K., and Jones, P. A. (2010). Epigenetics in cancer. *Carcinogenesis* **31**, 27-36.

Shi, Y. (2007). Histone lysine demethylases: emerging roles in development, physiology and disease. *Nat Rev Genet* **8**, 829-833.

Snow, E. T. (1991). A possible role for chromium(III) in genotoxicity. *Environ Health Perspect* **92**, 75-81.

Sun, H., Zhou, X., Chen, H., Li, Q., and Costa, M. (2009). Modulation of histone methylation and MLH1 gene silencing by hexavalent chromium. *Toxicol Appl Pharmacol* **237**, 258-266.

Takahashi, Y., Kondo, K., Hirose, T., Nakagawa, H., Tsuyuguchi, M., Hashimoto, M., Sano, T., Ochiai, A., and Monden, Y. (2005). Microsatellite instability and protein expression of the DNA mismatch repair gene, hMLH1, of lung cancer in chromate-exposed workers. *Mol. Carcinog.* **42**, 150-158.

Uddin, A. N., Burns, F. J., Rossman, T. G., Chen, H., Kluz, T., and Costa, M. (2007). Dietary chromium and nickel enhance UV-carcinogenesis in skin of hairless mice. *Toxicol Appl Pharmacol* **221**, 329-338.

Zhitkovich, A. (2005). Importance of chromium-DNA adducts in mutagenicity and toxicity of chromium(VI). *Chem. Res. Toxicol.* **18**, 3-11.

POST-MEETING COMMENTS SUBMITTED BY

John Pierce Wise Sr., Ph.D.

Toxicological Review of Hexavalent Chromium

PRE-MEETING WRITTEN COMMENTS

CHARGE QUESTIONS

General Charge Questions:

G1. ***Is the Toxicological Review logical, clear and concise? Has EPA clearly presented and synthesized the scientific evidence for noncancer and cancer hazard?***

The Toxicological Review is logical, clear and concise. However, overall the document is inconsistent and thus, EPA has not presented and synthesized the scientific evidence for noncancer and cancer hazard in a clear manner. Some sections, primarily the ones focused on animal data are clearly presented and synthesized. These sections present the primary literature and discuss the merits of each study with balance and insight.

Other sections, however, particularly those involving *in vitro* cell culture data and underpinning the mode of action are much less appropriately considered, are not well-presented, and do not synthesize the underlying data very well. Determining a mode of action is a key part of the risk assessment. The Toxicological Review would be a stronger document if it fully analyzed and synthesized the primary literature to ascertain the possible modes of action for Cr(VI). The strengths, weaknesses and data gaps for each could have been highlighted and discussed, and then a rationale for the chosen mode of action presented. However, as presented this approach is not apparent.

Instead, as presented, the document gives the impression that the mode of action was pre-determined from a select set of review articles and the best case for that mode of action presented. Decisions appear to have been made to agglomerate all of the genotoxicity data into positive or negative proof of mutagenesis rather than more careful consideration of individual lesions. Confounding factors, such as ascorbate levels, are cautioned against, but inadequately and inaccurately presented and unevenly applied, which undermines confidence that the primary data were adequately considered and contributes to a perception that decisions were predetermined. This perception is strongly reinforced by poor management and citation of the underlying literature and a heavy reliance on a few select review articles that unfortunately miscite the primary literature. As a result, many sections of the Toxicological Review lack clarity, accuracy, synthesis and rigor and the rationale for the choice of mode of action seems predetermined and forced. Each of these factors is elaborated on in more detail below.

1. Unnecessary agglomeration of genotoxicity data

The Toxicological Review essentially combines all of the lesions related to genotoxicity (stated on page 212 as "*…including DNA adduct formation, DNA damage, gene mutations, chromosomal aberrations, and micronuclei formation"*) into one bundle and refers to them as "mutagenicity". This decision is typically based on the presumption that the various genotoxic lesions will ultimately manifest as mutations in the primary DNA sequence. Hence, it is generally recognized that a crosslink or a strand break is not inherently a mutation, but that it may eventually manifest as one and thus, it is a mutagenic event. Based on the aggregation of all of these data, the Toxicological Profile declares Cr(VI) to be a mutagen and proposed a mutagenic mode of action.

This approach is consistent with older practice and perception of these genotoxicity assays and it may be a useful approach for a chemical with a limited data set. However, the genotoxicity data for Cr(VI) is a rich data set and deserves a more sophisticated consideration. The Cr(VI) literature often distinguishes in its presentation between mutagenic and genotoxic lesions. The Toxicological Review does not carry forward that distinction and does not explain the rationale for ignoring it. However, it is an important distinction because not all of these lesions are likely to be mutagenic after Cr(VI) exposure. Aggregating these lesions oversimplifies the interpretation of the data and masks the fact that much of the primary data suggest that in actuality, Cr(VI) is a very weak mutagen. Discussing each class of lesion on its own merit with a more careful consideration of the primary literature would have better framed the strengths and limitations of the genotoxicity studies and brought this discussion into a clearer light.

The fundamental problem underlying this section is a failure to clearly consider the primary literature to see that Cr(VI) is a weak mutagen when defined as an agent that can directly change the primary DNA sequence. Cr(VI)-induced mutations have indeed been observed in bacteria, cultured cells and animal studies. However, in most cases, one has to experimentally force the mutations to occur by using a high dose, a forced experimental system or a non-physiological exposure route.

There is no real synthesis of this literature beyond listing outcomes in a table. The Toxicological Review also considers the results as simply positive or negative. That certainly is one approach; however, it misses the opportunity to consider the data more thoroughly. Some consideration should be given to potency and its potential impact and the robustness of the underlying assays. If the experimental data show that Cr(VI) induces a 2-fold increase in mutations, then the Toxicological Review would call that outcome positive. However, if in that same assay an established mutagen induced a 50-fold increase in mutations, then does Cr(VI) still appear to be a mutagen? Or does it suggest a different mode of action, particularly when the frequency of mutations have not been reported to increase in Cr(VI)-induced human tumors? Does the fact

that ascorbate is higher in rodents make these mutations a rodent-specific event? A more careful and thoughtful presentation of the underlying data would have better informed the consideration of this mode of action.

In addition, before lumping all of these genotoxic endpoints together as all mutagenic outcomes, a careful review and discussion of the primary mutagenesis literature is needed. That review needs to determine, for Cr(VI), which of the various lesions (e.g. DNA adducts, DNA crosslinks, DNA strand breaks, gene mutations, chromosome damage, etc.) actually occur in cells (for example as discussed below the adducts may not actually form in cells) and to what extent they occur. Then, the review needs to determine which, if any, of these lesions actually lead to gene mutations. The discussion below illustrates that there are reasons to doubt that the various lesions are all mutagenic outcomes. After this analysis, those lesions that do form in cells and that do produce mutations could more reasonably be combined into a category of "mutagenicity".

Perhaps, the data will indicate Cr(VI) is a mutagen, but, perhaps, the data indicate that one only gets mutations in the DNA sequence when systems are forced experimentally to do so at very high concentrations, due to species specific factors or by non-physiological exposure routes. If the latter were true, this possibility would suggest that mutations are not likely to occur in humans, raising direct implications for the mode of action decision. A more thorough treatment of the primary mutation data is needed to clarify these important points.

There is concern that the discussion of some lesions is overstated while others are mentioned but not discussed. The section explaining DNA adducts is greatly overstated and also does not fully consider the primary literature. The section presents a case that implies the status and impact of the various potential adducts are known in cells and *in vivo*. The Toxicological Review even provides a structure of a Cr-DNA adduct. The major concern is that when the primary literature is fully considered, it becomes apparent that these adducts are all based on cell-free systems and no one has been able to clearly identify any specific adducts in cells, whole animals or humans beyond observing tangles of DNA, protein, and Cr that are considered to be DNA-DNA or DNA-protein crosslinks. The primary literature has only measured adduct levels in cells by isolating DNA and then measuring the amount of Cr associated with it or by nonspecific P32 postlabelling. These measures cannot ascertain how or if Cr is bound to the DNA, only that it is associated with it in some way. Some studies have synthesized adducts in cell free systems and applied them to cells, but that does not mean those specific adducts form in the cell.

Thus, it is unknown if specific DNA adduct events occur in cells, whole animals or humans. Nonspecific adducts have been detected by postlabelling, but specific adducts remain elusive. To lump these studies together as clear evidence of mutagenicity gives them a weight of evidence that seems premature and inaccurate. Overall, this section is very misleading in its portrayal of the status of adducts as more understood than they actually are.

Oxidative damage is also included as evidence of mutagenicity. However, discussion is missing to establish whether this oxidative damage is a direct effect of Cr(VI) causing oxidative damage to DNA and thus, potentially a mutagenic event, or if this damage is actually indirect, resulting from overall oxidative stress to cells caused by high doses of Cr(VI) depleting intracellular antioxidants.

Cr(VI)-induced strand breaks are cited as another type of mutagenic event. These lesions are discussed as post-replication-induced breaks. However, the discussion fails to question and discuss whether or not these are actually frank DNA breaks. As the Toxicological Review indicates, the studies have focused on gamma-H2A.X focus production as the measure of breaks. Data indicate that chromatin remodeling may also induce the production of gamma-H2A.X so they may not be frank DNA breaks after all. This possible explanation is missing from the section.

DNA-protein crosslinks are included as a type of mutagenicity in the tables and description despite the fact that the document states on page 186 that it is unknown if they are mutagenic: "*Tests for the mutagenicity of these crosslinks have proved inconclusive (reviewed in Macfie et al., 2010), but the bulkiness of these lesions indicates the potential for genotoxicity…*"

The most consistent genotoxic outcome is the production of damage to metaphase chromosomes manifested as aberrations, sister chromatid exchanges, and micronuclei. The fact that Cr(VI) induces these events was presented but not discussed. This lesion may be the key lesion as it is the most consistent and yet the mechanisms that may underlie it are ignored and not discussed. Chromosome damage could be a mutagenic lesion as assumed. Alternatively, it could be the consequence of epigenetic changes in the cell resulting from Cr binding to centrosomes in the mitotic spindle assembly apparatus or from bypass of the spindle assembly checkpoint. Cr(VI) has been shown to affect centrosomes and the spindle assembly checkpoint and perhaps it causes uneven pulling leading to breaks and errors in chromosome number. Cells with broken chromosomes may undergo apoptosis, while those with increased chromosome number may go on and survive as highly aneuploid cells. Cr(VI) has been shown to induce highly aneuploid cells that can clonally expand and survive. This outcome would not be consistent with a mutagenic mode of action.

These concerns above are only magnified by the problems with uneven consideration of confounders and in poor management of the underlying literature described below. Together these factors give the impression of excluding or avoiding different syntheses of the data. More care and balance are needed to discuss and consider the genotoxicity data separately and evaluate if they are mutagenic markers.

2. Uneven application of experimental confounders

The perception of bias caused by bundling all of the genotoxicity endpoints together is magnified by an apparent uneven consideration of experimental confounders in the document. It appears that the Toxicological Review does not fully consider and present all of the relevant *in vitro* cell culture data that inform possible modes of action. Instead, selected examples of primary literature that reinforce one point of view are presented. This approach undermines the synthesis of the literature and because of a marked unevenness in presentation creates a perception of bias that should not be part of the analysis.

For example, on page 184-185, the Toxicological Review gives the reader the impression that ascorbate-trivalent chromium-DNA adducts have been found to be highly mutagenic. However, the section did not describe the experimental detail that indicate the cells used in the study were abnormal and genetically modified so that they could not carry out proficient DNA repair or apoptosis, or that the cells were not actually treated directly with Cr(VI). These omissions stand in stark contrast to the experimental criticisms the Toxicological Review applies to other cell culture studies.

For example, on page 47, the document states:

> "*Caution should be used in interpreting cell culture data, as the cell culture medium could play a role in hexavalent chromium reduction, confounding the extent of intracellular hexavalent chromium reduction.*"

It then cites a couple of examples where Cr(V) was detected in extracellular cell culture medium. The use of the word "caution" leads the reader to conclude that many *in vitro* studies may be flawed due to this reduction. No explanation is offered as to why this outcome is a problem. No discussion is provided that points out whether this same type of reduction might be expected to occur outside of cells in the body and thus actually be normal. The practical reality is that reducing agents are present in the extracellular fluid and thus, some extracellular reduction probably occurs in the extracellular fluid. This factor is probably not appropriate as a cautionary one and may actually reflect physiological conditions. But, no balanced discussion is provided for the reader to decide if this factor is indeed a concern.

