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Combining *Drosophila melanogaster* somatic-mutation-recombination and electron-spin-resonance-spectroscopy data to interpret epidemiologic observations on chromium carcinogenicity

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Abstract

Lung cancers are significantly increased among workers exposed to chromate (Cr^{6+}, Cr^{3+}), chromium pigments (Cr^{6+}) and chromium plating (Cr^{6+}). Chromium lung burdens and cancer risk increase proportionately with duration of employment at long latencies. However, this epidemiologic information alone is insufficient in determining whether Cr^{6+} or Cr^{3+} are equally important in causing cancer. We have attempted to combine epidemiologic data with data from the *Drosophila melanogaster* somatic-mutation-recombination-test and from the *in vitro* electron-spin-resonance spectroscopy study to demonstrate that following somatic recombination plays a more important role than somatic mutation in chromium carcinogenesis. Cr^{4+} is more important than Cr^{5+} or Cr^{6+} in inducing somatic recombination while Cr^{6+} produces more and bigger clones than Cr^{4+} in somatic mutation. Cr^{3+} produces negative results in this fruit-fly wing-spot-assay. When the larvae and flies exposed to Cr^{6+} and Cr^{4+} are examined by ESR, only Cr^{5+} and Cr^{3+} are found. Thermodynamic parameters ΔE, ΔH, and ΔS are also estimated from these latter experiments to explain the relative importance of Cr^{6+}, Cr^{4+}, Cr^{3+} in chromium carcinogenesis among exposed industrial workers. (Mol Cell Biochem **222**: 61–68, 2001)

Key words: fruit fly, somatic-mutation-recombination test, electron-spin-resonance-spectroscopy, epidemiolog, chromium carcinogenicity

Introduction

Favorable physicochemical properties of chromate compounds (e.g. color, malleability, ductility, and tensile strength) have led to extensive use of this metal in various industries including chromate production, chromate pigment production, chrome plating, stainless steel welding, ferrochrome alloy production, and leather tanning. Epidemiologic studies of workers in various chromium processing and manufacturing industries have revealed significantly elevated cancer incidence and mortality rates for cancer sites including lung, nasopharyngeal and urinary bladder. Lung cancer mortality rates increase among chromate production workers exposed to Cr^{6+}/Cr^{3+} and among chromate pigment workers exposed to Cr^{6+}. Chrome platers similarly exposed to Cr^{6+} and nickel (Ni^{2+}) likewise have significantly elevated lung cancer risks.

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Stainless steel and ferrochrome workers have high Cr^{3+}, Cr^{0} exposures and to a lesser extent Cr^{6+} exposures. However, associations between exposure and lung cancer among these stainless steel and ferrochrome workers are inconclusive if based on surrogate measures of exposure intensity. In other words, thus far it is unlikely that carcinogenic potentials of Cr^{3+} and Cr^{0} can be specifically quantified from existing cohort data sets solely using epidemiologic methods. Likewise, cancer epidemiologic studies of smelter workers with exposures to Cr^{6+}, Cr^{3+}, Cr^{0} and lanthanum, La^{2+} are burdened by multiple confounders. The same can be said for studies of foundry workers exposed to particulate matter and Cr^{3+}, Cr^{0}.

Despite this there seems little doubt that chromium is carcinogenic with increased risk among exposed workers based on duration of employment. Biopsies of tissues from workers and autopsies of workers who have died of lung cancer also show accumulated chromium loads with increasing employment duration in chromium production and processing. Analyses of exposure data by time from first exposure and total exposure duration show that lung cancer increases, but with long latency times.

Reasoning from cell physiology, Cr^{6+} is readily taken up in cells where it is believed to be reduced to lower valence states (Cr^{5+}, Cr^{4+}) prior to exerting its genotoxic effects. Neither pentavalent nor tetravalent chromium interacts directly with DNA, but rather interacts with hydrogen peroxide to yield highly reactive hydroxyl free radicals. While hydroxyl free radicals likely are the ultimate mutagens, it is not known if Cr^{5+} or Cr^{4+} is the penultimate mutagen.

