

ITER White Paper—In Support of the Inhalation Cancer Risk Assessment for Hexavalent Chromium

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Executive Summary

This white paper provides supplementary information to inform the lung cancer risk assessment for airborne hexavalent chromium [Cr(VI)] exposure. The published studies that describe the mortality assessment, exposure reconstruction and quantitative cancer risk assessment from the Painesville, Ohio cohort of chromate production workers are proposed for review and inclusion in the ITER database (Luippold *et al.* 2003; Crump *et al.* 2003; Proctor *et al.* 2003, 2004). The unit risk estimate from these studies is based on far more current and robust data than the current U.S. Environmental Protection Agency (U.S. EPA) cancer risk assessment, which was prepared originally in 1984 and has not been substantially updated since. Several newer cancer risk assessments, most notably those developed by the Occupational Safety and Health Administration (OSHA) for its 2006 Cr(VI) Rule, and by the National Institute for Occupational Safety and Health (NIOSH) for occupational exposures to Cr(VI), as well as a 2011 assessment prepared by the Office of Environmental Health Hazard Assessment (OEHHA) of California EPA (CalEPA), are discussed as points of comparison. Notably, with the exception of the OEHHA values that were developed from a highly limited data set, the cancer potency estimates for inhaled Cr(VI) are remarkably similar, with less than a 5-fold difference between estimates provided by different studies and using different approaches. Finally, this white paper includes an analysis of the carcinogenic mode of action (MOA) underlying Cr(VI)-induced carcinogenicity based on the modified Hill Criteria, and important toxicokinetic considerations in risk assessment. The weight of evidence supports that Cr(VI)-induced carcinogenicity in the lung acts by a non-mutagenic MOA that involves oxidative stress and oxidative DNA damage, tissue injury and inflammation, occurring at the high exposure concentrations experienced historically in certain industries, and as demonstrated *in vitro* and in animal models. As such, the biological data indicate that the dose-response for lung cancer is expected to include a threshold; however the currently available epidemiologic data do not provide sufficient statistical power in the low dose range to distinguish non-linearities, if they exist. Finally, the toxicokinetic data demonstrate that particle solubility and size are important factors in carcinogenicity and may provide partial explanations as to why occupational exposure to Cr(VI) in some industries, such as aerospace, are not associated with an increased cancer risk, and inform the cancer risk assessment for other occupational as well as environmental exposures.

1.0 Overview of this White Paper

This white paper summarizes the findings of a published quantitative cancer risk assessment for lung cancer due to airborne Cr(VI) exposure. The risk assessment was conducted using occupational exposure and mortality data developed from a cohort of chromate production workers who were employed in the Painesville, Ohio plant starting from 1940-1972. The study of Painesville plant workers was conducted approximately 7 years ago and published in four peer-reviewed articles (Luippold *et al.* 2003; Crump *et al.* 2003; Proctor *et al.* 2003; 2004). In addition, this white paper provides supplemental risk assessment information that is relevant to current risk assessment practice and summarizes other risk assessments prepared since the publication of these articles.

In 2006, OSHA used Luippold *et al.* (2003) as one of two focus studies for quantitative cancer risk assessment and as justification for changing the Permissible Exposure Limit (PEL) for Cr(VI) from 52 to 5 $\mu\text{g}/\text{m}^3$ (OSHA 2006). This white paper discusses OSHA's analysis of the study results. The other focus study used for risk assessment by OSHA was the Gibb *et al.* (2000a) study, which is an occupational epidemiology study of chromate production workers from a facility in Baltimore. This white paper compares three risk assessment modeling analyses performed by OSHA, NIOSH, and OEHHA using the Gibb *et al.* (2000a) Baltimore study. OSHA used the risk assessment developed from the Painesville data set as published (Crump *et al.* 2003).

Because Cr(VI) may be detoxified by reduction to trivalent chromium [Cr(III)] prior to intracellular absorption, toxicokinetics are expected to play an important role in target tissue dose, and low-dose extrapolations in risk assessment. Reduction of Cr(VI) to Cr(III) prior to absorption has been proposed to provide a biological basis for a carcinogenic threshold (DeFlora 2000; Proctor *et al.* 2004). Thus, this white paper briefly discusses toxicokinetics of inhaled Cr(VI) including particle solubility and size. In addition, this white paper discusses differences in toxicokinetics with differing Cr(VI) exposure scenarios, i.e., occupational Cr(VI) exposures in the historical chromate production industry, which have been used for cancer risk assessment, compared to other occupational and environmental exposures. .

Finally, for consistency with current cancer risk assessment practice, this white paper includes a discussion of the data that informs development of a potential carcinogenic mode of action (MOA) for lung cancer induced by inhalation exposures to Cr(VI) (EPA 2005).

2.0 Summary of Published Studies that are the Basis of This Submission

Many occupational epidemiology studies have linked inhalation exposure to Cr(VI) with lung cancer, but very few studies provide reliable quantitative exposure data that may be used for cancer risk assessment. Luippold *et al.* (2003) used measures of cumulative and highest-monthly inhalation exposure to Cr(VI) and calculated standardized mortality ratios (SMRs), referenced to standardized populations, and thus represents substantial improvement compared to the Mancuso studies of workers from the Painesville plant. These latter studies are the basis for the current EPA inhalation unit risk factor and were only based on total Cr measures and consequently provide only crude mortality rates. Details regarding the mortality and exposure assessment are provided in four published studies that are included in the submission with this white paper (Luippold *et al.*, 2003; Crump *et al.*, 2003; Proctor *et al.*, 2003; 2004).

Specific advantages for risk assessment that the studies on the Painesville Plant workers offers as compared to other published studies include:

- A cohort with a significant fraction of decedents (63%) and adequate statistical power to observe a clear dose-response
- A cohort with a significant fraction of long-term workers (50th percentile of occupational tenure is 6 years, and 15% worked more than 20 years), representing a reasonably stable work force
- Detailed exposure reconstruction using Cr(VI)-speciated airborne monitoring data from 25 surveys conducted over nearly 30 years, more than 800 data points, collected and analyzed using methods that are similar to currently validated methods
- Mortality assessment conducted with standardized reference rates
- Dose-response using dose metrics of lifetime cumulative dose and highest 8-hour time-weighted average (TWA) daily dose, which is similar to the dose-metric of the Permissible Exposure Limit (PEL) and allows for evaluation of cancer dose-response in units of exposure concentration, in addition to cumulative exposure

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- Consistently positive dose-response relationship that can be used to quantify the relationship between exposure and excess lung cancer for high level exposures, as well as the exposure level at which no elevation in lung cancer was observed.

3.0 Other Risk Assessments of Cr(VI)

3.1 Occupational Exposure Risk Assessment by OSHA

In 2006, OSHA promulgated a new rule for occupational Cr(VI) exposure and revised the PEL from 52 to 5 $\mu\text{g}/\text{m}^3$. The final PEL value was assigned based on technical feasibility, rather than risk, because for some industries meeting a PEL based on an acceptable cancer risk would not be feasible.

OSHA conducted a thorough review of the literature, analyzing more than 40 occupational epidemiology studies, and developed quantitative cancer risk estimates for occupational exposure using all data sets that provided sufficient data, including the Mancuso studies (1975; 1997), the Painesville studies (Luippold *et al.*, 2003; Crump *et al.*, 2003; Proctor *et al.*, 2003 and 2004) described herein, and the Gibb *et al.* (2000a) study of the Baltimore cohort. The Painesville and Baltimore cohort studies were selected as focus studies because OSHA believed that these data provided the strongest data for risk assessment. Risk estimates developed by OSHA, in support of a revised PEL, relied on analyses from these cohorts.