A similar unevenness occurs during the presentation of the relative importance of ascorbate. The Toxicological Review states on page 50:

> "*An additional important note on these biotransformations regards the interpretation and reliability of data from in vitro assays. In vivo, the intracellular levels of ascorbate are quite high (about 1 mM). In contrast, the levels of ascorbate in tissue culture media are quite low since generally it is not added to the media so that the only source is supplemented fetal bovine serum (FBS). With 10% FBS, the level of ascorbate in tissue cultured cells is only about 50 μM which is 20 times lower than that which is found in vivo (Zhitkovich, 2005). Therefore, experiments on mutagenesis and other toxic effects of hexavalent chromium in tissue culture may underestimate its mutagenic, genotoxic, and cell-transforming activities (Zhitkovich, 2005; Costa and Klein, 2006).*"

No further discussion is presented. There is no presentation of the primary literature in cell culture showing the impact or lack of impact of this difference, just speculation that it might cause some underestimation. There is no presentation of the primary literature to establish what the ascorbate levels are in the relevant tissues of concern. There is just this one review article (Zhitkovich 2005) with some comment from a secondary review article (Costa and Klein 2006) cited. Closer inspection shows that the Costa and Klein review is actually citing the same Zhitkovich review so in the final consideration, this entire section relies only on Zhitkovich 2005 which, as discussed below in section 3.D., miscites the primary and secondary literature and draws a conclusion the primary literature does not closely support.

Thus, the Toxicological Review draws attention to the possible presence of extracellular metabolism and lack of intracellular ascorbate as confounding factors, which it may have used to exclude some cell culture studies. But, by contrast, it makes no mention and expresses no concern about studies done in abnormal compromised cells treated only indirectly with Cr(VI). This discrepancy makes the document and its treatment of the underlying literature seem uneven.

Moreover, in its discussion of the impact of ascorbate, the Toxicological Review does not discuss the potential impact of ascorbate differences on the bacterial mutation studies or the possible impact of ascorbate differences on the animal genotoxicity data. For example, in Table 4-23 on page 172, the Toxicological Review indicates a positive effect for mutagenicity in mice after intratracheal instillation. Mice, however, have more ascorbate in their lung tissue. If one accepts the speculation in the Toxicological Review that ascorbate–trivalent chromium–DNA adducts form and are highly mutagenic, then the elevated mutations in this study might simply be due to the elevated ascorbate levels in this species suggesting a species specific effect. Such a possibility would explain why there are mutations in rodents but not in human tumors.

Regardless of which conclusion is correct, the point is that the Toxicological Review does not appear to apply this confounder it stresses in the *in vitro* work evenly to all studies reinforcing a perception of selective bias.

Similarly, the Toxicological Review presents the primary research studies by Quievryn et al., 2003; Voitkun et al., 1998 as showing adduct effects, but both studies used the cell culture medium the Toxicological Review expresses concerns about and neither study addressed the ascorbate concern, but these aspects are not mentioned in the document. The absence of discussion of these confounders in these experiments give the impression that the Toxicological Review does not seem to apply its confounding criticisms evenly.

This inconsistent presentation of experimental expectations and application of confounding factors creates a perception of uneven evaluation of the primary literature. Considered together, the language and approach suggest a strong bias against *in vitro* studies and the cautionary language should be removed to eliminate that bias.

The discussion about ascorbate needs to be more balanced and thorough and the information better synthesized. The ascorbate section could be removed or if the EPA feels the issue needs to be considered, it should be fully vetted with a discussion of how differences in ascorbate might affect the interpretation of the bacterial mutagenesis studies and the rodent data. The discussion would need to also include the strengths and limitations of the primary literature. The relative merits of data from a primary normal human cell line without vitamin C supplementation versus data from a tumor-derived cell line with ascorbate supplementation would need to be presented and discussed. The various underlying phenotypic issues in cell lines would also need to be considered as a mitigating factor. Similarly, the technical limitations of ascorbate supplementation in culture would need to be considered including how long it is retained by the cell and the impact of its diffusion out of the cell and into the extracellular medium.

This discussion would need to include a full evaluation of ascorbate levels in tissues of interest in humans and animals and whether those levels are intracellular or extracellular or both. It should include a full discussion of any data that show whether or not there is an actual impact of different ascorbate levels on outcomes inside the cell from cell culture studies. It should also include a discussion about the fact that ascorbate in the cell becomes depleted over time after Cr(VI) exposure and whether the relevant exposure is when ascorbate levels are normal or depleted.

3. Poor management and citation of the literature

The above two concerns are further magnified by the presentation and management of the literature in the sections involving *in vitro* cell culture data and underpinning the mode of action. There is a tendency in the document to overstate the findings of the selected primary literature included in the document, raising questions about whether the primary literature was properly evaluated and weighed. There is inconsistent application of the phrases "*in vitro*" and "*in vivo*" resulting in substantial confusion regarding the underlying literature and reinforcing a perception of inaccuracy in the document. There are flaws in citations, extensive direct quoting and long stretches of general paraphrasing of a small number of review articles raising questions about the heavy reliance on those articles and points of view. Finally, there is often a failure to check the underlying primary research studies cited in these review articles reinforcing the perception that the document relies on the review and not the underlying primary research data. Considered together, these aspects raise questions about the process of how the conclusions were drawn, create confusion about whether the primary data were fully reviewed or whether the view of the authors of those few review articles was simply adopted, and raise significant questions about the credibility of the overall evaluation. Each concern is explained in more detail below.

A. Overstating the findings of the selected primary literature.

There are concerns that the Toxicological Review over-generalizes its presentation of the primary literature, particularly with respect to *in vitro* cell culture studies. One example of this problem is seen in its discussion of the literature concerning DNA adducts where the Toxicological Review states on pages 184-185 that:

> "*Although the ascorbate–trivalent chromium–DNA adducts are recovered less frequently in vitro due to the low concentrations of vitamin C present in commonly used tissue culture media (Zhitkovich, 2005), these adducts have been shown to be the most mutagenic of all the ternary adducts (Quievryn et al., 2003).*" … "*They have been detected in vitro in Chinese hamster ovary cells following exposure to hexavalent chromium, and account for up to 50% of all chromium–DNA adducts. The ternary adducts have been found to cause mutagenic and replication-blocking lesions in human fibroblasts in vitro (Quievryn et al., 2003; Voitkun et al., 1998).*"

Thus, the reader is led to believe that ascorbate-trivalent chromium-DNA adducts have been found in cells and these adducts have been shown to be highly mutagenic. Careful examination of the two cited references reveals that the statement in the Toxicological Review quote listed above that states:

> "*They have been detected in vitro in Chinese hamster ovary cells following exposure to hexavalent chromium…*"

is incorrect. Detection of adducts in Chinese hamster ovary cells was not actually presented as data in either paper or mentioned in the text of either paper. The claim is unsubstantiated as presented, which makes it potentially misleading.

Furthermore, when one considers the experimental detail in Quievryn et al., 2003 and Voitkun et al,, 1998, one learns that the adducts were synthesized in a cell free system. A sequence of DNA was treated with Cr(VI) and ascorbate in a cell free system. Then, the damaged DNA sequence was administered to cells. The cells then converted the damaged DNA sequence to a mutation that was revealed when the sequence was recovered and sequenced.

The detail also shows that the cells used were not normal human cells, but rather a SV40 immortalized cell line. SV40 is known to silence p53 activity, among other cellular and molecular changes, thus these cells were unable to carry out proficient DNA repair or apoptosis, as these are normally p53-dependent events.

Thus, the studies did not show that these adducts were normally present or able to form inside the cell. They could not account for the fate of the adduct structure after the transfection process and are only assuming it remained intact. The studies did not show that mutations would have occurred normally inside the cell as a consequence of Cr(VI) exposure or as a consequence of these lesions. Moreover, they do not show that these events would have happened in a repair proficient or apoptosis-proficient cell. It could be that the only reason mutations were seen is that the cells' ability to repair or eliminate them through apoptosis was artificially turned off beforehand.

An alternative interpretation of the studies could be that one can experimentally force a cell to generate a mutation in response to a Cr adduct if repair and apoptosis are silenced. Indeed, a step forward, but not one that establishes that adducts form or are mutagenic in cells.

It is unclear why these two studies were chosen to show that Cr induces adducts in cells. It is remarkable that given the emphasis the Toxicological Review places on the importance of physiologically relevant cell cultures, that it would fail to mention or discuss the integrity of the cell line itself, which in these studies were not robust cells. The document seems to be saying that there is a problem with cell culture studies that have some extracellular metabolism of Cr(VI) or that might not have enough ascorbate, but there are no problems with studies in cells with compromised DNA repair and cell death pathways.

There are more examples of this type of exaggeration of the implications of the primary literature in the Toxicological Review. These exaggerations obscure the meaning and applicability of the data and should be

corrected. These exaggerations also undermine the integrity of the document and raise questions about its accuracy and process. Other studies in the primary literature may reemerge as more relevant if treated more evenly and these studies should be reconsidered and possibly presented.

B. Flaws in citations, extensive direct quoting and long stretches of general paraphrasing

One concern is that the Toxicological Review actually appears to directly quote sources without indicating the comments are quotes of the original source. For example, in the Toxicological Review on page 46, lines 13-15, the writing states:

> "*Studies on the reduction of Cr(VI) by extracts of rat liver, lung, or kidney have found that ascorbate accounted for at least 80% of Cr(VI) metabolism in these tissues (Standeven et al., 1991,1992).*"

which is the exact same sentence that occurs in the Toxicological Review's Zhitkovich 2005 reference. That reference states on its page 5:

> "*Studies on the reduction of Cr-(VI) by extracts from rat lung, liver, or kidney have found that ascorbate accounted for at least 80% of Cr(VI) metabolism in these target tissues (45, 46).*"

Then on the same page, lines 20-22, the Toxicological Review states:

> "*Depending on the nature of the reducing agent and its concentration, this process can generate various amounts of unstable Cr(V) and Cr(IV) intermediates (Stearns et al., 1994).*"

which is the exact same sentence that is in Zhitkovich 2005. That reference states on its pages 5-6:

> "*Depending on the nature of the reducing agent and its concentration, this process can generate various amounts of unstable Cr(V) and Cr(IV) intermediates (14-16).*"

Neither of these sentences are indicated as being exact quotes of the original source and neither one is attributed to the original source. This omission is a concern as it is important to know when the document is choosing to quote from a source directly. These examples are not the only occurrences of this type of error and the entire document needs to be checked to identify other such problems.

In other instances, the Toxicological Review only changes a couple of words in a direct quote and fails to indicate it is a direct quote, which is also unacceptable. For example, the Toxicological Review states on page 50:

> "*Therefore, experiments on mutagenesis and other toxic effects of hexavalent chromium in tissue culture may underestimate its mutagenic, genotoxic, and cell-transforming activities (Zhitkovich, 2005; Costa and Klein, 2006).*"

The underlying reference by Costa and Klein 2006 states on its page 157:

> "*Thus, experiments on mutagenesis and other toxic effects of hexavalent Cr in tissue culture may underestimate its mutagenic, genotoxic, and cell-transforming activity (Zhitkovich, 2005).*"

The quote in the Toxicological Review and the Costa and Klein review differ by only substituting a "Therefore" for a "Thus" at the beginning and "activities" for "activity" near the end. Changing two words does not avoid the need to offset this sentence as a direct quote. As written, it is sufficiently in the original authors' words that it is considered a direct quote.

Similarly, in the Toxicological Review on page 46, the writing states:

> "*Ascorbate is also the fastest reducer in the in vitro reactions, and its rate of reduction at 1 mM exceeds that of cysteine and glutathione by approximately 13 and 61 times, respectively (Zhitkovich, 2005; Quivryn et al., 2001).*"

which is the exact same sentence that is in the Toxicological Review's Zhitkovich 2005 reference that states on its page 5:

> "*Ascorbate is also the fastest reducer of Cr(VI) in the in vitro reactions, and its rate of reduction at 1 mM concentration exceeds that of cysteine and glutathione approximately 13 and 61 times, respectively (48).*"

Again, this language is a direct quote and needs to be offset in quotation to make that clear.

This type of error also occurs with some frequency in the document and needs to be addressed.

Next, there are numerous instances when the Toxicological Review extensively paraphrases a review article and the meaning of the original passage is altered to another meaning that was not originally intended resulting in some overstatements and inaccuracies. For example, the Toxicological Review states on page 185:

> "*Reduction of hexavalent chromium in vitro produces a large proportion of binary trivalent chromium–DNA adducts, but these have not been detected in vivo. It has been theorized that*

> *the formation of the ternary adducts described above occurs far more frequently due to the high concentration of ligands capable of complexing with trivalent chromium before it can bind to DNA. (Zhitkovich, 2005).*"

Given its general use of "*in vitro*" to mean "in cell culture", the Toxicological Review appears to be stating that binary trivalent chromium-DNA adducts occur in cell culture but not in *in vivo* studies. However, the underlying Zhitkovich review reference actually states that the binary adducts have been detected in a test tube and not in cell culture. Specifically, it states (bold added here for emphasis):

> "*Reductive metabolism of Cr(VI) in vitro usually generates a large number of binary Cr(III)-DNA adducts (22, 37, 53), but the presence of these DNA modifications* ***in cells*** *has not yet been established. The formation of binary Cr-DNA complexes in cells is expected to be strongly inhibited due to the abundance of intracellular ligands capable of rapid coordination to Cr(III) prior to its binding to DNA.*"

This error occurs quite often and creates confusion about what the underlying literature is indicating.

There is a reference to "Salnikov and Zhitkovich, 2009" in a couple of places and no citation for this reference is provided. In the citation list, there is one reference listed as "Salnikow, K; Zhitkovich, A. (2008)" and another just below it as "Salnikov, K; Zhitkovich, A. (2008)" that looks to be exactly the same reference. These details should be straightened out and the entire reference section rechecked.