Since hexavalent chromium is rapidly converted in the lung to insoluble trivalent chromium, one questions whether the trivalent compound is equally carcinogenic. Thus, the potential carcinogenicity of hexavalent vs. trivalent chromium of exposed industrial workers is a research concern for industry and public health officials.

This paper reports on the evaluation of Cr^{6+}, Cr^{4+} and Cr^{3+} for genotoxic effects in somatic tissue of the fruit fly, *Drosophila melanogaster*. The results suggest that Cr^{4+} is highly genotoxic in flies via induction of mitotic recombination. Mitotic recombination leads to a *loss-of-heterozygosity* in an organism, which is suspected of playing a major role in the development of human cancers [1–5].

Materials and methods

Somatic mutation and recombination Test of Drosophila melanogaster

The somatic mutation and recombination test of *Drosophila* (i.e. somatic wing spot assay) was developed in the 1980s to provide researchers with a rapid and inexpensive assay for genotoxicity in somatic cells of higher eukaryotes [6–7]. Details of the assay can be found in Graf *et al.* [6]. The assay can detect a variety of genetic alterations, including gene mutations, deletions, aneuploidy/segmental aneuploidy and mitotic recombination. The detection of mitotic recombination is an important feature of the assay since mitotic recombination can lead to *loss-of-heterozygosity* [3–10]. The assay utilizes two loci located on the left arm of chromosome 3 – *mwh* (*multiple wing hair*) and flr^3 (*flare*). Both loci influence development of hair growth in each adult wing blade cell. An induced genetic alteration in a developing wing disc cell of a treated larva can result in a clone of cells possessing malformed hairs (i.e. a spot) on the adult wing blade.

Larvae exposed to a test substance are allowed to develop into adults, then their wings are removed and mounted in microscopic slides for scoring at 400× magnification. Three endpoints are distinguished in the assay: (1) small single spots of either *mwh* or flr^3 phenotype, which consist of just 1–2 cells; (2) large single spots of either *mwh* or flr^3 phenotype, which consist of 3 or more cells; and (3) twin spots, which consist of adjacent *mwh* and flr^3 spots. While small and large single spots can arise from a variety of genetic alterations, twin spots result *solely* from mitotic recombination. The vast majority (90–95%) of spontaneously arising spots on wings of negative control flies are of the small single type while large single spots and twin spots account for the remaining 5–10% of negative control spots.

Experimental and statistical methodology

Virgin females of genotype *mwh* were mated to males of genotype $flr^3/TM3, Ser$. The flr^3 allele is a homozygotic lethal maintained in a heterozygous state by using the multiply inverted third chromosome balancer *TM3,* which also contains the dominant wing mutation *Ser* (*serrate*). Female parental flies lay eggs for an 8-h interval on standard fly media in glass bottles. The parents were then removed and eggs allowed to continue normal development. Approximately 72 h after beginning egg-laying, the bottled 3rd instar larvae were collected, washed and randomly allocated to different treatments.

All treatments included feeding larvae for 6 h with a neutral cellulose powder or a test substance mixture both wetted with distilled water to form a slurry. Test substances included $K_2Cr_2O_7$ and GSH-Cr^{4+} evaluated at 20 and 40 mM concentrations, and $CrCl_3$ evaluated at 40 mM. This latter acute exposure treatment was used to overcome insolubility of GSH-Cr^{4+} in water. The negative control treatment was plain cellulose powder with water. Larvae were then collected after 6 h, washed and placed in glass vials containing Instant Drosophila Medium (Carolina Biological Supply Co., Burlington, NC, USA) wetted with distilled water. Following appearance in the vials, adult flies were removed and stored in

70% ethanol until needed. Culturing of strains and all experimental procedures were conducted at 25°C.