OSHA used the risk assessment results of the Painesville cohort as published by Crump *et al.* (2003) and the dose metric of cumulative exposure. Risk estimates from this cohort at the new PEL of 5 $\mu\text{g}/\text{m}^3$ were 10/1000 for a 45-year working lifetime. For purposes of risk assessment, OSHA obtained the original data from the Gibb *et al.* (2000a) study. OSHA calculated SMRs using Baltimore reference rates, in addition to SMRs calculated from Maryland rates which were used in the original publication. OSHA also developed a dose-response based on 10 exposure groupings, whereas only four groupings (quartiles of exposure) were presented in the Gibb *et al.* (2000a) publication. Baltimore reference rates were selected because lung cancer rates in Baltimore were approximately 25% higher than in the State of Maryland, and previous assessments of this cohort relied on Baltimore rates (Hayes *et al.* 1979; Braver *et al.* 1985). OSHA's risk estimates from the Baltimore cohort at the new PEL of 5 $\mu\text{g}/\text{m}^3$ were 45/1000 for a 45-year working lifetime.

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Hence, OSHA developed a range of risk estimates from 10/1000 to 45/1000 for a 45-year working lifetime and 4.3/1000 to 22/1000 for 20-year occupational exposures at the new PEL. The lower bound represents the maximum likelihood estimates (MLE) from the Painesville cohort and the upper bound represents that from OSHA's analysis of the Baltimore cohort. As noted above, because it was not feasible to meet a PEL that achieved a 1/1000 risk in certain industries, the PEL value was ultimately set for technical feasibility.

3.2 NIOSH quantitative risk assessments of lung cancer associated with occupational exposure to Cr(VI)

Since the Painesville study work was published, NIOSH (Park *et al.* 2004; Park and Stayner, 2006) and OEHHA (2011) have developed additional cancer potency estimates using the data from the Gibb *et al.* (2000a) cohort. While NIOSH had access to the original data to conduct their work, OEHHA's risk assessment was based on the data provided in Gibb *et al.* (2000a). The advantage of the NIOSH risk assessment work is that it could regroup the exposure data, and did not have to rely on the data presented in Gibb *et al.* (2000a). NIOSH could investigate alternative reference populations and quantitatively adjust for smoking behavior. The published Gibb *et al.* (2000a) study is limited for risk assessment in that SMRs by quartile of exposure (four groupings) were provided, and the values were referenced to US and Maryland rates. In multivariate analyses, Gibb *et al.* (2000a) concluded that the lung cancer risk estimates were not confounded by smoking; however such analysis cannot account for additive effects of smoking and any underestimation of risk associated with the use of reference rates that are for populations that do not have similar smoking patterns. It is notable that 91% of the Baltimore cohort workers were known to be smokers at their date of hire.

The risk assessment work conducted by NIOSH and OEHHA are briefly summarized below.

Park *et al.* (2004)

Park *et al.* (2004) estimated excess lifetime risk of lung cancer death resulting from occupational exposure to Cr(VI)-containing dusts and mists via the inhalation route. Six different Poisson regression models of exposure-response were evaluated using the mortality data collected from a cohort previously analyzed by Gibb *et al.* (2000a). As noted above, Park *et al.* (2004) had the original data from the Gibb *et al.* study from which to conduct the modeling.

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Of the 2,357 workers included for evaluation, 1,205 males were white (51%), 848 nonwhite (36%) were most likely African Americans, and 304 were unknown (13%). Smoking information at hire was available for 91% of the study population, and cumulative smoking exposure (in pack/days-yrs) was calculated assuming workers smoked from age 18 until the end of follow-up and using a five-year lag time. These estimated cumulative exposures from smoking were included in the regression model.

For Cr(VI) exposure, a retrospective exposure assessment was conducted based on area sampling; recorded observations on the fraction of time spent in the exposure zones by each job title and by employer-calculated exposures by job title; and full-shift personal samples that were collected after 1977. Cr(VI) concentrations (as chromium trioxide or CrO₃) were based on laboratory determinations of water-soluble chromate. Area sampling and time spent in exposure zones were adjusted to the personal sample results. For each worker, exposure history was calculated based on jobs held (defined by dates) and Cr(VI) exposure estimate specific for each job title and time period. The mean total cumulative exposure to Cr(VI) was found to be 0.134 mg/m³-yr with a maximum of 5.3 mg/m³-yr.

Lung cancer was the underlying cause for 122 deaths. Standardized mortality ratio for white (SMR= 1.85, 95% CI 1.45-2.31) and nonwhite (SMR= 1.87, 95% CI 1.39-2.46) were close to those reported in Gibb *et al.* (2000a). The preferred model was linear with cumulative Cr(VI) exposure and log-linear for age, smoking, and race. With this model, a cancer rate ratio for a 45-year cumulative exposure to 1 mg/m³ Cr(VI) was estimated to be 2.44 (95% CI 1.54–3.83). At the previous OSHA permissible level (PEL) of 0.052 mg/m³ Cr(VI), a lifetime excess risk of lung cancer death of 255 per 1,000 (95% CI: 109-416) was predicted. At the current OSHA PEL of 5 µg/m³ Cr(VI), this risk assessment results in a lifetime excess lung cancer death risk of 3 per 1000 (95% CI: 1.2-5.9) was predicted.

A significantly higher dose-response coefficient for nonwhite workers than for white workers was observed, but because the source of exposure-race interaction was unknown, race interaction was not included in the final risk assessment model. Asbestos was identified as a potential exposure but was not viewed as an important confounder in the study. Potential confounding factors that have been identified in Park *et al.* (2004) included asbestos exposure and the possibility of inadequate control for smoking. The study authors also identified misclassification of chromium exposure as a potential source of error.

Park and Stayner (2006)

In a subsequent analysis to Park *et al.* (2004), attempts were made to estimate possible threshold for exposure-response in the Baltimore cohort. Using Poisson regression, a simple model that represented a two-step carcinogenesis process was evaluated. Different measures of cumulative exposures were calculated in which only concentrations exceeding specified threshold value were summed over time.

A simple two-stage model of carcinogenesis did not provide improvement in fit. The best-fitting one-stage models used simple cumulative exposure with no threshold for exposure intensity and had sufficient power to rule out thresholds as large as 16 $\mu\text{g}/\text{m}^3$ as Cr(VI) (one-sided 95% confidence limit, likelihood ratio test). Park and Stayner (2006) reported that models with a threshold (0.01-2.0 $\text{mg}/\text{m}^3\text{-year}$) for linear cumulative exposure effect were not significantly better fitting than the model with no threshold. However, improved fit was observed in models with cumulative exposure thresholds of 0.03 and 0.5 $\text{mg-yr}/\text{m}^3$ with and without an exposure-race interaction term, respectively. The upper (two-side) 95% confidence limits for the estimated threshold from the two models were 1.09 and 0.39 $\text{mg}/\text{m}^3\text{-year}$ indicating that if there is a nonzero threshold, the value would likely be in the range of these exposure levels.

3.3 OEHHA risk assessment using Gibb *et al.* (2000a) study for environmental exposures

In the technical support document for the 2011 Cr(VI) Public Health Goal, OEHHA evaluated the Gibb *et al.* (2000a) study data, as published, and calculated an inhalation unit risk factor.¹ OEHHA mistakenly assumed that the Gibb *et al.* (2000a) study was an update of the Mancuso (1975 and 1997) study, which has been the basis of the CalEPA Cr(VI) inhalation unit risk factor since 1985. However, Gibb *et al.* studied workers of the Baltimore chromate production plant, and Mancuso studied workers of the Painesville plant; thus they are two entirely different cohorts. In 1985, OEHHA (then in the California Department of Health Services) based its cancer potency estimates on the Mancuso (1975) study.

Lung cancer exposure-response data for cumulative exposure quartiles as reported in Gibb *et al.* (2000a) were fit to a relative risk model adjusting for uncontrolled

¹ In the final derivation of the numerical value of the public health goal (PHG), the contribution from the inhalation route of exposure was not considered significant (OEHHA 2011) as compared to oral exposures via drinking water.

confounding bias. Reference rates for the state of Maryland were used. The number of observed lung cancer cases was assumed to be a Poisson random variable with a known distribution. The resulting dose-response did not conform to a linear model, but suggested a supra-linear dose-response wherein the greatest per-dose risk occurred at the lowest exposures. Using all four quartiles of exposure, the linear model overpredicted risk for the lowest exposure quartile, underpredicted for the two mid quartiles and was reasonably accurate for the highest exposure quartile. Because the lowest exposure range is of greatest interest, models were fit to two different subgroups of data reported by Gibb *et al.* (2000a)—the lowest three quartiles and the lower two quartiles. Not surprisingly, OEHHA reported that the linear model fit perfectly with the two lowest data points. However, the resulting potency estimate using the two lowest data points was approximately two orders of magnitude greater than the potency estimate from fitting the entire data set.