The occurrence of these various errors in citations undermines confidence in the Toxicological Review and raises significant concerns about process. It gives the impression that review articles formed the basis for the evaluation, rather than primary sources and, with the extensive quoting and paraphrasing, that some articles were simply integrated into the document. The use of these review articles in the document needs to be revised and addressed.

C. <u>Confusion due to inconsistent application of the phrases "in vitro" and "in vivo".</u>

The data in the Toxicological Review essentially fall into four groups. There are cell-free system studies, cell culture studies, whole animal studies and human studies. To describe these data, the phrases "*in vitro*" and "*in vivo*" are used. To most readers "*in vivo*" is thought to refer to studies in the body and so include whole animal and in some instances human studies. By contrast, "*in vitro*" is thought to refer to cell culture studies. There are inconsistencies in the use of these terms in the Toxicological Review as some of the underlying references use "*in vivo*" to mean in cell culture and "*in vitro*" to mean in cell free systems. The Toxicological Review has often failed to clarify the underlying studies and carried the underlying language forward into the review.

Two examples of this problem are presented in the preceding criticism, where the Toxicological Review elevated the cells in culture to an "*in vivo*" status, but there are many more occurrences in the document. There are two explanations for this outcome. One possibility is that in some places the Toxicological Review uses "*in vitro*" to mean in cell culture and in others to mean in cell free systems and in some places it uses "*in vivo*" to mean in whole animals and in others to mean in cell culture. The second is that the authors of the Toxicological Review did not realize that the underlying literature meant for "*in vivo*" to mean in cell culture and "*in vitro*" to mean in cell free systems. Regardless of which reason applies, as written the use of the terms is confusing and in some cases, such as the one explained above, misleading. The EPA needs to decide on a definition for these terms, present it, review the underlying literature to be sure they reflect what is meant and then apply them consistently in the document.

D. Failure to check the underlying primary research studies in review articles.

There was a failure to fully consider the underlying primary research articles in the review articles that are extensively cited. This failure creates a perception that the authors did not read beyond that review article, raising questions about process and whether primary data was evaluated at all. Where the document depends on a review article for its source, the underlying primary literature should be checked to confirm the integrity of the statements. One example of this problem is seen in the passage below from page 50 of the Toxicological Review:

> *"An additional important note on these biotransformations regards the interpretation and reliability of data from in vitro assays. In vivo, the intracellular levels of ascorbate are quite high (about 1 mM). In contrast, the levels of ascorbate in tissue culture media are quite low since generally it is not added to the media so that the only source is supplemented fetal bovine serum (FBS). With 10% FBS, the level of ascorbate in tissue cultured cells is only about 50 μM which is 20 times lower than that which is found in vivo (Zhitkovich, 2005). Therefore, experiments on mutagenesis and other toxic effects of hexavalent chromium in tissue culture may underestimate its mutagenic, genotoxic, and cell-transforming activities (Zhitkovich, 2005; Costa and Klein, 2006)."*

Thus, the Toxicological Review wants the reader to question cell culture studies if they do not contain intracellular levels of ascorbate in the mM range. However, the Zhitkovich review article that this section is entirely based upon miscites the primary literature on this matter and it appears the Toxicological Review did not check it. Specifically, as seen in the passage below from the Zhitkovich 2005 review, the claims about physiological levels of ascorbate being in the millimolar range rely on primary research papers that are its references 53 and 54. The Zhitkovich review states:

> *"Under standard tissue culture conditions, A549 and many other human and rodent cells either lack detectable ascorbate or contain it only at micromolar levels (physiological levels are in millimolar range) (53, 54) due to low concentrations of this vitamin in fetal bovine serum and its absence in the most commonly used types of growth media (DMEM, RPMI 1640, F10, F12)."*

These two references are:

> *"(53) Quievryn, G., Messer, J., and Zhitkovich, A. (2002) Carcinogenic chromium(VI) induces cross-linking of vitamin C to DNA in vitro and in human lung A549 cells. Biochemistry 41, 3156-3167.*
>
> *(54) Salnikow, K., Donald, S. P., Bruick, R. K., Zhitkovich, A., Phang, J. M., and Kasprzak, K. S. (2004) Depletion of intracellular ascorbate by the carcinogenic metals nickel and cobalt results in the induction of hypoxic stress. J. Biol. Chem. 279, 40337-40344."*

Examination of these two references shows, however, that neither one offers any data or evidence of physiological ascorbate levels being in the mM range. Salnikow et al. measures the amount of ascorbate loss in cultured cells treated with nickel and cobalt. They do measure ascorbate levels in untreated control cells to determine the background level of their experimental system. But their study does not measure any levels of vitamin C in any physiological setting. Nor does the citation make any reference at all to any study that does.

Quievryn et al., treats the human carcinoma cell line A549 with ascorbate and dihydroascorbate and then measures the amount of vitamin C inside the cell under these experimental conditions. But the study does not measure any levels of vitamin C in any physiological setting. The discussion section does make a comment that: "*Human cells in vivo contain a millimolar concentration of Asc (43)…*". However, that reference 43 is "*43. Meister, A. (1994) J. Biol. Chem. 269, 9397-9400*", which is a review article concerning glutathione and ascorbate in rodents. It contains no mention of ascorbate in human cells.

Thus, the EPA expresses a significant concern about *in vitro* cell culture studies based on a single review article that miscites the primary and secondary literature. This oversight implies that in the preparation of this Toxicological Review, the EPA did not access the primary literature and confirm the secondary and tertiary review articles.

If one were to look at primary literature for ascorbate levels, one would find that these claims are overstated. Ascorbate can reach mM levels in the body, but they are not universally mM levels. For example, Slade et

al., report lung ascorbate levels of 2.91 to 62.35 mg/100 g (Slade, R., Stead, A.G., Graham, J.A., and Hatch, G.E. (1985) Comparison of lung antioxidant levels in humans and laboratory animals. Am. Rev. Respir. Dis. 131(5), 742-6). This measure can be converted to a range of 165 uM to 3.5 mM. However, these are not intracellular levels, but rather the product of tissue homogenization and so a mixture of extracellular and intracellular sources. Thus, there is clearly variability in levels that span the uM and mM range, indicating that this factor may not be so essential.

E. Some exaggerations about Cr transport in cells.

Of less concern, but certainly in need of being addressed is some of the inaccurate language concerning Cr transport into the cell. In several places, cells are described as being impermeable to Cr(III). This characterization is too strong and inaccurate. Cr(III) will enter cells. It is just a slower uptake process than Cr(VI) uptake as Cr(III) moves by simple diffusion and it requires a higher dose to create the concentration gradient to get in the cell. This language should be adjusted.

Also, there is language implying Cr(VI) is actively transported into cells. Cr(VI) does enter rapidly by facilitated diffusion, but it is not an active transport process. These comments should be adjusted.

F. Some typographical errors.

There is also a mention on page 150 that states: 'As discussed in detail in Section 4.4.2 (Intracellular Reduction)…" It is actually section 4.5.2.

In sum, these factors all combine to give the appearance that the mode of action was not fully and consistently considered. To make the document and its conclusions much stronger and more accurate and the rationale behind its decisions more transparent, the following steps should be taken: 1) The EPA needs to separate the genotoxicity literature into discrete endpoints and consider them individually. This consideration should be based on the primary literature, which should be presented in a more careful and coherent fashion so that the reader can understand the strengths, weaknesses and data gaps. 2) Based on that analysis the EPA should choose which lesions are the key lesions and explain the rationale for that choice. 3) Once the key lesions are chosen, the EPA should consider the possible mechanisms of action that may cause those lesions and determine if there are data to support those mechanisms of action. 4) Once the key lesions are identified and the likely mechanisms described, the EPA should explain its rationale for the one chosen to be the mode of action. Of course, non-genotoxic modes of action should also receive similar analysis and presentation. This approach would help the EPA determine the most robust mode of action based on the primary literature. In addition, the EPA should decide what factors are truly confounders of concern and then apply them evenly to all of the literature, reduce its use of secondary and tertiary review articles and improve its management and citation of the literature.

G2. ***Please identify any additional studies that would make a significant impact on the conclusions of the Toxicological Review.***

Although this review is focused on oral exposures, some insight may be gleaned from the inhalation exposure data. Specifically, the data on mutations in lung tumors for Cr(VI)-exposed workers should be considered. These data show a lack of mutations in those tumors suggesting that mutagenicity as considered as a primary change in the DNA sequence is not a key event in the mechanism of action. They are consistent with the fact that one only sees these types of mutations in mammalian experimental models when one forces them by applying very high doses. These studies are:

Katabami M, Dosaka-Akita H, Mishina T, Honma K, Kimura K, Uchida Y, et al. Frequent cyclin D1 expression in chromate induced lung cancers. Hum Pathol 2000; 31 : 973-9.

Kondo K, Hino N, Sasa M, Kamamura Y, Sakiyama S,Tsuyuguchi M, et al. Mutations of the p53 gene in human lung cancer from chromate-exposed workers. Biochem Biophy Res Commun 1997; 239 : 95-100.

Ewis AA, Kondo K, Lee J, Tsuyuguchi M, Hashimoto M, Yokose T, et al. Occupational cancer genetics: Infrequent ras oncogenes point mutation in lung cancer samples from chromate workers. Am J Ind Med 2001; 40 : 92-7.

Hirose T, Kondo K, Takahashi Y, Ishikura H, Fujino H, Tsuyuguchi M, et al. Frequent microsatellite instability in lung cancer from chromate-exposed workers. Mol Carcinog 2002; 33 : 172-80.

Takahashi Y, Kondo K, Hirose T, Nakagawa H, Tsuyuguchi M, Hashimoto M, et al. Microsatellite instability and protein expression of the DNA mismatch repair gene, hMLH1, of lung cancer in chromate-exposed workers. Mol Carinog 2005; 42: 150-8.

Kondo K, Takahashi Y, Hirose Y, Nagao T, Tsuyuguchi M, Hashimoto M, et al. The reduced expression and aberrant methylation of p16INK4a in chromate workers with lung cancer. Lung Cancer 2006; 53 : 295-302.

Ewis AA, Kondo K, Dang F, Nakahori Y, Shinohara Y, Ishikawa M, et al. Surfactant protein B gene variations and susceptibility to lung cancer in chromate workers. Am J Ind Med 2006; 49 : 267-73.

There are also studies of Cr(VI)-induced neoplastic transformation of cells in culture. These need to be considered and included. In particular, reports by Xie et. al., show that cells must acquire a DNA double strand break repair phenotype to undergo transformation indicating escape from repair may be a key event in the mode of action. These studies are:

Patierno SR, Banh D, Landolph JR. Transformation of C3H/10T1/2 mouse embryo cells to focus formation and anchorage independence by insoluble lead chromate but not soluble calcium chromate: relationship to mutagenesis and internalization of lead chromate particles. Cancer Res 1988; 47 : 3815-23.

Xie H, Holmes AL, Wise SS, Huang S, Peng C, Wise Sr JP. Neoplastic transformation of human bronchial cells by lead chromate particles. Am J Respir Cell Mol Biol 2007; 37: 544- 52.

Xie H, Wise SS, Wise Sr. JP. Deficient repair of particulate chromate-induced DNA double strand breaks leads to neoplastic transformation. Mutat Res 2008; 649 : 230-8.

The document only considered mismatch repair, but there are important data showing that other DNA repair pathways must be overcome to induce genotoxicity and carcinogenesis. Double strand breaks and their repair, in particular, are important. The following studies should be added to the repair/DNA double strand break discussion:

Xie, H., Holmes, A.L., Young, J.L., Qin, Q., Joyce, K, Pelsue, S.C., Peng, C., Wise, S.S., Jeevarajan, A., Wallace, W.T., Hammond, D. and Wise, Sr., J.P. Zinc Chromate Induces Chromosome Instability and DNA Double Strand Breaks in Human Lung Cells. Toxicology and Applied Pharmacology, 234: 293–299, 2009.

Xie H, Wise SS, Holmes AL, Xu B, Wakeman T, Pelsue SC, et al. Carcinogenic lead chromate induces DNA double-strand breaks and activates ATM kinase in human lung cells. Mutat Res 2005; 586 : 160-72.

Xie H, Wise SS, Wise Sr. JP. Deficient repair of particulate chromate-induced DNA double strand breaks leads to neoplastic transformation. Mutat Res 2008; 649 : 230-8.

Stackpole MM, Wise SS, Goodale BC, Duzevik EG, Munroe RC, Thompson WD, et al. Homologous recombination protects against particulate chromate-induced genomic instability in Chinese hamster cells. Mutat Res 2007; 625:145-54.

Camrye E, Wise SS, Milligan P, Gordon N, Goodale B, Stackpole M, et al. Ku80 deficiency does not affect particulate chromate-induced chromosome damage and cytotoxicity in Chinese hamster ovary cells. Toxicol Sci 2007; 97: 348-54.

Bryant HE, Ying S, Helleday T. Homologous recombination is involved in repair of chromium-induced DNA damage in mammalian cells. Mutat Res 2006;599:116-23.

