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Dear Dr. Bowes:

Attached is my review of the chromium document. I have some serious reservations about the mechanistic aspects, as you will see.

Yours truly,

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Review: Public Health Goal for Hexavalent Chromium in Drinking Water

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1. Accuracy of the information presented on metabolism, toxicity, mode(s) of action and exposure, including potential for carcinogenicity and reproductive toxicity.

There are a number of issues here, especially in the areas of genotoxicity and mode of action (MOA). These sections are very much out of date and inaccurate.

The concept of "genotoxicity": This represents positive results in a group of assays that measure a number of very different things. On page 41, lumped together under "a wide range of DNA damage" are DNA adducts, DNA-protein crosslinks, DNA-DNA crosslinks, DNA strand breaks, oxidized DNA bases, chromosome aberrations, mutations, sister chromatid exchanges and micronuclei. Only some of these represent actual DNA damage (underlined). DNA damage *per se* does not inform us about eventual heritable change, which is the true issue. Assays that do not depend on the survival of genetically-altered offspring (i.e. chromosome aberrations, SCE, micronuclei) are only suggestive.

The description of an agent as a "genotoxic carcinogen" is out of date. What we really need to know is whether an agent has a mutagenic mode of action (MOA).

Cr(VI) 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., Chemico-Biological Interactions in press: doi:10.1016/j.cbi.2010.04.018; see also other references below). Mutations can result from DNA damage, but can also result from loss of mismatch repair and other types of genomic instability, and in some cases "mutations" are actually epimutations resulting from altered DNA methylation. These are important considerations for Cr(VI), since cells grown in the presence of Cr(VI) show selection for mismatch repair-deficient cells that are Cr(VI)-resistant (reviewed in Salnikow and Zhitkovich, Chem. Rev. Toxicol. 21:28-44, 2008). These cells are mutators (having a high spontaneous mutation rate) and show microsatellite instability, as do chromium-induced lung cancer cells (Takahashi et al., Mol. Carcinog. 281:150-158, 2005). Salnikow and Zhitkovich also discuss the lack of p53 mutations in Cr-induced lung tumors (which usually have p53 mutations when 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.

There are also other mechanisms for the appearance of Cr(VI)-resistance in exposed cells. These include 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.)

In addition, using a cell line capable of detecting both mutations and epimutations, Klein et al. (Environ. Health Perspect. 110, Suppl. 5:739-743) showed that treatment with 40 μM Cr(VI)

(resulting in about 40% survival) induced apparent mutations at 3-times background levels. Further analysis showed that 53% of the apparent mutations were deletions and the most of the rest were epimutations due to DNA methylation. Thus, the actual increase in mutant fraction reached only about 1.5-fold over background. The ability of Cr(VI) to cause changes in DNA methylation can also be related to the selection of mismatch repair-deficient cells, since turning off mismatch repair genes by methylation is known to be an important mechanism in carcinogenesis. A discussion of Cr(VI)-induced epigenetic alterations can also be found in Nickens et al.)

Chromosome aberrations and DNA strand breaks can occur as a result of cytotoxicity. Unless assays for cytotoxicity are performed, it is not possible to know whether DNA damage occurs in the relatively nontoxic dose-range that one can relate to carcinogenesis. It should be noted that Cr(VI)-induced cytotoxicity can take a long time to become manifest, and thus methods to detect cytotoxicity should include clonal survival or at least apoptosis at later times after exposure, assays rarely carried out in cytogenetic experiments. It is of interest that most of the *in vivo* drinking water experiment described in the document gave negative results for genotoxicity, except for the high dose of 100-200 ppm (Coogan et al., 1991). The positive results are seen with exposure gavage (bolus), more likely to result in toxicity.

Micronuclei can result from DNA damage or from malsegregation of chromosomes. In the case of Cr(VI), most of the micronuclei are kinetecore-positive, meaning that they arise from malsegregation (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(VI) induces aneuploidy rather than DNA damage at lower concentrations. Aneugenesis is caused by alterations in proteins, not DNA, and has thresholds.

Other concerns:
p. 6: There is evidence that food contains Cr(VI) as well as Cr(III). In fact, all parts of grain contain Cr(VI) and 10% of the Cr in bread is Cr(VI) (Mishra et al., Food Chem. Toxicol. 33:393-397, 1995; Soares et al., J. Agric. Food Chem. 58:1366-1370). It is possible that dietary Cr(VI) is significant and should be evaluated.

Bottom of p. 41: It is incorrect to say that the relative contribution of the various species to DNA damage is unknown when the most recent reference given is 2000. Besides the Salnikow and Zhitkovich, 2008 and Nickens et al., 2010 references, see also Guttmann et al., Chem. Res. Toxicol. 21:2188-2194, 2008.

p.42: The intracellular reduction of Cr(VI) is non-enzymatic. Reductants are ascorbate (major), GSH, other thiols, maybe NADH. See references above.

The second paragraph on p. 42 should be deleted and replaced with up-to-date material.

p.86-The experiment by Davidson et al. is not a non-oral route, it is a cocarcinogenesis experiment with solar UV and Cr(VI) in drinking water.

2. Selection of the NTP data set and supporting tumor data for the risk extrapolation to humans, particularly regarding interpretations of carcinogenicity data and mechanisms.

The data set is probably the best available. However, the calculations are confusing.

p.51-53: Concerning the dose/response relationship in Fig. 13: What are the units on the axes? Where are the error bars or 95% C.I.?

p.52: The paragraph on the historic rate of small intestine tumors is confusing. There are 2 sets of numbers each for male and female. Is one for control and the other for all exposed groups? It appears as if the tumor rate in control mice in the NTP experiment is much higher than in the historic controls (if I am interpreting this correctly). In any case, the final sentence in the first paragraph is nonsense. Statistical analysis decides.

3. Appropriateness of the risk assessment methodology used for extrapolation to human exposures to Cr6 in drinking water.

The assumption is that Cr(VI) in drinking water has a mutagenic MOA with no threshold. This is not valid for the following reasons:

1. A “genotoxic” agent does not necessarily cause tumors by a mutagenic MOA. Cr(VI) is only weakly mutagenic in animal cells (it is more mutagenic to bacteria). Furthermore, the mutagenicity occurs only at toxic doses in a narrow dose range (i.e. it has a threshold). (I am referring here to mutations in genes that are integrated into chromosomes, not shuttle vector studies where naked DNA is treated and then inserted into cells. The latter are useful for testing hypotheses about the mutagenic potential of specific adducts, but they do not reflect the mutability of agents given to whole cells).

2. Other MOA’s have not been considered. These include, for example, selection for Cr-resistance (involving epigenetic changes) and aneuploidy. These events generally show thresholds.

In the NTP study, there is no statistically significant increase in tumors below 85.7 mg/L. Is this taken into account in deriving the slope? What would happen if a threshold were included?

Please consider the recent meta-analysis of cancers of the G.I. tract among those occupationally exposed to Cr(VI), which 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 also leads to G.I. exposure, so this also suggests a possible threshold if the ingested dose can be estimated.

Using the LED_{10} is overly conservative. When added to a lack of threshold, an uncertainty factor of 3,000, and an upper 95-percentile estimate of water intake, the results indicate 0.06 ppb for PHG. This value is unrealistically low. Most sources of drinking water will be above this.

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

4. Identifications of the uncertainties in the risk assessment and proposed PHG calculation.

I think this has been answered above. I would just add that Cr(VI) in food may be more significant than assumed. The fact that Cr is essential also implies that oral Cr(VI) could supply the necessary Cr(III), again implying a thrshold.