OEHHA also conducted an analysis stratified by age and exposure quartile. The background hazard for a given age category was derived from the general population lung cancer deaths for Maryland, divided by person years at risk. To express the model slope in units of a unit risk value, the slope parameter was multiplied by 70 years and the product multiplied by the background risk of lung cancer in the population of California. The results were further adjusted to account for the occupational exposure ($0.33 = (10 \text{ m}^3/20 \text{ m}^3) \times (240/360 \text{ days per year})$).

Potency estimates generated using the age-stratified and simple models were relatively consistent. Potency estimates generated with the two lowest exposure quartiles—which were approximately 100-fold higher than those with the full data set—were also similar to that developed by OEHHA using the Mancuso (1975) study and thus were deemed supportive of the 1985 assessment.

OEHHA considered the Luippold *et al.* (2003) study in its derivation of an inhalation unit risk, but did not quote the quantitative risk assessment published using these data (Crump *et al.* 2003). Rather, OEHHA used the data in Luippold *et al.* (2003) to calculate a slope and potency, which OEHHA found was approximately equal to its calculation using the full Gibb *et al.* (2000a) data set. OEHHA decided that the analysis based on the two lower quartiles of the Gibb *et al.* (2000a) study was preferable because it was based on exposures that are nearer to environmental exposures. OEHHA ultimately concluded to retain the unit risk obtained in its 1985 analysis using the crude model and the Mancuso (1975) study because the older value was in the range of values developed using the Gibb *et al.* (2000a) study, when the top quartile and the top two quartiles were excluded.

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3.4 Considerations Regarding Cumulative Exposure as a Dose Metric

Each the risk assessments discussed above rely on the dose metric of cumulative exposure, and the assumption that short-term high concentration exposure can be extrapolated to long-term low level exposure on the basis of cumulative exposure. However, it is well recognized that the human body has physiological defense mechanisms that protect against toxicity of chemical agents, including Cr(VI), at low exposure levels (DeFlora 2000). These defense mechanisms include metabolism, immune response and repair of damaged cells (ATSDR 2008). As such, there is likely a relevant low concentration at which most of inhaled Cr(VI) will be detoxified and the target tissue dose of Cr(VI) will not be sufficient to initiate a cascade of key events in the MOA leading to lung cancer. As discussed to follow in the MOA discussion, evidence supporting a threshold dose response is building. Unfortunately, the currently available epidemiologic data do not provide sufficient statistical power in the low dose range to distinguish non-linearities. Further, the use of cumulative exposure as the only available dose-metric in many of the available studies, including Gibb *et al.* (2000a) clearly limits discernment of a threshold dose-response that is more likely observed using measures of exposure intensity.

3.5 Comparison of Unit Risk Factors Developed from the Painesville and Baltimore Study Data

Crump *et al.* (2003) is the only study that provided a lifetable analysis for lifetime exposure. The NIOSH and OSHA risk assessments evaluated risk using occupational lifetable analyses and the OEHHA risk assessments used a crude modeling approach to estimate unit risk estimates for the California population. For the sake of comparisons, unit risk estimates for continuous lifetime exposure as published by Crump *et al.* (2003) are compared to estimated values based on the occupational unit risk values published by Park *et al.* and OSHA (2006). The unit risk values calculated using the Painesville and Baltimore cohort study data are summarized in **Table 1** and compared with the current EPA value (U.S. EPA 1984) which is based on an earlier analysis of the data in Mancuso 1975. Interestingly, with the exception of the unit risk estimates developed by OEHHA after dropping highest and top two highest quartiles, unit risk estimates from these studies are all relatively consistent, with less than 5-fold variance.

Table 1. Summary of Unit Risk Values Developed from the Painesville and Baltimore Cohort Data

Study Cohort— Source of Evaluation	Environmental Unit Risk (risk at MLE ($\mu\text{g}/\text{m}^3$) ⁻¹)	Basis of Value	Comment
Painesville ¹	0.0098	As published	Value also used by OSHA in its risk assessment for this cohort; developed using Life-table analysis for current US population
Baltimore ² —As Evaluated by OSHA (2006)	0.045	Estimated based on occupational unit risk	OSHA regrouped SMR data, used Baltimore reference rates
Baltimore ² —As Evaluated by Park <i>et al.</i> (2004)	0.025	Estimated based on occupational unit risk	Park <i>et al.</i> regrouped SMR data and quantitatively accounted for smoking
Baltimore ² —As Evaluated by OEHHA (2011)	0.0128 0.162 1.4	-Including all exposure groups -After dropping the highest quartile (based on 3 data points) - After dropping the highest quartile (based on 2 data points)	OEHHA modeled the published study data (Gibb <i>et al.</i> 2000a) whereas OSHA and NIOSH used the original data
Current EPA value based on Mancuso (1975)	0.012	As published on IRIS currently and in U.S. EPA (1984)	EPA developed the value in 1984 (U.S. EPA, 1984). Mancuso study presented crude mortality rates and exposure estimates for total Cr and was thus highly uncertain.

¹ Painesville values published in Crump *et al.* (2003).

² Baltimore mortality data published by Gibb *et al.* (2000a).

MLE = Maximum Likelihood Estimate

4.0 Current Considerations for Cancer Risk Assessment

4.1 Mode of action data for Cr(VI)-induced lung cancer

The main focus of EPA's current cancer risk assessment guidelines (EPA 2005) is on the use of mode of action (MOA) data to evaluate early key events in tumorigenesis. MOA data are also used to inform the most appropriate approach for low dose extrapolation. Thus, an examination of a plausible MOA underlying tumor formation in the lung is an important consideration in the current inhalation risk assessment of Cr(VI). Using the modified Hill Criteria outlined in EPA cancer risk assessment guidance, we have provided a review of the human, animal and mechanistic data supporting a MOA, evaluated plausibility, dose-response and temporal concordance and considered alternative MOAs (EPA 2005).

To date, there is no published or well-accepted MOA for Cr(VI)-induced lung cancer. Cr(VI) inhalation exposure is associated with increased risk of lung cancer among historical workers of certain industries—specifically chromate production, pigment production, and chrome plating, but there is little or no evidence that inhaled Cr(VI) causes cancers in other industries with significant potential for exposure (welding, aerospace, ferrochrome, tanning, glassware cleaning) or is a risk at typical environmental exposure levels (IARC 1990). Several recent review articles have concluded that Cr(VI) has only weak mutagenic potential, and thus suggest that other MOAs may be operational in Cr(VI)-induced lung cancer (Holmes *et al.*, 2008; Nickens *et al.*, 2010). Moreover, recent data from an in-depth MOA study of the oral carcinogenicity of Cr(VI) suggest that Cr(VI)-induced carcinogenesis is not likely to be due to a mutagenic MOA. Although the MOA for Cr(VI)-induced lung cancer has not been established, a review of the available human, animal, and *in vitro* data support that it is unlikely to operate via a mutagenic MOA (*vide infra*).

To assess an inhalation MOA, select studies were reviewed, particularly those that investigate the *in vivo* effects of Cr(VI) in the target tissue of the lung, as well as those that address genotoxicity. The body of literature on the *in vitro* genotoxicity of Cr(VI) is vast and has been the subject of several recent review articles and thus will not be reviewed in depth here (Chiu *et al.*, 2010; Holmes *et al.*, 2008; Nickens *et al.*, 2010; Zhitkovich, 2011). This report focuses primarily on *in vivo* genotoxicity data as well as recently published *in vitro* data (Thompson *et al.*, 2012b).