Grlickova-Duzevik EG, Wise SS, Munroe RC, Thompson WD, Wise Sr. JP XRCC1 protects cells against particulate chromate-induced chromosome damage and cytotoxicity in Chinese hamster ovary cells. Tox Sci 2006a;92(2):409-15.

Grlickova-Duzevik E, Wise SS, Munroe RC, Thompson WD, Wise Sr JP. XRCC1 protects cells from chromate-induced chromosome damage, but does not affect cytotoxicity. Mutat Res 2006; 610(1-2):31-7.

Vilcheck SK, Ceryak S, O'Brien TJ, Patierno SR. FANCD2 monoubiquitination and activation by hexavalent chromium [Cr(VI)] exposure: Activation is not required for repair of chromium(VI)-induced DSBs. Mutat Res 2006;610:21-30.

Savery LC, Grlickova-Duzevik E, Wise SS, Thompson WD, Hinz JM, Thompson LH, Wise Sr. JP. Role of the Fancg gene in protecting cells from particulate chromate-induced chromosome instability. Mutat Res 2007, 626(1-2):120-127.

There needs to be a stronger and clearer discussion about aneuploidy as a potential mechanism. These studies should be added to that discussion (some are in the document already):
Holmes, A.L., Wise, S.S., Pelsue, S.C., Aboueissa, A., Lingle, W., Salisbury, S., Gallaher, J. and Wise, Sr., J.P. Chronic exposure to zinc chromate induces centrosome amplification and spindle assembly checkpoint bypass in human lung fibroblasts. Chemical Research in Toxicology, 23(2): 386-395, 2010.
Guerci A, Seoane A, Dulout FN. Aneugenic effects of some metal compounds assessed by chromosome counting in MRC-5 human cells. Mutat Res 2000; 469 : 35-40.

Seoane AL, Guerci AM, Dulout FN. Malsegregation as a possible mechanism of aneuploidy induction by metal salts in MRC-5 human cells. Environ Mol Mutagen 2002; 40 : 200-6.

Holmes AL, Wise SS, Sandwick SJ, Lingle WL, Negron VC, Thompson WD, et al. Chronic exposure to lead chromate causes centrosome abnormalities and aneuploidy in human lung cells. Cancer Res 2006; 66: 4041-8.

Wise SS, Holmes AL, Xie H, Thompson WD, Wise Sr JP. Chronic exposure to particulate chromate induces spindle assembly checkpoint bypass in human lung cells. Chem Res Toxicol 2006; 19 : 1492-8.

The following two studies should be added to the clastogenicity results, particularly in light of one reviewer's comments that telomerase may be important as the second paper suggests telomerase does not affect Cr genotoxicity:

Xie, H., Holmes, A.L., Wise, S.S., Gordon, N. and Wise, Sr., J.P. Lead chromate-induced chromosome damage requires extracellular dissolution to liberate chromium ions but does not require particle internalization or intracellular dissolution. Chemical Research in Toxicology, 17(10): 1362-1367, 2004.
Wise SS, Elmore LW, Holt SE, Little JE, Antonucci PG, Bryant BH, et al. Telomerase-mediated lifespan extension of human bronchial cells does not affect hexavalent chromium-induced cytotoxicity or genotoxicity. Mol Cell Biochem 2004; 255: 103-11.

Finally, there are misleading comments about DNA-DNA crosslinks. The Toxicological Review states they are unlikely to form *in vivo*. When one studies the underlying review cited as evidence by Salnikow and Zhitkovich, it becomes apparent that by *in vivo* they mean cells in culture or whole animals. Thus, the review implies that Cr -DNA-DNA crosslinks would not be predicted to occur in cells or whole animals, however, data from Josh Hamilton and Karen Wetterhahn show Cr DNA-DNA crosslinks do form *in vivo*. The inclusion of Josh Hamilton's study showing DNA-DNA crosslinks *in vivo* would correct the inaccurate conclusion in the Toxicological Review that these lesions do not occur *in vivo*. I cannot locate that paper in the time frame available, but Josh Hamilton is a reviewer and should be able to provide it.

Chemical-Specific Charge Questions:

(A) Oral Reference Dose (RfD) for Hexavalent Chromium

A1. ***A two-year drinking water study of sodium dichromate dihydrate in rats and mice (NTP, 2008) was selected as the basis for the derivation of the RfD. Please comment on whether the selection of this study as the principal study is scientifically supported and clearly described. Please identify and provide the rationale for any other studies that should be selected as the principal study.***

This study is the proper study based on the available data. The study is flawed because only very high doses were considered in the study, thus, there is concern that it may not reflect events at lower doses. The EPA is in the unique position that a study that repeats the one above and extends it to lower doses is almost completed. The EPA should wait for the final results of that study to make the most informed analysis.

A2. ***Diffuse epithelial hyperplasia in the duodenum of female mice was selected as the critical effect for the RfD. Please comment on whether the selection of this critical effect is scientifically supported and clearly described. Please identify and provide the rationale for any other endpoints that should be selected as the critical effect.***

This endpoint is a proper endpoint based on the available data, but is not necessarily a toxic outcome. To allow for better understanding, RfD's for other endpoints should be done and presented including

some continuous endpoints. The EPA may conclude this endpoint is the critical effect, but this approach makes the analysis more transparent, open and clear.

A3. ***Benchmark dose (BMD) modeling was applied to the incidence of diffuse epithelial hyperplasia in the duodenum of female mice to derive the point of departure (POD) for the RfD. Has the BMD modeling been appropriately conducted and clearly described? Is the benchmark response (BMR) selected for use in deriving the POD (i.e., a 10% increase in the incidence of diffuse epithelial hyperplasia) scientifically supported and clearly described?***

The BMD modeling has been appropriately conducted and clearly described. To allow for better understanding, BMR modeling at 5% and 1% should be done and presented. This approach makes the analysis more transparent, open and clear.

A4. ***Please comment on the rationale for the selection of the uncertainty factors (UFs) applied to the POD for the derivation of the RfD. Are the UFs scientifically supported and clearly described? If changes to the selected UFs are proposed, please identify and provide a rationale.***

The rationale for the selection of the uncertainty factors (UFs) applied to the POD for the derivation of the RfD are appropriate. It was suggested that an UF for children and those with different conditions be used. People with different conditions (e.g. antacid use) are already contemplated in the UF applied for interindividual variation. Currently, such a factor for children is not included in the EPA guidelines. It could be that the same interindividual variation may apply in that case as well.

(B) Carcinogenicity of Hexavalent Chromium

B1. ***Under EPA's 2005 Guidelines for Carcinogen Risk Assessment (www.epa.gov/iris/backgrd.html), hexavalent chromium is likely to be carcinogenic to humans by the oral route of exposure. Is the cancer weight of evidence characterization scientifically supported and clearly described?***

The general lack of accuracy in the document in its handling of citing, paraphrasing and considering the underlying literature is of some concern in this presentation. There is a lot of cell culture and animal data showing genotoxicity and clastogenicity, however, the motivation for these studies were largely inhalational exposure-induced cancer. It seems if they can support one route, they could support the other, but it functionally means the data underpinning the oral route of exposure is the one NTP study.

That NTP study is flawed because only very high doses were considered in the study, thus, there is significant concern that it may not reflect events at lower doses. The EPA requirement for a "likely to

be carcinogenic to humans" classification defined as "…appropriate when the weight of the evidence is adequate to demonstrate carcinogenic potential to humans but does not reach the weight of evidence for the descriptor "Carcinogenic to Humans." It is understandable why an initial assessment of "likely to be carcinogenic" was chosen as the guidance states: " Supporting data for this descriptor may include: … an agent that has tested positive in animal experiments in more than one species, sex, strain, site, or exposure route, with or without evidence of carcinogenicity in humans".

The next possible descriptor is "suggestive evidence of carcinogenic potential". It is indicated as "…appropriate when the weight of evidence is suggestive of carcinogenicity; a concern for potential carcinogenic effects in humans is raised, but the data are judged not sufficient for a stronger conclusion." It is also said to cover "…evidence associated with varying levels of concern for carcinogenicity, ranging from a positive cancer result in the only study on an agent…".

Thus, there is conflicting guidance for Cr(VI) on these descriptors. On the one hand, there is a study of multiple species and genders possibly qualifying it for a descriptor of "likely to be carcinogenic to humans". On the other hand there is only one study showing this outcome making it suitable for a descriptor of "suggestive evidence of carcinogenic potential".

In studying the data and descriptors, it appears to be premature to conclude that the weight of the evidence is nearly adequate for demonstrating carcinogenic potential to humans. Moreover, in considering the spirit of the guidelines for the two descriptors, it is clear that "likely to be carcinogenic" descriptor is contemplating that the data concerning multiple species, genders, strains etc. will come from multiple studies not just one. It is also clear that the "suggestive evidence of carcinogenic potential" descriptor is intended for a database with flaws. This database is flawed by the lack of multiple studies and the fact that the one study available relied on very high doses. Accordingly, a descriptor of "suggestive evidence of carcinogenic potential" is more appropriate at this time.

However, the EPA is in the unique position to soon have another study that repeats the NTP study is almost completed. The EPA should wait for the final results of that study to make the most informed analysis. If it too shows tumors at all doses, then the stronger descriptor would be justified.

B2. ***A mutagenic mode of carcinogenic action by all routes of exposure is proposed as the primary mode of action for hexavalent chromium. Please comment on whether this determination is scientifically supported and clearly described. Please comment on data available for hexavalent chromium that may support an alternative primary mode of action.***

This document is supposed to be limited to an oral drinking water exposure. It is inappropriate to extend any finding to "all routes of exposure" in this document and such evidence has not been considered or presented for dermal or inhalation routes.

In its defense of a mutagenic mode of action, the Toxicological Review states on page 213 that:

> "*In addition to the evidence supporting a mutagenic mode of action in test animals, alternative or additional hypothesized modes of action for hexavalent chromium carcinogenicity have not been demonstrated.*"

There are three concerns with this statement. First, it seems to imply that other modes of action need to be "demonstrated" not simply supported. The frank reality is that no mode of action for Cr(VI) has been demonstrated, even the mutagenic mode of action is only supported and not demonstrated. There should not be a double standard here where the mutagenic mode of action needs to only be supported, while other modes must be demonstrated. The word "demonstrated" should be changed to "supported" to be consistent with the beginning of the passage.

The second concern is that the statement is inaccurate. We supported an alternative mode of action to the induction of mutations in our paper that is cited in the Toxicological Review. Specifically, in Holmes, A.; Wise, SS; Wise, Sr., JP (2008) Carcinogenicity of hexavalent chromium. Indian J Med Res 128:353 – 372, we argue that the mechanism for Cr(VI) does not involve mutations in the primary sequence of the DNA as it is a weak mutagen. Instead, we argue for a genotoxic mechanism leading not to mutations but changes in chromosome number and structure. This point of view is not considered much in the Toxicological Review, but is well-supported in the review and does offer an alternative mode of action that should have been discussed. It is as well-supported as the mutagenic mode of action and so it is inaccurate to state other views have not been demonstrated to the extent a mutagenic mode of action has been.

The third concern with the statement is that it seems to imply that the approach taken in determining the mode of action was to consider those possibilities suggested in reviews of the literature. Therefore, because there is no review article synthesizing the literature to suggest a mode of action, there are no other modes of actions to consider. A better approach would have been to consider the

primary literature and consider some possible modes of action that emerge from the data, but that have not yet emerged as a review article. There are other modes of action that emerge and two possibilities are presented below. There are data to support these modes that should be synthesized, evaluated and considered.

The mutagenic mode of action as the primary mode of action is not sufficiently scientifically supported or described in the Toxicological Review. Many concerns in the presentation with respect to proper citation of results, bias against cell culture studies, and an incomplete consideration of the primary literature are discussed above. The only approach taken was to consider all of these lesions in bulk as simply all representative of mutagenic events and not consider the possible confounding factors for each that may indicate they are not mutagenic events.

A more careful consideration of the primary literature, considering each endpoint on its own merit could argue against a mutagenic mode of action that involves changes to the primary sequence of the DNA strand resulting in mutations. Cr(VI)-induced human tumors rarely contain such mutations and Cr(VI)-induced mutations are most often generated in experimental systems when one artificially forces them to occur by using extraordinarily high doses or systems with compromised repair and cell death pathways or by non-physiological exposure routes. It is unlikely that Cr(VI) is a mutagen at low doses.

The most consistent outcome in the primary literature appears to be impacts on metaphase chromosomes. These outcomes occur at relatively low doses, in intact healthy human cells and across species in cell culture, whole animal and human worker studies. The question remains and this document does not address the underlying mechanism for this outcome. Induction of aneuploidy is another promising mode of action.

One mode of action could involve direct damage to the DNA strand resulting in an alteration in chromosome structure or number. This mode would have key events that include: 1) Uptake of Cr(VI), 2) intracellular reduction of Cr(VI), 3) interaction of reductant products with DNA strands, 4) production of chromosomal changes, 5) escape of DNA repair and apoptosis and 6) expansion of damaged cells.