Experimental matings produced two types of progeny: (1) trans-heterozygous flies (*mwh flr*$^{+}$*/mwh*$^{+}$ *flr*^{3}) identified as adults by their wild-type (non-Serrate) wings, and (2) inversion-heterozygous flies (*mwh/TM3, Ser*) identified by their Serrate wings. While all three endpoints (small singles, large singles and twin spots) develop on wings of the trans-heterozygous flies, only small and large *mwh* single spots develop on wings of inversion-heterozygous flies. This is because only *mwh* spots can occur in inversion-heterozygous flies since *flr*^{3} is not present in the strain, and furthermore, mitotic recombination is suppressed by the multiple-inverted *TM3* chromosome. Among trans-heterozygous flies, 40 negative control wings were scored along with 20 wings from each mutagen-treated group. For inversion-heterozygous flies, 20 wings were scored for effects of the 40 mM treatments with $K_2Cr^{6+}{}_2O_7$ and GSH-Cr^{4+}.

Statistical analyses were conducted for tested endpoint variables. Data analysis yielded mean, range and standard deviations of spots observed in treated and negative control trans-heterozygous wings. Then a multiple-decision methodology described by Frei and Würgler [8] was applied to these descriptive statistics determining the test mutagen's capacity to induce specific endpoints as either (1) positive, (2) weakly positive, (3) inconclusive or (4) negative. Statistical significance was assessed at the p = 0.05 level.

Electron spin resonance or paramagnetic resonance spectroscopy

Following 6 h feeding of 3rd instar larvae that included test substances Cr^{6+}, Cr^{4+}, Cr^{3+}, some larvae were collected, washed and preserved in chloroform for later ESR analysis. Cr^{3+} signals can be measured on a Varian E-109E EPR spectrometer (9.6 GHz, X-band) since the machine can detect a wide 'scan' range (2000 gauss) with relatively high sensitivity. Spectrometer settings were: magnetic field, 3240 gauss; incident microwave power, 5 mW; modulation frequency, 100 kHz; and modulation amplitude, 2.0 gauss. Cr^{5+} and Cr^{4+} were a flat cell assembly. The settings were: receiver gain, 3.2×10^3; time constant, 0.3 sec; incident microwave power, 20 mW; modulation amplitude, 4.0 G; center field, 3,520 G; sweep width, 400 G for Cr^{4+} and 2,000 G for Cr^{3+}. Settings for the Cr^{5+} measurements were:1.2 GHz, L-band; 425 gauss, magnetic field; incident microwave power, 30 mW; scan time, 1 min; and modulation frequency, 27 kHz [6–8]. A summary of the ESR study parameters is presented in Table 1.

Table 1. Parameters of the ESR analyses

Ionic species	Centerfield	Range	Modulation frequency	Modulation amplitude
Cr^{3+}	3520 gauss	2000 gauss	100 kHz (9.6 GHz)	2.0 gauss
Cr^{4+}	3520 gauss	400 gauss	100 kHz (9.6 GHz)	4.0 gauss
Cr^{5+}	3520 gauss	425 gauss	27 kHz (1.2 GHz)	0.2 gauss

Results

Somatic mutation and recombination test

A summary of genotoxic effects in trans-heterozygous fruit flies for various valences of chromium is presented in Table 2. Numbers and means of observed spots per wing are listed for endpoints (types of spots) at each test compound concentration. Cr^{6+} was a positive inducer of all endpoints at high and low dose concentrations.

Similarly, Cr^{4+} was found to be a positive inducer of all three endpoints at the higher concentration, but was judged inconclusive for inducing small and large single spots at the lower concentration. However, Cr^{4+} was a positive inducer of twin spots at the lower concentration. Findings that Cr^{6+} and Cr^{4+} induce twin spots demonstrate that chromium in these valence states induce mitotic recombinations. Cr^{3+} was inconclusive for inducing small, large single and twin spots. In other words, there was no Cr^{3+} induced-somatic-mutation or mitotic-recombination.