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4.1.1 *In vivo* mutation studies

According to U.S. EPA (2007), *in vivo* mutation data is the highest level of evidence in the hierarchy of data needed to establish a mutagenic MOA. Given the interest in Cr(VI), a handful of studies have examined Cr(VI)-induced mutations *in vivo*. Two studies have reported positive findings for gene mutation following i.p. injection of high doses of Cr(VI) (Itoh and Shimada, 1998; Knudsen, 1980); however, these exposure routes are not physiologically relevant. Two studies have examined *in vivo* mutations following oral exposure to Cr(VI). NTP (2007) conducted studies in Big Blue mice exposed to Cr(VI) for in drinking water for 3 months; however, the mutation analyses were apparently not conducted for technical reasons. O'Brien *et al.* (in prep), exposed mice to Cr(VI) in drinking water for 3 months and found no increases in the mutation frequency (MF) of *Kras* in the mouse small intestine. Genes from the ras family are highly relevant to both intestinal and lung cancers.

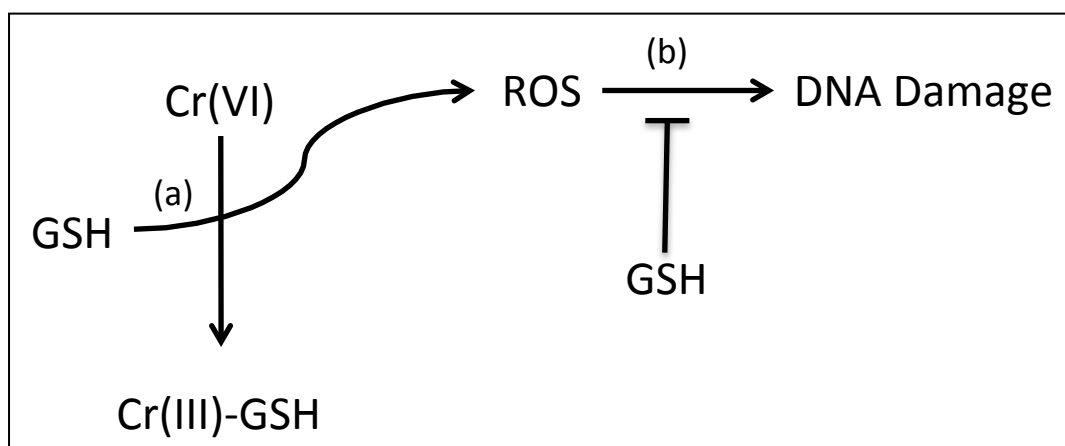
One *in vivo* study (Cheng *et al.*, 2000) deserves special attention because Cr(VI) increased the MF in the lungs of Big Blue mice following intratracheal instillation. At 2 and 4 weeks post exposure, 2-fold and 4-fold increases in MF, respectively, were reported. However, there are several caveats to this study, and the applicability of these mutation results is highly uncertain. First, Cr(VI) was administered surgically. Second, exposures were carried out at 6.75, 9, and 22.5 mg/kg; lethality was observed in 3/3 mice at 22.5 mg/kg and 1/3 mice at 9 mg/kg. Thus, the dose employed to induce mutations, 2.4 mg/kg Cr (6.75 mg/kg x 35%) was likely very toxic. This dose was instilled in a volume of 25 μ l, and thus at a concentration of 2.4 g/L (2.4 mg/kg x 0.025 kg \div 25 μ l). Third, MFs at 2 and 4 weeks were compared to the MF in saline-treated animals conducted at 1 week post exposure; thus, one cannot be certain whether the time-dependent increase in MF in Cr(VI)-treated animals also occurs in saline-treated animals, perhaps as a result of the intratracheal instillation procedure. Notably, the MF was only increased from 3.4 to 4.6 x 10⁻⁵ (1.3-fold) at 1 week post exposure, thus the lack of saline controls at 2 and 4 weeks post treatment render interpretation of increased MF in the Cr(VI)-treated animals at these later time points questionable.

Interestingly, Cheng *et al.* (2000) reported that depletion of GSH prior to Cr(VI) instillation decreased Cr(VI)-induced MF from a 4-fold increase to only 2-fold increase (at 4 weeks post exposure). One problem with such depletion assays is that it is often assumed that GSH depletion simply affects the pharmacokinetics of scavenging. However, GSH (and the GSH/GSSH ratio) regulate protein oxidation status and many proteins are regulated by their redox state (e.g. S-glutathionylation of protein thiols). Thus, changing cellular GSH levels might alter transporters involved in Cr(VI) uptake, the cell cycle, DNA repair, and numerous enzyme-

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mediated processes – all of which might influence responses to Cr(VI). It is conceivable that depleting GSH levels several hours (or days²) before exposure to Cr(VI) may induce a set of adaptive responses that mitigate cytotoxicity and genotoxicity (perhaps by inducing an oxidative stress response); thus the changes in the MF might have nothing to do with altered interactions between Cr(VI) and GSH.

Notwithstanding the above caveats, there are interesting implications for the Cheng *et al.* (2000) findings. Considering the simplified **Scheme 1** below, depletion of GSH would increase oxidative DNA damage (and MF) if it were not also playing a role in generating reactive oxygen species (ROS) by reducing Cr(VI) to Cr(III). Thus, the mitigation of MF by GSH depletion implies that GSH is indeed playing a role in Cr(VI) reduction, and that GSH depletion is protective because intracellular Cr(VI) would remain as such and be unable to interact with DNA or generate ROS. However, it has been argued that ascorbate is a far better reducer of Cr(VI) than GSH, and that Cr-ascorbate binary ligands are more mutagenic than Cr-GSH ligands (Zhitkovich, 2011; Zhitkovich, 2005). Therefore, depletion of GSH should allow for both an increase in ascorbate-mediated Cr(VI) reduction via formation of Cr-ascorbate ligands, as well as increased oxidative stress and oxidative DNA damage. That GSH depletion does not increase the MF suggests that GSH indeed plays a major role in both reducing Cr(VI) to Cr(III) and scavenging ROS generated in the process. Taken together, this suggests that the MOA for Cr(VI)-induced mutations involves oxidative DNA damage, and that exposures that do not elicit significant increases in cellular oxidative stress are unlikely to be mutagenic.



² In fact, Cheng *et al.* injected mice with BSO twice daily for three days prior to the Cr(VI) exposure in order to deplete GSH; this is not likely to be without some adaptive response.

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Scheme 1. Glutathione (GSH) both facilitates (a) and mitigates (b) Cr(VI)-induced oxidative DNA damage. (a) Cr(VI) reduction to Cr(III) is mediated by GSH (shown) and other cellular constituents (not shown), and this process can generate reactive oxygen species (ROS). (b) ROS-mediate oxidative DNA damage can be mitigated by GSH.

4.1.2 Cr(VI) *in vivo* DNA modification studies

Although the evidence for chromium-induced DNA damage *in vitro* is extensive, the evidence for Cr(VI)-induced genotoxicity *in vivo* is comparatively weak, particularly when administered via drinking water. Recent reviews on the carcinogenicity of Cr(VI) cite several subchronic studies as providing evidence for *in vivo* genotoxicity (McCarroll *et al.*, 2010; Sedman *et al.*, 2006). Many of these studies, however, employed relatively high doses of Cr(VI) and were often administered by i.p. injection, which has very limited application for understanding the MOA of Cr(VI) from typical environmental exposures. Several studies involving oral exposures to Cr(VI) in rats have reported positive responses for genotoxicity in tissues such as bone marrow, liver, brain or blood cells (Bigaliev *et al.*, 1977; Coogan *et al.*, 1991; Bagchi *et al.*, 1995a; Bagchi *et al.*, 1995b; Bagchi *et al.*, 1997). However, a 2-year bioassay conducted with very high concentrations of Cr(VI) (5-182 mg/L), yielded no tumors in these tissues (NTP, 2008). Moreover, three of the aforementioned studies attributed the genotoxicity to oxidative mechanisms, not direct DNA reactivity (Bagchi *et al.*, 1995a; Bagchi *et al.*, 1995b; Bagchi *et al.*, 1997).