Alternatively, the mode of action might not involve direct damage to the DNA. Instead, it could involve direct interactions with the mitotic spindle apparatus and be more of an epigenetic event. This mode would have key events that include: 1) Uptake of Cr(VI), 2) intracellular reduction of

Cr(VI), 3) accumulation of intracellular reductant products, 4) interaction of reductant products with mitotic spindle apparatus, perhaps binding to the centrosomes, 5) production of chromosomal changes, 6) bypass of the spindle assembly checkpoint, 7) escape of apoptosis and 8) expansion of damaged cells.

B3. A two-year drinking water study in rats and mice (NTP, 2008) was selected for the derivation of an oral slope factor. Please comment on whether the selection of this study for quantification is scientifically supported and clearly described. Please identify and provide the rationale for any other studies that should be considered.

This study is the proper study based on the available data. The study is flawed because only very high doses were considered in the study, thus, there is concern that it may not reflect events at lower doses. The EPA is in the unique position that a study that repeats the one above and extends it to lower doses is almost completed. The EPA should wait for the final results of that study to make the most informed analysis.

B4. The incidence of adenomas and carcinomas combined in the small intestine of male mice from the NTP (2008) two-year drinking water study were selected to serve as the basis for the quantitative cancer assessment. Please comment on whether this selection is scientifically supported and clearly described. Please identify and provide the rationale for any other endpoints that should be selected to serve as the basis for the quantitative cancer assessment.

If one is going to rely on this NTP study, the selection of the incidence of adenomas and carcinomas in the small intestine of male mice from the NTP (2008) two-year drinking water study to serve as the basis for the quantitative cancer assessment is appropriate. However, scientifically these lesions are not the same and are not necessarily linked. Thus, one or the other should be used.

B5. The oral slope factor was calculated by linear extrapolation from the POD (i.e., the lower 95% confidence limit on the dose associated with 10% extra risk of tumors of the small intestine in male mice). Has the modeling been appropriately conducted and clearly described?

The modeling has been appropriately conducted and clearly described. More methods are needed to make it clearer. It is possible that Cr(VI) acts via a threshold. The EPA is in the unique position that a study that repeats the NTP study and extends it to lower doses is almost completed. This study will help clarify if there is a threshold. The EPA should wait for the final results of that study to make the most informed analysis.

POST-MEETING COMMENTS SUBMITTED BY

Anatoly Zhitkovich, Ph.D.

Review of a draft of *Toxicological Review of Hexavalent Chromium* prepared by the US-EPA in Support of Summary Information on the Integrated Risk Information System (IRIS)

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General Charge Questions:

G1. Is the Toxicological Review logical, clear and concise? Has EPA clearly presented and synthesized the scientific evidence for noncancer and cancer hazard?

In general, I found the Draft to be well prepared and balanced in its presentation of various aspects of chromium-6 toxicology and carcinogenesis. It has a logical structure, leading a reader from the basics of redox chemistry of chromium-3 and chromium-6 and their interactions with biosystems to the detailed description of *in vivo* studies on bioavailability, tissue disposition and finally, toxic and carcinogenic effects. Weaknesses and strengths of the key *in vivo* studies along with the reasons for the inclusion or exclusion of specific findings were also clearly presented. Different sections vary somewhat in their degree of emphasis on the importance of one or another mechanistic aspect of Cr(VI) toxicology, which is also reflective of divergent opinions in the field. As typical for any large document covering a complex topic, the Draft contains some information that is not up-to-date and would benefit from additional editorial work.

Suggested modifications and corrections:

1) Section 2.1 "*Environmental Sources and Occurrence*" appears to draw a large amount of information from decades-old literature. The analytical approaches for the detection of both total Cr and Cr-6 underwent major improvements in 1980s and the older references to the amount of Cr-6 in various environmental media and biological samples should be looked at with a healthy degree of skepticism and scrutinized for potential overestimations. My specific concerns are related to the included values for Cr-6 levels in soil, freshwater and seawater. All three sets of values are too high for the typical samples from the noncontaminated areas/sites.

2) Table 2-1 "*Industrial uses of hexavalent chromium compounds*" is missing uses of sodium/potassium chromate and dichromate. The addition of information on sodium dichromate is particularly important in light of its testing for carcinogenicity by the NTP.

3) p.14, last para: Cr(III) oxidation to Cr(VI) by atmospheric oxygen can also occur in the presence of calcium oxide (Pillay et al. 2003).

4) Table 2-4 "*Detection limits for methods…*" reports outdated values. The EPA's Method 218.6 for Cr(VI) in water has a detection limit which is ~100 times lower than detection limits listed in Table 2-4. A recent modification of this method affords detection of Cr(VI) at the 0.003 ppb level (Application Update 179 from Dionex). The detection sensitivity of flame AAS is also underestimated. Based on the discussion of work by Levine (2007) on p.16 and other available literature, the detection limit for total Cr in water samples by ICP-MS reported in Table 2-4 is probably lower by a couple orders of magnitude.

5) p.25, lines 5-6: in Donaldson and Barreras (1966), urinary excretion for orally administered Cr(VI) and Cr(III) were 2.1 and 0.5%, respectively (not 2.1 versus 1.5%).

6) Table 3-5 and the discussion of these results appear contradictory.

7) Table on p.39 is confusing: it reports daily doses of sodium dichromate dihydrate in the NTP-2008 study but the ratio mice:rats looks incorrect based on the data in the top two rows. It is also unclear why Cr(VI) consumption was compared between male rats and female mice and not between animals of the same sex.

8) Finley et al. (1996) delivered a Cr-6 dose of 0.005 mg/kg/day, not 0.005 mg (p.45).

9) Section 3.3 describes Cr(VI) reduction by microsomal enzymes in detail on three pages. This degree of attention may create an erroneous impression about the importance of the specialized enzymatic processes in Cr-6 metabolism. There is a strong consensus in the field that Cr(VI) reduction in mammalian cells is primarily accomplished nonenzymatically by ascorbate and small thiols such as glutathione and cysteine. As acknowledged in other sections of the draft, ascorbate alone accounts for reduction of 80-95% Cr(VI) depending on the tissue (Standeven and Wetterhahn, 1991,1992). A combined contribution of ascorbate and thiols is responsible for more than 95% Cr(VI) reduction. These estimates from tissue preparations were confirmed by the measurements of individual reduction rates (Quievryn et al. 2003). It is clearly important to present mechanistic aspects of Cr(VI) reduction but the detailed focus should be on ascorbate and non-protein thiols, not enzymatic systems with a minimal contribution to the overall Cr(VI) metabolism *in vivo*. The absence of Cr(V) intermediate during Cr(VI) reduction by ascorbate is especially important.

10) Last sentence on p. 50: The description of vitamin C accumulation by cells is not entirely correct. Cellular accumulation of vitamin C via uptake of its oxidized form dehydroascorbic acid is a *physiological* mechanism that functions in all mammalian cells. It is particularly active in human cells, which leads to very efficient recycling and much lower daily requirements for vitamin C in humans compared to rodents (Nualart et al. 2003, Montel-Hagen et al. 2008). These differences between humans and rodents are relevant for the interspecies extrapolation.

11) Figure 3-6 needs to be modified:

    a) Depiction of the cation channel with the comment “No effect” could be interpreted as indicating some nontoxic delivery route for chromium. Unlike some other toxic metals, cation channels play no role in uptake of Cr ions and the cation route should be deleted from this Figure.

    b) Although some Cr(III)-ligand complexes can exhibit a limited ability to enter cells, there is no evidence that they can react with DNA and cause mutagenic/genotoxic ternary Cr-DNA adducts, as shown in the Figure. The Figure should be modified by removing this nonexistent route of DNA damage.

    c) The route for the formation of DSB by mismatch repair needs to be revised. As demonstrated in a recent study by Reynolds MF et al (2009), ternary Cr-DNA adducts are directly bound by mismatch repair proteins followed by DSB formation in G2 phase without stalling replication forks in the preceding S-phase.

12) Summary Table 4-20 should add the *+(M) designation* for mutagenesis of sodium dichromate in laboratory animal, as demonstrated by Cheng et al. 2000 (this study was later cited in Table 4-23).

13) Table 4-23 “In vivo genotoxicity studies… Mutations section” is missing references to two positive mutagenesis studies in vivo by Itoh and Shimada (1997, 1998).

14) Section 4.7.3.1. *Hypothesized Mode of Action*:

a) Ref. to Salnikow et al. (1992) in support of ternary complexes is inappropriate and could be replaced by Voitkun et al. (1998).

b) Neither Zhitkovich (2005) nor Voitkun et al. (1998) dealt with "intrastrand DNA-DNA crosslinks". A study by Lloyd et al. (1998) is the only original report describing putative intrastrand crosslinks, which were generated in a buffer solution with massive concentrations of hydrogen peroxide. Tested under the same reaction conditions, essential metals copper and cobalt were even more potent inducers of these presumed crosslinks. There is no evidence for the formation of these crosslinks by chromium-6 in cells or in acellular systems containing its main biological reducers.

15) Tables 4-22 and 4-23 failed to include any references to studies reporting the formation of chromium-DNA adducts in cultured mammalian cells and *in vivo*. This is a critical omission as the presence of chromium-DNA adducts demonstrates a direct DNA-damaging mechanism for Cr(VI) genotoxicity. The formation of DNA adducts was briefly discussed in other sections of the Draft.

16) Discussion of a negative report on DNA damage by DeFlora et al. (2008) on pp.206-207:

This study found no evidence of DNA damage in forestomach, glandular stomach and duodenum of female SKH-1 mice after a 9-month long exposure to 5 and 20 mg/L Cr(VI) in drinking water. Based on the study by DeFlora et al. (2008), a high safety threshold argument was also made in some of the submitted public comments. The Draft argued that a shorter duration of exposure (9 months vs. 2 years in the NTP study) made the DeFlora 2008 study "infeasible" for the comparison. With the exception of mutations and a potential accumulation of unrepaired damage in a population of the long-lived crypt stem cells, there are no other obvious factors suggesting that the formation of DNA damage by Cr(VI) in the entire duodenum during the first half of the 2-year exposure would be significantly different from damage occurring at the end of the 2 years.

However, the study by DeFlora et al. (1998) is uninformative for other reasons. The authors assayed tissues for two forms of DNA damage: DNA-protein crosslinks and 8-oxo-dG (the Draft incorrectly described 8-oxo-dG as a DNA adduct; it is actually a base oxidation product). Both types of damage showed no increases above background in tissues of exposed animals; however, these negative results were predictable based on the technical limitations of their analytical methodologies. Since Cr(VI) tumorigenesis occurred in the duodenum, I will limit my discussion to this tissue.

1) DNA-protein crosslinks:

A positive control consisting of mouse duodenal cells treated *ex vivo* with 1.6 mM Cr(VI) (83.2 mg/L) generated a 2.5-fold response. Such a low responsiveness was clearly insufficient to detect DNA damage for exposures with 4.2- and 16.6-times lower Cr(VI) levels in the 20 mg/L and 5 mg/L test groups, respectively, even in the unlikely scenario of no reduction and no dilution of Cr(VI) with stomach juices before reaching the duodenum. Although chronic exposures frequently leads to the accumulation of unrepairable damage, a dramatic increase in DNA-protein crosslinks during chronic Cr(VI) exposures would be not very likely. DNA-protein crosslinks are repairable lesions in mammalian cells *in vivo* and culture (Tsapakos et al. 1983, Sugiyama et al. 1986, Zecevic et al. 2010) with the possible exception of human peripheral blood lymphocytes (Quievryn and Zhitkovich 2000). Furthermore, ongoing proliferation and shedding of cells in the duodenal villi would result in continuous dilution of damage and loss of previously exposed cells.

2) 8-oxo-dG measurements:

A positive control generated by exposure of mouse duodenal cells to 1.6 mM Cr(VI) (83.2 mg/L) produced a 3.8-fold increase in the levels of 8-oxo-dG. It is doubtful that this assay sensitivity was sufficient to detect significant increases in 8-oxo-dG levels for even undiluted/unreduced 20mg/L and 5mg/L Cr(VI) concentrations that were used in the treatment groups. 8-oxo-dG as a biomarker of DNA damage has one critical limitation – a short lifetime due to its rapid removal by base excision repair. Repair of 50% 8-oxo-dG occur within 30 min and is complete within 2 hr (Lan et al. 2004). Not only would this short lifetime prevent any accumulation of 8-oxo-dG during chronic exposures, but it would also make it very difficult to detect this lesion even after recently ingested water with a sufficiently high dose producing positive responses under *ex vivo* conditions.

G2. Please identify any additional studies that would make a significant impact on the conclusions of the Toxicological Review.

The Draft included information from all major studies that have a significant impact on the main conclusions. It does not list or discuss every study published on Cr(VI) but there was also no systematic exclusion. In Section 4.4, I would recommend adding an important report by Gibb et al. (2000), which is the largest epidemiological study of cancer risk due to inhalation exposure to Cr(VI). While the omission or inclusion of this study does not change the overall conclusion about Cr(VI) carcinogenicity to humans via inhalation, Gibb et al. (2000) provided strong evidence of chromate dose-dependence for lung cancer risk and its independence of the common confounder, tobacco smoking.

Chemical-Specific Charge Questions:

(A) Oral Reference Dose (RfD) for Hexavalent Chromium

A1. A two-year drinking water study of sodium dichromate in rats and mice (NTP, 2008) was selected as the basis for the derivation of the RfD. Please comment on whether the selection of this study as the principal study is scientifically supported and clearly described. Please identify and provide the rationale for any other studies that should be selected as the principal study.

The NTP-2008 is the best available study of chromium-6 toxicity via oral exposure and its choice for the calculation of the RfD is scientifically sound and was clearly explained in the Draft.

A2. Diffuse epithelial hyperplasia in the duodenum of female mice was selected as the critical effect for the RfD. Please comment on whether the selection of this critical effect is scientifically supported and clearly described. Please identify and provide the rationale for any other endpoints that should be selected as the critical effect.

The incidence of diffuse epithelial hyperplasia in the duodenum of female mice was the most sensitive histological response observed in Cr(VI)-exposed groups and therefore, it was appropriately selected as the critical effect for the RfD.

A3. Benchmark dose (BMD) modeling was applied to the incidence of diffuse epithelial hyperplasia in the duodenum of female mice to derive the point of departure (POD) for the RfD. Has the BMD modeling been appropriately conducted and clearly described. Is the benchmark response (BMR) selected for use in deriving the POD (i.e., a 10% increase in the incidence of diffuse epithelial hyperplasia) scientifically supported and clearly described?

BMD modeling and the calculations of the POD both appear to be appropriately performed.

A4. Please comment on the rationale for the selection of the uncertainty factors (UFs) applied to the POD for the derivation of the RfD. Are the UFs scientifically supported and clearly described? If changes to the selected UFs are proposed, please identify and provide the rationale.

Two UFs were applied: UF=10 for interspecies extrapolation to humans and UF=10 for interindividual variability in the human population. The interspecies UF was used because there is no available information to quantitatively assess the true differences in chromium-6 toxicokinetics and toxicodynamics between humans and laboratory rodents. There are, however, two biological factors that point to a potentially greater sensitivity of humans relative to mice. The first is related to the fact that telomerase was shown to suppress genetic damage by chromium-6 (Glaviano et al. 2006). All mouse cells express telomerase while only stem cells retain telomerase expression in human tissues. The second factor is the difference in ascorbate metabolism. Human cells actively recycle ascorbate (Nualart et al. 2003, Montel-Hagen et al. 2008), resulting in ~100 times lower requirements for this vitamin by humans relative to rodents. A more economical use of vitamin C by humans also results in lower ascorbate concentrations in the extracellular fluid (for example, as reported for bronchoalveolar lavage fluid by Slade et al. 1993), which would more rapidly detoxify chromium-6 via extracellular reduction. However, No specific information about ascorbate concentrations in the extracellular environment of the duodenum and jejunum of mice and humans is currently available.

The interindividual variability in sensitivity to chromium-6 was not studied and the application of the safety coefficient (UF) is definitely appropriate in this case. However, the proposed UF=10 likely underestimates the range of the interindividual variability. The Draft has a brief discussion on the common presence of genetic polymorphism in DNA repair genes as one source of interindividual differences. Four major DNA repair pathways (mismatch repair, nucleotide excision repair, base excision repair and homologous recombination) are known to impact the extent of genetic damage and cytotoxicity by Cr(VI), and the use of UF=10 to account for interindividual differences in the overall DNA repair would assume a quite low degree of variability for each repair process (overall 10-fold variation would result from a very narrow 1.8-fold variation in each process: $1.8^4 = 10.5$).

Chromium-6 toxicity can be affected on three levels: 1) differences in extracellular detoxification, 2) differences in cellular uptake and 3) differences in cellular/genomic defense mechanisms. A 5-fold variation at each stage would give a potential 125-fold variation in the general population. A study by Donaldson and Barreras (1966) showed that individuals with pernicious anemia had 4-times higher systemic uptake of chromium-6 due to its lower detoxification in the stomach. Widespread use of antacid medications has a clear potential to diminish reduction rates of chromium-6. No systematic studies on potential variations in chromium-6 uptake have been performed yet, but two human lung carcinoma lines, H460 and A549, displayed a 5-fold difference in chromium-6 accumulation (Macfie et al. 2010). A caveat of using information from these two cell lines is that they are malignant and therefore it is not possible to determine whether their differences were present in the initial normal cells or whether it is a side effect of different transformation processes.

The Draft correctly stated on p.214 that there is no information about susceptibility of children to chromium-6 toxicity. In this case, it would be clearly appropriate to use additional UF=10 to account for a potential early life susceptibility. If EPA considers it unnecessary, then the exclusion of this UF should be justified in Section 5.1.3. My recommendation would be to use UF=100 to account for the combined effects of the interindividual variability in susceptibility and early life exposures.

(B) Carcinogenicity of Hexavalent Chromium

B1. Under EPA's 2005 Guidelines for Carcinogen Risk Assessment, hexavalent chromium is likely to be carcinogenic to humans by the oral route of exposure. Is the cancer weight of evidence characterization scientifically supported and clearly described.

The classification of hexavalent chromium as "likely to be carcinogenic to humans" via the oral route of exposure is supported by evidence of its tumorigenicity in the oral cavity of female and male rats and in the small intestine of female and male mice. An increased incidence of stomach cancers in the JinZhou area (China), which was contaminated with high concentrations of chromium-6 in drinking water, is supportive of the selected classification. Even if the ecological study from China is excluded, the weight of evidence from animal studies is adequate to designate hexavalent chromium as "likely to be carcinogenic to humans" via the oral exposure.

B2. A mutagenic mode of carcinogenic action by all routes of exposure is proposed as the primary mode of action for hexavalent chromium. Please comment whether this determination is scientifically supported and clearly described. Please comment on data available for hexavalent chromium that may support an alternative primary mode of action.

Mutagenic mode of action: Hexavalent chromium was overwhelmingly positive for genotoxicity in a large variety of cells and organisms. It was also consistently mutagenic in bacterial and mammalian test systems. The mutagenicity and genotoxicity of Cr-6 result from a direct DNA-damaging mechanism, as evidenced by the induction of mutagenic chromium-DNA adducts and other forms of DNA damage. Formation of chromium-specific DNA lesions at environmentally relevant Cr-6 concentrations and sensitivity of genotoxic responses to manipulations of cellular DNA repair further support the role of direct DNA damage as a primary cause of genotoxicity. Since Cr-6 is taken up via ubiquitously expressed sulfate transporters and its metabolism in cells occur via ubiquitously present ascorbate, glutathione and cysteine, there is no reason to believe that the formation of DNA damage in the small intestinal cells and in more extensively studied cell types would be significantly different. Thus, diverse lines of evidence are fully consistent with a mutagenic mode of carcinogenic action for hexavalent chromium. The Draft clearly presented the main arguments for this designation. However, as pointed out above, Tables 4-22 and 4-23 need to be supplemented with information on Cr-DNA adducts.

Supporting the importance of Cr-DNA adducts in chromate tumorigenicity are findings from the MOA study by the ACC in which levels of adducts were dramatically higher in the duodenum and jejunum of mice vs. rats. This result mirrors species differences in the intestinal carcinogenesis by chromate and it could not be explained by differences in tissue accumulation of chromium.

In contrast to clear positive mutagenicity and genotoxicity data from *in vivo* studies and ascorbate-restored mammalian cell cultures, aneuploidy and epigenetic responses have not yet been tested in animal models and so far have been observed only in ascorbate-deficient cells. In fact, Sun et el. (2009) have found that the induction of epigenetic changes by chromate in human cultured cells occurs only under ascorbate-depleted conditions.

The measurements of mutations in KRAS and p53 genes as part of the MOA study sponsored by the American Chemistry Council would not necessarily provide a clear answer about the mutagenic mode of action. A short 3-months duration of this study vs. 2 years for the NTP bioassay certainly diminishes its ability to detect mutations. Among the proposed mutation readouts, three KRAS codons represent a very small and consequently, insensitive mutagenic target. This gene was only rarely mutated in chromate-associated human lung cancers (Ewis et al. 2001). The p53 gene is also uncommonly mutated in cancers

among chromate workers (Kondo et al. 1997). The presence or absence of KRAS or p53 mutations do not serve as a strong test for the validity of the mutagenic mode of carcinogenic action, as the frequency of cells with mutated KRAS or p53 can increase through selection of the pre-existing mutant clones whereas transformation process can result from mutagenic events in other components of KRAS and p53 pathways. For example, a large wave of early thyroid cancers among Chernobyl radioiodine-exposed children was caused by translocations in growth factor receptors with almost no RAS and p53 mutations (Nikiforov et al. 1996, Suchy et al. 1998, Williams 2002).

Potential alternative modes of action: Two lines of in vivo evidence have been presented to point to a potentially nonmutagenic mode of carcinogenic action. One is based on the drinking water study by DeFlora et al. 2008, which found negative results for DNA damage in the duodenum of mice. However, as discussed in detail above, this study used assays that were insensitive for detection of DNA damage by the employed doses of Cr(VI)in drinking water. Therefore, the negative results of this work were expected and therefore, uninformative.

The other observation leading to the discussion of nonmutagenic or indirectly mutagenic mechanisms of carcinogenicity was the presence of diffuse epithelial hyperplasia in the NTP bioassay. Although the NTP study has not found significant necrosis in the small intestine of exposed mice, it is quite possible that the observed hyperplasia was a typical manifestation of regenerative responses. A combination of increased proliferation and inflammation could be presented as an alternative mechanism for indirect induction of mutations due to higher rates of cell division and by reactive oxygen species released by the recruited inflammatory cells. This carcinogenic pathway would exhibit a strongly sublinear, threshold-type dose dependence, as it relies on the induction of cell death and small doses would not kill cells. Inflammatory events could also be linked to cell death of chromium-damaged cells, which release pro-inflammatory molecules. The extent of hyperproliferation in chromium-exposed groups was modest, and considering the overall very high rate of cell division in the small intestine, it is hard to see how somewhat faster replication would provide dramatically more spontaneous mutations required for cancer development. At best, the cytotoxicity-induced compensatory proliferation mechanism and the mutagenic mode should co-exist at high tumorigenic doses.

The results from the MOA study sponsored by the ACC argue against significant inflammatory responses in the duodenum of chromate-exposed mice, as no increases in the levels of 8-oxodG and in the panel of 22 cytokines have been observed. A statistically significant drop in the ratio of GSH/GSSG was small in its magnitude, further demonstrating that tumorigenic doses were not associated with the state of strongly elevated oxidative stress and inflammation.

The presence of chromium-induced hyperplasia could also be viewed as a manifestation of cancer-protective responses by the small intestine. Elimination of genetically damaged cells by apoptosis or another form of cell death is a firmly established protective mechanism against cancer. Thus, there are two opposing interpretations for the toxicological significance of the observed hyperplasia: one is pro-tumorigenic and another is anti-tumorigenic. The supralinear shape of dose-tumor incidence responses in the NTP-2008 studies for female mice is consistent with the engagement of cancer-protective mechanisms. Tumor incidence vs. dose in male mice visually displayed a linear dose-dependence (as shown in Stern 2010). Thus, a hypothetical cytotoxicity-based mechanism with the expected dose-response sublinearity is contradicted by the available evidence.

B3. A two-year drinking water study in rats and mice (NTP, 2008) was selected for the derivation of an oral slope factor. Please comment on whether the selection of this study for quantitation is scientifically supported and clearly described. Please identify and provide the rationale for any other studies that should be considered.

The selection of a two-year drinking water study in rats and mice by the NTP (2008) for the calculation of an oral slope factor is appropriate. The NTP study was well designed and well executed. No other multiple-dose chronic oral carcinogenicity study in animals is available and the dose-dependence from the single ecological study linking chromium-6 in drinking water to human stomach cancers cannot be reliably estimated.

B4. The incidence of adenomas and carcinomas combined in the small intestine of male mice from the NTP (2008) two-year drinking water study were selected to serve as the basis for the quantitative cancer assessment. Please comment on whether the selection is scientifically supported and clearly described. Please identify and provide the rationale for any other endpoints that should be selected to serve as the basis for the quantitative cancer assessment.

The choice of the combined incidence of adenomas and carcinomas in the small intestine of male mice from the NTP-2008 study for the quantitative cancer assessment was based on a better fit of the multistage model for the male mouse data than for the female mouse data. However, it was unclear why a combination of male and female mouse data sets was not used.

B5. The oral slope factor was calculated by linear extrapolation from the POD. Has the modeling been appropriately conducted and clearly described.

The calculation of the oral slope factor from the POD was appropriately performed. As per US-EPA 2005 Guidelines for Carcinogen Risk Assessment, a linear extrapolation to low doses was used based on the selection of the mutagenic mode of carcinogenic action for chromium-6.

The ability of ingested chromium-6 to cause adverse effects at both environmentally relevant and much higher doses has been questioned, given the reported high chromate reducing capacity of the gastric juice and a limited systemic penetration of chromium after oral exposure (as extensively reviewed by the Draft). These considerations led to the formulation of the threshold model of chromium-6 carcinogenesis, which postulates that only doses that exceed the reducing capacity of the tissue (stomach for ingestion exposures) would be carcinogenic (DeFlora 2000). This model would argue that despite the selection of a mutagenic mode of action with the resulting recommendation for default linear extrapolation, the complete detoxification of low-to-moderate chromium-6 doses in the stomach makes it inappropriate to perform linear extrapolation from the POD.