More relevant to the lung, Izzotti *et al.* (1998) showed that three consecutive days of intratracheal administration of 0.25 mg/kg Cr(VI) (as sodium dichromate dehydrate, SDD) increased DNA fragmentation, DNA-protein crosslinks, nucleotide modifications, and 8-hydroxydeoxyguanosine (8-OHdG) in rat lungs relative to the lungs of sham treated animals. The authors concluded that the nucleotide modifications were consistent with oxidative DNA damage; moreover, they showed that oral administration of N-acetylcysteine, a precursor of cysteine and GSH, prevented the aforementioned lesions (Izzotti *et al.*, 1998). As Izzotti *et al.* point out, 0.25 mg/kg as SDD is a dose that was shown not to be carcinogenic to rats when administered either once or five times per week for 30 months (Steinhoff *et al.*, 1986). These findings suggest that the damage observed in the Izzotti study was not sufficient to increase cancer even over a lifetime of exposure, and further imply that the damage induced was not mutagenic in nature, but more likely due to repairable oxidative DNA damage.

4.1.3 Cr(VI) *in vitro* micronucleus assays

The micronucleus (MN) assay is one test used to assess the genotoxicity of a compound. Previous studies report that Cr(VI) induced MN in human bronchial epithelial cells (Reynolds *et al.*, 2006), but only after pre-incubation of cells with ascorbate. Other studies have reported that Cr(VI) induced MN in human fibroblasts through aneuploidic (rather than clastogenic) mechanisms (Seoane and Dulout, 2001; Seoane *et al.*, 2002). More recently, Cr(VI) genotoxicity was assessed by high content screening methods in the CHO-K1 cell line (Thompson *et al.*, 2012b), which is a cell model recommended for genotoxicity testing by the Organisation for Economic Co-operation and Development (OECD, 2010). Exposure to Cr(VI) induced MN formation only at concentrations ($\geq 32 \mu\text{M}$) that significantly reduced cell viability and the number of bi-nucleated cells. Additional assays in A549, a human lung adenocarcinoma epithelial cell line, caused relatively small but statistically significant ($p < 0.05$) increases in cytotoxicity and decreases in the percentage of bi-nucleated cells at $3.2 \mu\text{M}$. At this concentration, the frequency of MN in bi-nucleated cells was slightly increased from 1.47 ± 0.50 to $2.12 \pm 0.41\%$. At higher Cr(VI) concentrations, cell death was extensive. These data indicate that Cr(VI) has weak genotoxic potential (Thompson *et al.*, 2012b).

4.1.4 Animal bioassays

There are two repeated dose studies for the carcinogenicity of Cr(VI) in the rodent lung (Glaser *et al.*, 1986; Steinhoff *et al.*, 1986). Steinhoff *et al.* (1986) reported repeated intratracheal administration of Cr(VI) up to 0.25 mg/kg five times per week for 30 months did not increase lung tumors. In contrast, a single intratracheal administration of 1.25 mg/kg per week for 30 months induced lung tumors in 17.5% of the rats. In rats exposed to a single weekly dose of 1.25 mg/kg, there were signs of chronic inflammation including the presence of alveolar macrophages, proliferation of bronchiolar epithelium, and chronic inflammatory thickening of alveolar septa. These lesions were much milder in rats exposed to the same weekly dose but in five installments of 0.25 mg/kg, as well as rats receiving five installments of 0.05 or 0.01 mg/kg, or single installments of 0.5 or 0.05 mg/kg/week for 30 months. Steinhoff *et al.* concluded that concentration and irritancy/inflammation were more important in tumor formation than overall weekly dose. Similar effects were associated with lung tumors induced by calcium chromate (Steinhoff *et al.*, 1986) (**Table 2**).

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Table 2. Lung Tumors Among Rats Exposed to Sodium Dichromate and Calcium Dichromate by Intratracheal Instillation (from Steinhoff et al. 1986¹)

Exposure Group ²	Number of Rats	Number of Rats with Lung Tumors
Sodium dichromate		
5 x 0.01 mg/kg/week	85	0
5 x 0.05 mg/kg/week	85	0
5 x 0.25 mg/kg/week	90	0
1 x 0.05 mg/kg/week	80	0
1 x 0.25 mg/kg/week	80	1
1 x 1.25 mg/kg/week	80	14
Calcium chromate		
5 x 0.25 mg/kg/week	90	6
1 x 1.25 mg/kg/week	80	13

1 There were 260 rats in the control group and none developed lung tumors

2 Exposure groups are expressed in the number of doses per week x the dose given, e.g., 5 x 0.25 mg/kg/week means that 5 doses were given during the week and each dose was 0.25 mg/kg.

Glaser *et al.* (1986) conducted an inhalation study with Cr(VI) as sodium dichromate, and a chromium oxide mixture of 3 Cr(VI):2Cr(III) (Cr₅O₁₂). Rats were exposed to 25, 50 and 100 µg/m³ dichromate and 100 µg/m³ Cr₅O₁₂ for 18 months followed by a 12-month observation period. The incidence of lung tumor formation was 16% (3/19) in the 100 µg/m³ dichromate group, but no tumors were observed in the 50 µg/m³, 25 µg/m³, or control groups. Exposure to Cr₅O₁₂ at 100 µg/m³ (~63 µg/m³ Cr(VI)) also increased lung tumor incidence from 0 to 6% (1/18). Glaser *et al.* characterized these findings as indicating weak carcinogenicity of Cr(VI). Interestingly, Glaser *et al.* (1986) reported similar lesions as Steinhoff *et al.* (1986) specifically, accumulation of macrophages in lungs, eosinophilic substances inside the alveolar lumens, focal thickened septa, and fibrosis. However, these lesions were only reported in animals exposed to Cr₅O₁₂, and were attributed by the authors to the depressed lung clearance function. These findings appear to contradict the higher rate of tumor formation observed in rats exposed to 100 µg/m³ dichromate. However, Glaser *et al.* also showed that the chromium lung burden in Cr₅O₁₂ treated rats was 10-fold higher than in rats exposed to 100 µg/m³

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dichromate (when measured 12 months after termination of exposures). These data indicate that the less soluble Cr_5O_{12} was more slowly cleared from the lung, and likely explains the presence of nonneoplastic histological lesions in animals exposed to Cr_5O_{12} but not in rats exposed to dichromate 12 months after exposure termination. This study demonstrates that Cr(VI) induces inflammatory responses in the lung that likely contribute to the carcinogenic response, and that soluble sodium dichromate is more rapidly cleared from the lung than Cr_5O_{12} .

Over two decades later, the role of inflammation appears to have been confirmed by a series of studies conducted by Beaver and colleagues. Mice exposed to 0.6 mg/mL zinc chromate via intranasal instillation either once or repeatedly (every 14 days for 64 days) exhibited clear signs of peribronchiolar, alveolar, and interstitial inflammation, as well as elevated and aberrant cell proliferation in the airway lining (Beaver *et al.*, 2009b). Beaver *et al.* concluded that this Cr(VI)-induced inflammation contributes to the initiation and promotion of neoplastic growth in the lung.

Recent studies conducted on oral exposure to high concentrations of Cr(VI) indicate that Cr(VI) induces oxidative stress and proliferative responses in the mouse small intestine (Kopeck *et al.*, 2012; Thompson *et al.*, 2011). Toxicogenomic profiling also suggest that the gene changes elicited by Cr(VI) are more consistent with other known nonmutagenic carcinogens than mutagenic carcinogens (Thompson *et al.*, 2012a). Although it is difficult to extrapolate the findings in the mouse small intestine to the mouse lung, the data suggest that the intestinal tumors in mice are a result of cytotoxicity-induced regenerative hyperplasia and perhaps oxidative stress.