The ability of gastric juices to reduce/detoxify chromium-6 is generally accepted in the field; however, studies with human volunteers and other considerations argue against the completeness of the detoxification process. For example, the bioavailability for Cr(VI) was ~10-times higher than for Cr(VI) reduced with orange juice prior to ingestion (Kuykendall et al. 1996). The extent of chromium-6 reduction in the stomach is influenced by three factors: its reduction capacity, reduction rate and stomach emptying time. Based on the reported high reduction capacity of the stomach (>80 mg/day, DeFlora et al. 1997), the rate of reduction by gastric juice under fasting conditions could exhibit pseudo-first order kinetics for a broad range of chromium-6 concentrations. This means that the percentage of reduced chromium-6 could be the same for both very small amounts and much larger amounts. Reduction of chromium-6 by artificial gastric juice has been found to follow first order reaction kinetics (Gammelgaard et al. 1999). Consistent with the first-order reaction kinetics, Donaldson and Barreras (1966) found that human subjects excreted in the 24-hr urine 2.1% of ingested 20 ng radioactive chromium-6 whereas Kerger et al. (1997) found that ingestion of 5 mg chromium-

6 by human volunteers led to about 1.43% excretion during the first 24 hr (1.43% excretion is a conservative 1/4th estimate from the average 4-day excretion value of 5.7%). Thus, the bioavailability of 20 ng and 5 mg chromium-6 (250,000-fold range) looks quite similar.

Donaldson and Barreras (1966) also performed a very important experiment on the bioavailability of 20 ng radioactive chromium-6 that was directly delivered into the duodenum of human subjects. In this case, they found that 10.6% of chromium was excreted in the urine. Since the duodenal delivery represent 100% nonreduced chromium-6, then the amount of nonreduced chromium-6 in their oral route experiment can be estimated from the urinary excretion of 2.1% divided by 0.106 = 19.8%. For the study by Kerger et al (2007), the same type of calculations gives an estimate of 14.3% nonreduced chromium-6 reaching the duodenum. Gammelgaard et al. (1999) calculated a half-life of 23 min for reduction of 0.1mg/L chromium-6 (current MCL for total chromium) by artificial gastric juice. After 1 hr, this reduction rate would leave 16.5% chromium-6.

REFERENCES

Cheng L, Sonntag DM, de Boer J, Dixon K. Chromium(VI)-induced mutagenesis in the lungs of big blue transgenic mice. J Environ Pathol Toxicol Oncol. 19(3): 239-249 (2000).

De Flora S. Threshold mechanisms and site specificity in chromium(VI) carcinogenesis. Carcinogenesis 21(4): 533-541 (2000).

De Flora S, Camoirano A, Bagnasco M, Bennicelli C, Corbett GE, Kerger BD. Estimates of the chromium(VI) reducing capacity in human body compartments as a mechanism for attenuating its potential toxicity and carcinogenicity. Carcinogenesis 18(3): 531-537 (1997).

De Flora S, D'Agostini F, Balansky R, Micale R, Baluce B, Izzotti A. Lack of genotoxic effects in hematopoietic and gastrointestinal cells of mice receiving chromium(VI) with the drinking water. Mutat Res. 659(1-2): 60-67 (2008).

Donaldson RM Jr, Barreras RF. Intestinal absorption of trace quantities of chromium. J Lab Clin Med. 68(3): 484-493 (1966).

Ewis AA, Kondo K, Lee J, Tsuyuguchi M, Hashimoto M, Yokose T, Mukai K, Kodama T, Shinka T, Monden Y, Nakahori Y. Occupational cancer genetics: infrequent ras oncogenes point mutations in lung cancer samples from chromate workers. Am J Ind Med. 40: 92-97 (2001).

Gammelgaard B, Jensen K, Steffansen B. In vitro metabolism and permeation studies in rat jejunum: organic chromium compared to inorganic chromium. J Trace Elem Med Biol.13(1-2): 82-88 (1999).

Gibb H.J., Lees P.S.J., Pinsky P.F. and Rooney B.C. Lung cancer among workers in chromium chemical production. Am. J. Ind. Med., **38,** 115-126 (2000).

Glaviano A, Nayak V, Cabuy E, Baird DM, Yin Z, Newson R, et al. Effects on hTERT on metal ion-induced genomic instability. Oncogene 25: 3424-3435 (2006).

Itoh S, Shimada H. Clastogenicity and mutagenicity of hexavalent chromium in lacZ transgenic mice. Toxicol. Lett. 91(3): 229-233 (1997).

Itoh S, Shimada H. Bone marrow and liver mutagenesis in lacZ transgenic mice treated with hexavalent chromium. Mutat. Res. 412(1): 63-67 (1998).

Kerger BD, Finley BL, Corbett GE, Dodge DG, Paustenbach DJ. Ingestion of chromium(VI) in drinking water by human volunteers: absorption, distribution, and excretion of single and repeated doses. J Toxicol Environ Health 50(1): 67-95 (1997).

Kondo, K., Hino, N., Sasa, M., Kamamura, Y., Sakiyama, S., Tsuyuguchi, M., Hashimoto, M., Uyama, T., and Monden, Y. Mutations of the p53 gene in human lung cancer from chromate-exposed workers. Biochem. Biophys. Res. Commun. 239: 95-100 (1997).

Kuykendall JR, Kerger BD, Jarvi EJ, Corbett GE, Paustenbach DJ. Measurement of DNA-protein cross-links in human leukocytes following acute ingestion of chromium in drinking water. Carcinogenesis 17: 1971-1977 (1996).

Lan L, Nakajima S, Oohata Y, Takao M, Okano S, Masutani M, Wilson SH, Yasui A. In situ analysis of repair processes for oxidative DNA damage in mammalian cells. Proc Natl Acad Sci USA 101(38): 13738-13743 (2004).

Macfie A., Hagan E. and Zhitkovich A. Mechanism of DNA-protein cross-linking by chromium. Chem. Res. Toxicol. 23: 341-347 (2010).

Montel-Hagen A, Kinet S, Manel N, Mongellaz C, Prohaska R, Battini JL, Delaunay J, Sitbon M, Taylor N. Erythrocyte Glut1 triggers dehydroascorbic acid uptake in mammals unable to synthesize vitamin C. Cell 132: 1039-1048 (2008).

Nikiforov YE, Nikiforova MN, Gnepp DR, Fagin JA. Prevalence of mutations of ras and p53 in benign and malignant thyroid tumors from children exposed to radiation after the Chernobyl nuclear accident. Oncogene 13: 687-693 (1996).

Nualart FJ, Rivas CI, Montecinos VP, Godoy AS, Guaiquil VH, Golde DW, Vera JC. Recycling of vitamin C by a bystander effect. J Biol Chem. 278(12): 10128-10133 (2003).

Pillay K, von Blottnitz H, Petersen J. Ageing of chromium(III)-bearing slag and its relation to the atmospheric oxidation of solid chromium(III)-oxide in the presence of calcium oxide. Chemosphere 52(10): 1771-1779 (2003).

Quievryn G. and Zhitkovich A. Loss of DNA-protein crosslinks from formaldehyde-exposed cells occurs through spontaneous hydrolysis and an active repair process linked to proteosome function. Carcinogenesis 21: 1573-1580 (2000).

Quievryn G., Peterson E., Messer J. and Zhitkovich A. Genotoxicity and mutagenicity of chromium(VI)/ascorbate-generated DNA adducts in human and bacterial cells. Biochemistry 42: 1062-1070 (2003).

Reynolds MF, Peterson-Roth EC, Bespalov IA, Johnston T, Gurel VM, Menard HL, Zhitkovich A. Rapid DNA double-strand breaks resulting from processing of Cr-DNA cross-links by both MutS dimers. Cancer Res. 69(3): 1071-1079 (2009).

Slade R, Crissman K, Norwood J, Hatch G. Comparison of antioxidant substances in bronchoalveolar lavage cells and fluid from humans, guinea pigs, and rats. Exp Lung Res. 19(4): 469-484 (1993).

Standeven A.M. and Wetterhahn K.E. Ascorbate is the principal reductant of chromium (VI) in rat liver and kidney ultrafiltrates. Carcinogenesis 12: 1733-1737 (1991).

Standeven A.M. and Wetterhahn K.E. Ascorbate is the principal reductant of chromium(VI) in rat lung ultrafiltrates and cytosols, and mediates chromium-DNA binding in vitro. Carcinogenesis 13: 1319-1324 (1992).

Stern A.H. A quantitative assessment of the carcinogenicity of hexavalent chromium by the oral route and its relevance to human exposure. Env. Res. 110: 798-807 (2010).

Suchy B, Waldmann V, Klugbauer S, Rabes HM. Absence of RAS and p53 mutations in thyroid carcinomas of children after Chernobyl in contrast to adult thyroid tumours. Br J Cancer. 77(6): 952-955 (1998).

Sugiyama M., Patierno S. R., Cantoni O. and Costa M. Characterization of DNA lesions induced by CaCrO4 in synchronous and asynchronous cultured mammalian cells. Mol. Pharmacol. 29: 606–613 (1986).

Sun H, Zhou X, Chen H, Li Q, Costa M. Modulation of histone methylation and MLH1 gene silencing by hexavalent chromium. Toxicol. Appl. Pharmacol. 237(3): 258-266 (2009).

Tsapakos M. J., Hampton T. H. and Wetterhahn K. E. Chromium(VI)-induced DNA lesions and chromium distribution in rat kidney, liver, and lung. Cancer Res. 43: 5662–5667 (1983).

Williams D. Cancer after nuclear fallout: lessons from the Chernobyl accident. Nat Rev Cancer. 2(7): 543-549 (2002).

Zecevic A., Hagan E., Reynolds M., Poage G., Johnston T. and Zhitkovich A. XPA impacts formation but not proteasome-sensitive repair of DNA-protein crosslinks induced by chromate. Mutagenesis 25: 381-388 (2010).

POST-MEETING COMMENTS SUBMITTED BY

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Post Meeting Review Comments on EPA's

Toxicological Review of Hexavalent Chromium

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G1. Is the Toxicological Review logical, clear and concise? Has EPA clearly presented and synthesized the scientific evidence for noncancer and cancer hazard?

This EPA's Review is well organized overall and for most part well presented. The literature review is extensive and thorough. However, the Review does not contain a set of clearly-stated criteria under which the literature was searched, critiqued, and synthesized. Specifically, was each published study judged with respect to design (including sample size), exposure assessment, choice of dose metrics, choice of endpoints, adequate dose-response data, dose-response modeling, and positive findings? Whereas some of these criteria may have been used in the Review, the lack of a systematic approach may have compromised the consistency and transparency of this review process. In its independent review of the EPA's IRIS Documents on Formaldehyde and Dioxins, for example, the National Academies of Science and National Research Council have strongly advocated the adoption of a systematic review approach to EPA's IRIS risk assessment process (NAS 2006, 2011). The present Review of Hexavalent Chromium once again demonstrates the need for adopting a systematic review approach.

G2. Please identify any additional studies that would make a significant impact on the conclusions of the Toxicological Review.

NA

Chemical-Specific Charge Questions:

(A) Oral Reference Dose (RfD) for Hexavalent Chromium

A1. A two-year drinking water study of sodium dichromate dihydrate in rats and mice (NTP, 2008) was selected as the basis for the derivation of the RfD. Please comment on whether the selection of this study as the principal study is scientifically supported and clearly described. Please identify and provide the rationale for any other studies that should be selected as the principal study.

The Review offers EPA's rational for selecting the NTP's two-year drinking water study of sodium dichromate dihydrate in rats and mice (NTP, 2008). EPA's justification includes the lack of reliable epidemiological data, solid design of the NTP's experiment, its controlled exposure regimens, the sensitivity of the endpoint, the availability of dose-response data, and consistency with hypothesized

genotoxicity MOA. These justifications are acceptable. There are still merits to include other studies, particularly the 3-month sodium dichromate dehydrate drinking water exposure of rats and mice (NTP 2007) in calculating RfDs. Inclusion of additional and all qualified studies is especially beneficial for better quantifying uncertainties and variations arising from different studies due to different study designs, strain/species of animals, and exposure regimens. As in a systematic review, studies meeting selection criteria should all be included for review and for analysis. Selecting a final RfD then becomes a risk management decision.

A2. Diffuse epithelial hyperplasia in the duodenum of female mice was selected as the critical effect for the RfD. Please comment on whether the selection of this critical effect is scientifically supported and clearly described. Please identify and provide the rationale for any other endpoints that should be selected as the critical effect.

EPA considered seven non-cancer endpoints for deriving RfDs (Table 5-1): chronic liver inflammation in female rats, histiocytic cellular infiltration in the liver of female mice, diffuse epithelial hyperplasia in the duodenum of the male and female mice, histiocytic cellular infiltration in the mesenteric lymph nodes of male and female mice, and cytoplasmic cellular alteration of acinar epithelial cells in the pancreas of female mice. All seven are quantal response from the NTP's 2-year chronic exposure study. The selections were largely driven by the dose-response data these effects exhibited. After dose-response modeling and the estimation of benchmark dose (BMD) for each of these select effects, diffuse epithelial hyperplasia in the duodenum of the female mice was chosen as the critical endpoint simply because it yielded the smallest BMD and its corresponding lower confidence limit (BMDL). It must be noted that the dose-response model for this critical effect was done only after deleting the two highest doses. As a result, the dose-response modeling relied on only three dose level (including the control), leaving little room for any flexible dose-response forms other than the "linear" multi-stage model with a polynomial of 1 degree of freedom.