4.1.5 Hypothesized MOA of Cr(VI) via the inhalation route of exposure

Taken together, the animal, human and mechanistic data suggest that Cr(VI)-induced carcinogenesis is due to nonmutagenic mechanisms that exhibit some similarities (and some differences) across tissues. In particular, clear histological signs of inflammation were not evident in the mouse small intestine; however, tissue injury, oxidative stress and crypt cell proliferation were observed as early precursors at carcinogenic doses. In the lung, Cr(VI) induced clear signs of tissue injury, inflammation, and cell proliferation. Notably, inflammation is often accompanied by oxidative stress.

The occupational epidemiologic studies support the role of irritation and inflammation in carcinogenicity. In the Baltimore cohort, clinical findings of irritation, such as nasal septum perforation and bleeding, irritated or ulcerated skin,

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and nasal irritation and ulceration, were identified through routine examinations and visits of the cohort members to the company clinic (Gibb *et al.* 2000b). Nasal irritation and ulceration were the most common clinical findings, occurring in more than 60% of the cohort. Average time from the start of employment (and exposure) to first occurrence of these findings was less than 3 months. Exposure to median concentration of Cr(VI) at the time of symptomatic occurrence was approximately $10 \mu\text{g Cr(VI)}/\text{m}^3$, and mean exposure concentration was approximately $25 \mu\text{g Cr(VI)}/\text{m}^3$. Similarly for the early Painesville cohort, Miller (1950) studied 100 randomly chosen workers including some Luippold *et al.* cohort members. Miller (1950) reported that 92% of the workers had nasal septum ulceration, and 65% had nasal septum perforation. Further, 98% and 93%, respectively, had engorgement and hypertrophy of the nasal turbinates. Clearly, the Cr(VI) air concentrations in the Painesville chromate production plant during this time period were very high, so as to result in frank effects of respiratory irritation and distress. At these exposure levels, increased incidence of lung cancer was observed (i.e., SMR of 365 from Luippold *et al.* cohort exposed during the 1940s), and it is reasonable to surmise that respiratory system tissue damage resulting from high Cr(VI) concentrations exposures may play a role in the MOA.

Human data also support the likelihood that Cr(VI)-induced lung cancers arise from an epigenetic MOA. Increased genomic instability – characterized by chromosomal instability (CIN) and/or microsatellite instability (MSI) have been reported in the tumors of Cr(VI)-exposed workers. The latter can be observed in tumor cells where there is either shortening or lengthening of repetitive DNA sequences that are prone to replication error (Geigl *et al.*, 2008; Grady and Carethers, 2008). MSI is often a result of the loss or hindrance of DNA mismatch repair (MMR) genes such as MutL homolog 1 (MLH1). Indeed, some tumors exhibit hypermethylation of MLH1, and thus epigenetic silencing of MLH1 can result in an MSI phenotype without mutation in MMR genes (Geigl *et al.*, 2008; Grady and Carethers, 2008). Lung tumor biopsies from workers occupationally exposed to chromate exhibit fewer p53 point mutations than normally expected in lung tumors (20% vs. ~50%); however 79% of the tumors examined had signs of MSI as compared to 15% (4/26) in tumors from unexposed individuals (Hirose *et al.*, 2002; Kondo *et al.*, 1997). Relative to lung tumors from non-exposed individuals, tumors from chromate workers also exhibit reduced expression of MLH1 as well as signs of MLH1 hypermethylation (Takahashi *et al.*, 2005).

Lending further support for epigenetic mechanisms, *in vitro* studies suggest involvement of altered DNA methylation and changes in MMR genes. Transgenic gpt⁺ Chinese hamster V79 fibroblasts treated with soluble potassium chromate

induced mutant colonies with apparent transgene (*gpt*) deletions, many of which turned out to be silenced through hypermethylation in the promoter region; moreover, many mutants could be reverted by inhibition of DNA methylation (Klein *et al.*, 2002). Treatment of cultured A549 with potassium chromate was shown to increase cellular methylation of histone H3 lysine 9 (H3K9), which was inhibited by pretreatment of cells with the antioxidant ascorbate (Sun *et al.*, 2009). Immunoprecipitation of methylated H3K9 following Cr(VI) exposure was shown to co-precipitate the MLH1 promoter; moreover, the treatment of cells with Cr(VI) produced time- and dose-dependent decreases in mRNA levels of MLH1 (Sun *et al.*, 2009). Other studies have shown that cell lines deficient in MMR genes were resistant to Cr(VI) induced toxicity (Peterson-Roth *et al.*, 2005). Salnikow and Zhitkovich (2008) argue that exposure to Cr(VI) might selectively inhibit the growth or promote the death of normal cells, but that cells deficient in MMR “due to spontaneous mutagenesis and epigenetic changes” would continue to proliferate and acquire more mutations due to their insensitivity to Cr(VI). This “selective outgrowth” would result in an MSI phenotype as observed in the tumors from chromate workers (Hirose *et al.*, 2002; Kondo *et al.*, 1997; Takahashi *et al.*, 2005).

4.1.6 Dose-Response and Temporal Concordance

A critical aspect of MOA analysis is dose-response and temporal concordance. Chronic inhalation studies indicate that Cr(VI) causes lung tumors in rodents in a highly nonlinear fashion. These findings are inconsistent with a linear MOA such as is hypothesized with a mutagenic MOA.

Evidence of early respiratory irritation, inflammation and hyperplasia may be found in the 90-day inhalation studies conducted by Glaser and colleagues (1985, 1990) in rats exposed to 25 to 400 $\mu\text{g Cr(VI)/m}^3$, 22 hours per day, 7 days per week. These studies examined sensitive measures of tissue damage in the lung and inflammatory responses through assessment of bronchial alveolar lavage fluid (BALF). At these exposures, lung, body, and spleen weights were measured, and at exposures greater than 50 $\mu\text{g Cr(VI)/m}^3$, bronchoalveolar hyperplasia was observed. Changes in BALF indicative of tissue damage (e.g., increased levels of protein) were observed among rats exposed at and above 50 $\mu\text{g Cr(VI)/m}^3$. In rats exposed to Cr(VI) at 25 $\mu\text{g Cr(VI)/m}^3$, the number of macrophages in BALF was not increased, but macrophage diameter was increased, as well as the percent of lymphocytes present (Glaser *et al.* 1985).

Temporal concordance of dose and response has been demonstrated. Beaver *et al.* have shown that Cr(VI) induces persistent inflammatory responses as early as one

day after single or repeated exposures to Cr(VI), and lung epithelial cell proliferation after at least five repeated exposures (Beaver *et al.*, 2009a; Beaver *et al.*, 2009b). It is well known that inflammation can induce oxidative stress and that chronic inflammation and cell proliferation both contribute to carcinogenesis.

4.1.7 Plausibility

Cytotoxicity and consequent regeneration is a well-known MOA for cancer (Ames *et al.*, 1993; U.S. EPA 2001, 2005; Meek, 2003). In fact it has been suggested that cytotoxic and proliferative effects observable by 13 weeks of exposure can be predictive of effects in 2-year cancer rodent bioassays (Cohen, 2010; Gaylor, 2005; Slikker *et al.*, 2004). Inflammation is also involved in the pathogenesis of cancer, including lung cancer. Thus, the hypothesized MOA presented above is highly plausible. As previously discussed, analyses of tumors from chromate workers suggests they have unique characteristics – including fewer than expected mutations in cancer-associated genes such as p53. *In vivo* studies on Cr(VI) genotoxicity and mutagenicity are generally negative and limited to toxicologically irrelevant doses and exposure routes. Such studies measured damage in tissues that have not been shown to develop cancer following lifetime exposures to Cr(VI). Several lines of evidence from multiple routes of exposure suggest that Cr(VI) may cause oxidative stress, tissue injury, and cell proliferation. Thus, the MOA for Cr(VI)-induced cancers of the lung likely involve a nonmutagenic MOA.

4.1.8 Alternative MOAs: Evidence Cited for a Mutagenic MOA

McCarroll *et al.* (2010) used Cr(VI) as a case study for applying the MOA framework described for carcinogenic MOA analysis in U.S. EPA (2005). McCarroll *et al.* (2010) concluded that Cr(VI) acts through a mutagenic MOA in rodents based on evidence for *in vivo* DNA damage in two studies (Coogan *et al.*, 1991; Dana Devi *et al.*, 2001), as well as *in vivo* evidence of gene mutations, chromosomal aberrations and micronuclei in three studies (Itoh and Shimada, 1998; Sarkar *et al.*, 1993; Shindo *et al.*, 1989). However, none of these studies directly show mutagenesis in the target tissue (lung or intestine); and significant additional data has been developed since the review was conducted.

In Coogan *et al.* (1991), a drinking water study, the endpoint observed was DNA-protein crosslinks in liver. In Dana Devi *et al.* (2001), an oral gavage study, the endpoint observed was DNA single and double strand breaks. Shindo *et al.* (1989) detected increases in bone marrow micronuclei after gavage i.p. doses of 10-320 mg/kg potassium chromate, but no increase in MN after gavage administration of

the same doses. Regardless, MN are not direct measures of mutagenicity. Itoh and Shimada (1998) reported increases in gene mutation in liver and bone marrow of transgenic mice administered 40 mg/kg potassium chromate via intraperitoneal (i.p.) injection. These mutations were not in the target tissue, and were induced following a non-relevant route of exposure that bypasses normal reductive mechanisms. Sarkar *et al.* (1993) showed chromosomal aberrations in bone marrow of mice following gavage of 20 mg/kg CrO₃. Notably, this damage was mitigated by treatment with the antioxidant chlorophyllin – suggesting that the DNA damage was a result of oxidative mechanisms. It should also be noted that a single gavage dose of 20 mg/kg Cr greatly exceeds the average daily drinking water dose of Cr(VI) that were carcinogenic to the mouse small intestine (3.1-8.7 mg/kg) (Stout *et al.* (2009).

Of the studies cited as providing evidence for mutagenicity, the study by Sarkar *et al.* is the most relevant. However, it is not supported by other similar studies that have failed to provide evidence of cytogenetic damage. Mirsalis *et al.* (1996) reported that Cr(VI) did not induce micronucleus formation in mice exposed to 1–20 mg Cr(VI)/l by drinking water or gavage. NTP (2007) reported that mice exposed to ~20 to 350 mg/l Cr(VI) for 90 days showed no significant increase in MN in blood cells. NTP (2007) also conducted a comparative study with three strains of male mice that were exposed to 20, 45, and 90 mg/l Cr(VI) for 90 days. Results in two strains were negative, whereas MN were significantly increased in one strain at the highest dose. De Flora *et al.* (2008) also reported negative MN following oral exposure to Cr(VI).

As support for Cr(VI)-induced mutagenesis in humans, McCarroll *et al.* (2010) cite evidence for “mutagenic DNA adducts” in human fibroblasts, and MN in lymphocytes and buccal cells from Cr(VI) workers (Benova *et al.*, 2002; Dana Devi *et al.*, 2004; Voitkun *et al.*, 1998). Dana Devi *et al.* (2004) reported increases in DNA damage (measured by Comet assay and MN formation) in blood leukocytes and buccal cells of welders relative to control subjects. This study does not demonstrate DNA mutagenesis. Voitkun *et al.* (1998) reported that Cr(VI) increases MF in shuttle vector systems. In brief, CrCl₃ was reacted with GSH and other amino acids at 50 °C, then incubated with plasmid DNA, then transfected into human fibroblasts, and the plasmids were then recovered 48 h later. These plasmids were then transformed into E.coli that were subsequently plated on selection media to determine MF in the plasmids. As its name implies, this system is highly artificial: adducts were generated at 50 °C *in vitro* (specifically acellularly), the plasmids were then inserted into the host genome of an immortalized (i.e. mutant) mammalian cell line, extracted and reintroduced into bacteria for analysis. Although this assay is potentially informative, the test system is highly artificial.

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Benova *et al.* (2003) reported increases in MN in lymphocyte and buccal cells from chromium workers; these MN were both centromere positive and negative – suggesting both aneuploidic and clastogenic mechanisms. These results do not directly demonstrate mutation. Moreover, these findings have been questioned (Nersesyan, 2003) because Benova *et al.* were unable to detect increases in chromosomal aberrations, which usually accompany MN.

4.1.9 Conclusions Regarding MOA and Dose-Response Modeling

Although it is well accepted that Cr(VI) can be genotoxic and mutagenic, the evidence for such is primarily derived from *in vitro* data at cytotoxic exposures and artificial routes of administration. Although the observation of genotoxic lesions is frequently taken to indicate that a chemical acts by a mutagenic MOA, the vast literature regarding Cr(VI) carcinogenicity supports that Cr(VI) is weakly mutagenic and is more likely to act by a non-mutagenic MOA in the formation of lung cancer.

In vivo, tumors are preceded in dose and time by tissue irritation and inflammation associated with high concentration exposures, and mechanistic data support an oxidative stress/oxidative DNA damage MOA. Also, Cr(VI) induced lung tumors are observed following an extended latency period of approximately 20-35 years from first exposure, e.g. 88% of lung cancer deaths occurred more than 20 years following first exposure in the Painesville cohort (Luippold *et al.* 2003). As discussed in detail in the preceding sections, multiple lines of evidence plausibly support the proposed MOA, and the key events that are arguably necessary for tumor formation, precede tumor formation in both dose and time.

Although the weight of evidence supports a nonmutagenic MOA, and a biological basis for a threshold dose-response, the lung cancer epidemiological data are of insufficient statistical power in the low dose range to clearly establish statistical non-linearities in the dose-response (Crump *et al.* 2003; Proctor *et al.* 2004; Park *et al.* 2005; Park and Stayner, 2006). Further, these studies—with the exception of the Painesville study—are limited in that they rely on only cumulative exposure as a dose-metric. It is more biologically plausible that, if a threshold exists and can be discerned within a population based on statistical deviance from a linear dose-response, a measure of exposure intensity appears to be more appropriate.

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4.2 Consideration of Toxicokinetics and Physical/Chemical Properties in Lung Cancer Risk Assessment

Cr(VI) is not one chemical but exists in many chemical forms of varying solubility and reactivity/stability. Further, when considering Cr(VI) particulate exposure, it is important to recognize that particle size affects deposition in the lung and target tissue dose. Thus, toxicokinetics are affected by physical/chemical properties and, as described to follow, will also affect the cancer risk associated with inhalation exposure to Cr(VI).

Cr(VI)-induced pulmonary carcinogenesis generally involves localized regions of tissue that sustain high Cr(VI) exposure and chronic cellular toxicity, primarily in bronchial bifurcations of the lung because particles predominantly deposit in these regions (Nickens *et al.* 2010; Ishikawa *et al.* 1994). Further, animal research demonstrates that sparingly soluble forms of Cr(VI), which have a longer residence time in the lung than soluble forms, have greater carcinogenic potential (Steinhoff *et al.* 1986; Levy *et al.* 1986). This is consistent with the observation that Cr(VI) carcinogenicity is most pronounced in the chromate production and chromate pigment production industries, where workers are exposed to sparingly soluble chromates, including calcium, zinc and strontium and lead chromates (OSHA 2006; Proctor *et al.* 2003; 2004; IARC 1990). Further, when lime was removed from the chromate production process in the mid to late 1950s, which resulted in decreased exposure to calcium chromate in this industry, cancer risks were also significantly reduced (Davies *et al.* 1991; Luippold *et al.* 2003; 2005; Birk *et al.* 2006).

Thus, both the animal and human data support that the forms of Cr(VI) with the longest residency time in the lung, i.e., the sparingly soluble forms, pose the more significant cancer hazard. This information supports that tissue dose in the lung, which is the sum of inhaled and retained dose less eliminated dose, is the dose metric most predictive of lung cancer risk. To date, no model is available to estimate tissue dose among Cr(VI) exposed workers at an increased risk of lung cancer; however such a model would allow for significant refinement of cancer risk assessment of Cr(VI) and allow for better extrapolation of risk across dose and by chemical form.