Instead of relying on a select "critical" effect, EPA could report a range of RfDs based on a set of qualified and select effects. As a result, EPA will be able to a range of RfDs, projecting the uncertainty and variation of RfDs arising from man y sources and affording risk management the opportunity to make an informed choice of a final RfD (NAS, 2010). This is important as EPA is moving towards enhancing analysis of uncertainty and variation in risk assessment.

To this end, EPA could have benefited greatly by including additional endpoints from this principle study as well as other qualified studies. Potential candidates include histiocytic cellular infiltration in the duodenum of female rats and male mice, histiocytic cellular inflammation in pancreatic lymph nodes of male rats, and histiocytic cellular infiltration in the liver of female rats. EPS considered only the quantal responses in this Review for the purpose of computing RfDs. It is unclear why effects of continuous measurement scale were not considered. Many of these effects show

unequivocal dose-response (e.g. Tables 4-12 and 4-13) and seem to be relevant to the hypothesized MOA of hexavalent chromium. The availability of EPA's software BMDS for dose-response modeling and benchmark dose computation makes it practical and useful to consider continuous effects for RfD derivation as well.

A3. Benchmark dose (BMD) modeling was applied to the incidence of diffuse epithelial hyperplasia in the duodenum of female mice to derive the point of departure (POD) for the RfD. Has the BMD modeling been appropriately conducted and clearly described? Is the benchmark response (BMR) selected for use in deriving the POD (i.e., a 10% increase in the incidence of diffuse epithelial hyperplasia) scientifically supported and clearly described?

EPA should be commended for conducting BMD modeling for multiple effects with different model forms. For the modeling of the incidence of diffuse epithelial hyperplasia in the duodenum of female mice, EPA should provide a more detailed discussion on the limitation of the dose-response modeling (See A2). Uncertainties due to model choice, variation in the shape of seemingly equally well-fit models also can be quantified to a degree by considering multiple benchmark response levels (BMR) for each model. EPA used only BMR=10%. It makes perfect sense to also consider BMR=5% or even BMR=1% when such a choice is supported by the data. This is the general recommendation of EPA's own guideline (EPA, 2000). By doing so EPA would be able to quantitatively demonstrate uncertainty and variations due to the choice of different models and different BMR levels. Additionally, EPA should briefly but clearly define the BMD concept and methodology in an appendix to improve the readability for readers unfamiliar with the process.

A4. Please comment on the rationale for the selection of the uncertainty factors (UFs) applied to the POD for the derivation of the RfD. Are the UFs scientifically supported and clearly described? If changes to the selected UFs are proposed, please identify and provide a rationale.

The use of uncertainty factors (UFs) in this review is well described and is consistent with EPA's guidance documents for RfDs. Exposure in earlier stage of life was discussed.

(B) Carcinogenicity of Hexavalent Chromium

B1. Under EPA's 2005 *Guidelines for Carcinogen Risk Assessment* (www.epa.gov/iris/backgrd.html), hexavalent chromium is *likely to be carcinogenic to humans* by the oral route of exposure. Is the cancer weight of evidence characterization scientifically supported and clearly described?

The descriptor of "likely to be carcinogenic to humans" for hexavalent chromium is consistent with EPA's Guidelines for Carcinogen Risk Assessment. EPA gave a clear description of the hypothesized MOA.

B2. A mutagenic mode of carcinogenic action by all routes of exposure is proposed as the primary mode of action for hexavalent chromium. Please comment on whether this determination is scientifically supported and clearly described. Please comment on data available for hexavalent chromium that may support an alternative primary mode of action.

A mutagenic mode of action was proposed as the primary mode of action. On the one hand, EPA discussed data gap and uncertainties about the mutagenic MOA and other possible MOAs. On the other hand, EPA defended the mutagenic MOA despite the lack of data evidence. For example, the only animal study that investigated target tissue genotoxicity (De Flora et al. 2008) reported negative results for DNA-protein crosslinks and DNA adducts in forestomach, glandular stomach, and duodenum of mice exposed to hexavalent chromium in drinking water for 9 month. EPA dismisses the negative finding on the basis of a shorter duration of the study compared with the 2-year NTP study.

B3. A two-year drinking water study in rats and mice (NTP, 2008) was selected for the derivation of an oral slope factor. Please comment on whether the selection of this study for quantification is scientifically supported and clearly described. Please identify and provide the rationale for any other studies that should be considered.

The selection of the NTP's 2-year drinking water study in rats and mice (NTP, 2008) is justified. Reasons for why existing epidemiological data were not used for estimating cancer slope factor are acceptable.

B4. The incidence of adenomas and carcinomas combined in the small intestine of male mice from the NTP (2008) two-year drinking water study were selected to serve as the basis for the quantitative cancer assessment. Please comment on whether this selection is scientifically supported and clearly described. Please identify and provide the rationale for any other endpoints that should be selected to serve as the basis for the quantitative cancer assessment.

For dose-response assessment EPA considered the incidences of adenoma and carcinoma combined in the small intestine of male and female B6C3F mice (Tables 5.3 and 5.4), in the oral cavity (mucosa and tongue) (Tables 5.3 and 5.4) in rats (NTP 2008). (Note the denominators that determine the tumor incidence in small intestine are not consistent with those in Table 4.19). EPA did not consider the incidence of other neoplasm because the incidence is not dose-dependent.

The incidence of adenoma or carcinoma in the oral cavity in both male and female rats elevated only at the highest dose, but not at the three lower doses (up to 2.1 and 2.4 mg/kg-d for male and female respectively). To fit a dose-response model to these incidence data that exhibited hockey-sticker shape of dose-response requires a nonlinear (curve-linear) functional form or even a threshold model. Such curve-linear pattern seems inconsistent with the hypothesized genotoxic MOA. The lack of dose-response in these two endpoints was cited as reason for not advancing these two endpoints for final dose-response analysis. Better justification is needed.

B5. The oral slope factor was calculated by linear extrapolation from the POD (i.e., the lower 95% confidence limit on the dose associated with 10% extra risk of tumors of the small intestine in male mice). Has the modeling been appropriately conducted and clearly described?

EPA carried out dose-response modeling and BMD estimation for the incidences of adenoma and carcinoma of small intestine in male and female mice separately. EPA stated that it relied on the multi-stage model because the model is preferred by the agency, but gave no justification or explanation. It went on to report an estimated slope of 0.09 (mg/kg-day) and 0.10 (mg/kg-day) for male and female mice respectively. In section 5.3.4 of the Review, EPA reported the CSFs derived on the basis of the cancer incidence of small intestine in male and female mice, and chooses that of male mice because of "the poor fit of the multistage model to the female mouse data". EPA did not provided adequate detail on the modeling efforts, or a discussion and justifications for its final selection (section 5.3.3 and 5.3.4). EPA did provide some detail in Appendix B2, which is essentially the direct output from running the BMDS software, but again no discussion or explanation of the output. It would be helpful and necessary that EPA substantially expand sections 5.3.3 and 5.3.4 to report in greater detail the modeling process, the issues encountered, and justify the decision and choice therein.

On a more technical side, examination of Appendix B2 reveals that (1) no standard error is reported for the estimate of model coefficient, and (2) the coefficient of the second order in the polynomial was set to be zero, not estimated. The model did not fit the data at all despite a non-significant p-value for goodness-of-fit test. EPA did not give any explanation or discussion in this regard. The significance of a goodness-fit-test depends on sample size, and a non-significant result does not imply a correct model, especially within a range where there are no data. EPA should explore different options: trying different models, considering omitting the highest dose, or considering combine male and female mice.

Inclusion of multiple studies, multiple endpoints, multiple model choices, and various BMR levels for deriving a POD is increasingly desirable towards a more systematic and quantitative risk assessment paradigm. It will afford an opportunity to quantify the underlying uncertainties and variations associated with the choices and options made each many stages of the risk assessment process. Within the context of CSF for hexavalent chromium, EPA is in a position to conduct a more thorough and comprehensive assessment by including multiple endpoints, different model forms that allow for nonlinear dose-response, various BMR levels. The outcome will then demonstrate a range for POD and CSF to permit a better quantification and better understanding of uncertainties and variations.

Appendix B: List of Reviewers

United States
Environmental Protection Agency
National Center for Environmental Assessment

Peer Review Workshop for EPA's Draft Toxicological Review of Hexavalent Chromium

Hilton Crystal City Hotel
Arlington, VA
May 12, 2011

Peer Reviewers

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Appendix C: List of Observers

United States
Environmental Protection Agency
National Center for Environmental Assessment

Peer Review Workshop for EPA's Draft Toxicological Review of Hexavalent Chromium

Hilton Crystal City Hotel
Arlington, VA
May 12, 2011

Final List of Observers

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Appendix D: Agenda

United States
Environmental Protection Agency
National Center for Environmental Assessment

Peer Review Workshop for EPA's Draft Toxicological Review of Hexavalent Chromium

Hilton Crystal City Hotel
Arlington, VA
May 12, 2011

Agenda

8:00 a.m. Registration/check in

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

8:40 a.m. **EPA Welcome Remarks** .....*Vincent Cogliano, IRIS Program Director, EPA NCEA*

8:45 a.m. **Public Comment** ..... *Jan Connery*

9:30 a.m. **General Questions**..... *Anatoly Zhitkovich* (*Chair) & Panel*

G1. Is the Toxicological Review logical, clear and concise? Has EPA clearly presented and synthesized the scientific evidence for noncancer and cancer hazard?

G2. Please identify any additional studies that would make a significant impact on the conclusions of the Toxicological Review.

10:30 a.m. .....BREAK -

Chemical-Specific Charge Questions

10:45 a.m. **(A) Oral Reference Dose (RfD) for Hexavalent Chromium**..... *Anatoly Zhitkovich & Panel*

A1. A two-year drinking water study of sodium dichromate dihydrate in rats and mice (NTP, 2008) was selected as the basis for the derivation of the RfD. Please comment on whether the selection of this study as the principal study is scientifically supported and clearly described. Please identify and provide the rationale for any other studies that should be selected as the principal study.

A2. Diffuse epithelial hyperplasia in the duodenum of female mice was selected as the critical effect for the RfD. Please comment on whether the selection of this critical effect is scientifically supported and clearly described. Please identify and provide the rationale for any other endpoints that should be selected as the critical effect.

A3. Benchmark dose (BMD) modeling was applied to the incidence of diffuse epithelial hyperplasia in the duodenum of female mice to derive the point of departure (POD) for the RfD. Has the BMD modeling been appropriately conducted and clearly described? Is the benchmark response (BMR) selected for

use in deriving the POD (i.e., a 10% increase in the incidence of diffuse epithelial hyperplasia) scientifically supported and clearly described?

A4. Please comment on the rationale for the selection of the uncertainty factors (UFs) applied to the POD for the derivation of the RfD. Are the UFs scientifically supported and clearly described? If changes to the selected UFs are proposed, please identify and provide a rationale.

Noon LUNCH

1:00 p.m. **(B) Carcinogenicity of Hexavalent Chromium**..... *Anatoly Zhitkovich & Panel*

B1. Under EPA's 2005 *Guidelines for Carcinogen Risk Assessment* (www.epa.gov/iris/backgrd.html), hexavalent chromium is *likely to be carcinogenic to humans* by the oral route of exposure. Is the cancer weight of evidence characterization scientifically supported and clearly described?

B2. A mutagenic mode of carcinogenic action by all routes of exposure is proposed as the primary mode of action for hexavalent chromium. Please comment on whether this determination is scientifically supported and clearly described. Please comment on data available for hexavalent chromium that may support an alternative primary mode of action.

B3. A two-year drinking water study in rats and mice (NTP, 2008) was selected for the derivation of an oral slope factor. Please comment on whether the selection of this study for quantification is scientifically supported and clearly described. Please identify and provide the rationale for any other studies that should be considered.

3:00 p.m. BREAK

3:15 p.m. **(B) Carcinogenicity of Hexavalent Chromium** *(continued)* ..... *Anatoly Zhitkovich & Panel*

B4. The incidence of adenomas and carcinomas combined in the small intestine of male mice from the NTP (2008) two-year drinking water study were selected to serve as the basis for the quantitative cancer assessment. Please comment on whether this selection is scientifically supported and clearly described. Please identify and provide the rationale for any other endpoints that should be selected to serve as the basis for the quantitative cancer assessment.

B5. The oral slope factor was calculated by linear extrapolation from the POD (i.e., the lower 95% confidence limit on the dose associated with 10% extra risk of tumors of the small intestine in male mice). Has the modeling been appropriately conducted and clearly described?

4:30 p.m. **Reviewer Final Comments** ..... *Anatoly Zhitkovich & Panel*

4:55 p.m. **Closing Remarks** ..... *Jan Connery*

5:00 p.m. ADJOURN