Nonetheless, it is important to consider and compare, even if only in a qualitative manner, the physical/chemical properties that influence toxicokinetics for the Cr(VI) exposures. We have focused on data for the worker cohorts used as a basis for quantitative cancer risk assessment.

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4.2.1 Particle Size

While only very limited data are available on the particle size of airborne Cr(VI) in the historical chromate production industry, the data that do exist from the Luippold *et al.* cohort of workers indicates that the aerodynamic equivalent diameter (AED) of the dust was 1.7 μm (Proctor *et al.* 2003). Also, the U.S Public Health Service conducted an evaluation of worker health in the early 1950s in the chromate production industry (PHS 1953). This survey included workers of both the Painesville and Baltimore Chromate Production Plants. Similar to the particle size reported for the Painesville plant, PHS (1953) reported median particle sizes in the range of 1.0 μm . Finally, there is intuitive evidence from the chromate production industry that the particle sizes of the Cr(VI) exposures were in the range that affects the tracheobronchial and alveolar regions of the lung, in that these cohorts experienced high rates of lung cancer.

Large particle size may be at least partially responsible for the lack of increased lung cancer risk in the aerospace industry. Although Cr(VI) exposures in the aerospace industry have been to comparable (high) with those of the chromate production workers in terms of total airborne Cr(VI) concentration, and aerospace painters are exposed to sparingly soluble forms of Cr(VI), there has not been an increased lung cancer risk reported in the vast majority of studies. This is likely due to the larger particle sizes to which these workers are exposed.

Sabty-Daily *et al.* (2004) evaluated the size distribution of paint spray aerosol particles containing Cr(VI) at an aerospace facility. The sampled paint products consisted of strontium chromate in an epoxy resin matrix. In paint aerosol, particles containing total chromium had a mass median aerodynamic diameter (MMAD) of 7.5 μm ; for particles containing Cr(VI), MMAD was 8.5 μm . On average, 62% of the chromium and Cr(VI) mass of the paint aerosol was in particles >10 μm . In this study, the investigators also reported that about 72% of the Cr(VI) mass inhaled by a painter as particles from paint aerosol was deposited in the head airways region and about 1.4% of the Cr(VI) mass may potentially deposit in the tracheobronchial region. This is an important consideration because lung cancer among Cr(VI)-exposed workers is most typically bronchogenic carcinoma. Only 2% of the Cr(VI) mass was potentially deposited in the alveolar region (Sabty-Daily *et al.* 2004).

Further, LaPuma *et al.* (2001, 2002) quantified the Cr(VI) content and mass of dry chromate paint particles of varying sizes. Cascade impactors were used to collect and separate paint particles based on their aerodynamic diameter. The particles were found to range from 0.7 to 34.1 μm , Particles less than 7 μm in size had

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disproportionately less Cr(VI) per mass of dry paint compared to larger particles. The chromium content per mass of dry paint decreased substantially with decreasing particle size. The smallest particles, which were about 0.7 μm in size, contained about 10% of the chromium content per mass of dry paint as the larger particles. Therefore, the smaller particles contain less chromium compared to larger particles, due to their smaller size (mass varies with the cube of the radius, i.e. if the radius is reduced to one-tenth, mass reduces to one-thousandth), and they also have less chromium content per mass of dry paint. These findings indicate that exposure to Cr(VI) particle sizes may differ between the painters and workers exposed in other industries.

4.2.2 Solubility

It has been recognized for decades that the toxicity of Cr(VI) compounds can vary by the solubility of the salt. Strontium chromate is sparingly soluble in water at 1,200 mg/L at 25°C. Barium chromate and lead chromate, on the other hand, are even less soluble (barium, 4.4 mg/L; lead, 0.58 mg/L), and although calcium chromate is much more soluble (163,000 mg/L) than the strontium salt, the forms of calcium chromate in the chromate production industry are not simple salts but complex molecules of sparing solubility (Proctor *et al.* 2003). Thus, the calcium chromate compounds to which the workers of the historical chromate production industry were exposed from kiln dust and roast were likely far less soluble than pure calcium chromate.

The studies by Levy *et al.* (1986a,b) found an incidence of 43% and 62% bronchial carcinomas in rats with two different samples of strontium chromate, which is sparingly soluble. By comparison, sodium dichromate, a highly water-soluble compound, did not cause a significant increase in tumor incidence. These studies were performed using an intrabronchial pellet implantation system whereby pellets loaded with the test compound were surgically implanted into the bronchi of the animals. Implanting a pellet creates a high level of the compound in a small, localized area, which is more likely to overwhelm the body's defense mechanisms and results in tissue irritation and inflammation, as well as genetic damage.

Finally, it is important to note that the historical chromate production workers were exposed to a wide range of Cr(VI) particulates and aerosols of varying solubility. These include sparingly soluble forms of calcium chromate that are generated in the production kilns and the highly water soluble chromates and dichromates which were produced in the production process and the product of this industry (Proctor *et al.* 2003, 2004). Further, both the Baltimore and Painesville plants operated

chromic acid production processes, and in Baltimore the plant also produced Cr(VI)-containing pigments such as zinc and lead chromate. Several studies of chromate production worker cohorts have demonstrated that the excess cancer risk is reduced when less lime is added to the roast mixture, reducing worker exposure to the sparingly soluble calcium chromate compounds (Luippold *et al.* 2003). Unfortunately, the analytical procedures used to characterize exposure for most of the time periods during which both the Painesville and Baltimore cohorts members worked involved a water extraction of Cr(VI). Thus, exposure to sparingly soluble forms of Cr(VI) may not have been accurately characterized. Rather the measured concentrations were mostly of Cr(VI) as a soluble salt. Although the carcinogenicity of sparingly soluble forms is likely great than that of soluble forms, the dose-response between water-soluble Cr(VI) measured in the Painesville and Baltimore chromate production plants and increased lung cancer risk is positive.

In summary, exposures to Cr(VI) in the historical chromate production industry (both Painesville and Baltimore) are expected to provide a worst-case scenario, i.e., overestimate of risk as compared to current occupational and environmental exposures. Bioavailability to the most potent Cr(VI) compounds is expected to increase with smaller particle size, lesser solubility, and increased residence time in the lung. The bioavailability of Cr(VI) particulates from environmental exposures may be significantly different from those experienced in historical workplace environments that were used as the basis for cancer risk assessment. However, quantifying that differences is not possible with the currently available information.

5.0 Conclusions

The toxicokinetic data demonstrate that particle solubility and size are important factors in carcinogenicity and may provide partial explanations as to why occupational exposure to Cr(VI) in some industries, but not others, are not associated with an increased lung cancer risk. Exposures of the Painesville worker cohort are expected to represent a worst-case scenario and risk estimates derived from these data are likely to overestimate risk from environmental exposures.

The weight of evidence, as evaluated using the modified Hill Criteria (EPA 2005) supports that Cr(VI)-induced carcinogenicity in the lung acts by a non-mutagenic MOA that involves oxidative stress and oxidative DNA damage, tissue injury and inflammation. There is also considerable evidence for epigenetic DNA modifications. This MOA is consistency with the observed increased cancer risk in workers associated with the high exposure concentrations experienced historically in certain industries, and as demonstrated *in vitro* and in animal models.

Thus, the biological data indicate that the dose-response for lung cancer is expected to include a threshold, but the currently available epidemiologic data do not provide sufficient statistical power in the low dose range to distinguish non-linearities. The Painesville study provides significant advantages for use in environmental risk assessment. Specifically, as compared to the other available risk assessments, these studies include a life-table analysis for estimating potency for lifetime exposures, and have evaluated dose-response in terms of exposure intensity, in addition to cumulative exposure. The risk assessment developed from these data could be improved through the development of a physiologically based pharmacokinetic (PBPK) model that includes a compartment for the respiratory system and takes into account particle size and variability. However such a model is not currently available and the risk assessment relies on conventional dose metrics.

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