These above results will now be interpreted in light of established principles of biochemical reaction kinetics. It is well known that in a series of reactions, if the concentration of intermediate chemical species, e.g. in this case Cr^{4+} markedly increases above the concentration of the initial chemical reactant species (Cr^{6+}), there will be a corresponding faster and more concentrated buildup of the terminal product (somatic recombination) compared to the initial reactants.

Table 2. Frequency N and means per wings of induced spots in treated trans-heterozygous (*mwh flr*$^{+}$*/mwh*$^{+}$ *flr*^{3}) flies*

Treatment dose	Wings, number	Small spots N	Small spots per wing	Large spots N	Large spots per wing	Twin spots N	Twin spots per wing
H_2O	40	5	0.125	1	0.025	0	0
Cr^{6+}, $K_2Cr_2O_7$							
40 mM	20	20	1.00 (POS)	82	4.10 (POS)	66	3.30 (POS)
20 mM	20	9	0.45 (POS)	39	1.95 (POS)	53	2.65 (POS)
Cr^{4+}, GSH-Cr							
40 mM	20	22	1.10 (POS)	23	1.15 (POS)	26	1.30 (POS)
20 mM	20	5	0.25 (INC)	5	0.20 (INC)	8	0.45 (POS)
Cr^{3+}, $CrCl_3$							
40 mM	20	3	0.15 (INC)	1	0.05 (INC)	0	0

*Comparison of statistical significance between H_2O-negative-control flies *vs* chromium treated flies where POS = positive result or INC = inconclusive result.

Table 3. Total wing spots in trans-heterozygous flies (*mwh flr*$^{+}$/*mwh*$^{+}$ *flr*^{3}) treated with Cr^{6+} and Cr^{4+}

Treatment	Total number spots	Mean number spots/wing
40 mM Cr^{6+}	168	8.40
20 mM Cr^{6+}	101	5.05
40 mM Cr^{4+}	71	3.55
20 mM Cr^{4+}	18	0.90

$$Cr^{6+} \Leftrightarrow Cr^{5+} \Leftrightarrow Cr^{4+} \Leftrightarrow \text{somatic recombination}$$
$$\Downarrow \qquad \Downarrow$$
$$\text{somatic mutation}$$

The total number of wing spots induced by Cr^{6+} and Cr^{4+} treatments at 20 and 40 mM in trans-heterozygous flies are summarized in Table 3. It shows that Cr^{6+} induced 8.40 spots per wing at 40 mM and 5.05 spots per wing at 20 mM, a 1.66 increase in mean spots per wing (i.e. 8.40/5.05 = 1.66 more for the higher treatment concentration. On the other hand, there was a 3.94 mean increase of Cr^{4+} induced spots (3.55/0.90 = 3.94) comparing the ratio of higher vs. lower treatment concentrations. The higher ratio of response associated with Cr^{4+} over Cr^{6+} with a doubling of mutagen concentration treatments and Cr^{4+} ability to increase twin spots or somatic recombination, therefore loss-of-heterozygosity contrast the results from Cr^{6+} treatment. These two observations suggest that Cr^{4+} *is* the penultimate genotoxic/carcinogenic agent.

A wing scoring summary of inversion-heterozygous flies treated with Cr^{6+} and Cr^{4+} is presented in Table 4. Only small and large single spots of phenotype *mwh* can arise in these flies (*flr*^{3} single spots and twin spots are not possible). For comparison purposes, the number of *mwh* single spots observed in trans-heterozygous flies are also presented, along with the total number of *mwh* single spots observed in the two strains.

The smaller number of *mwh* spots observed in inversion heterozygous flies compared to those in trans-heterozygous flies is due to suppression of mitotic recombination in the inversion-heterozygous strain. This is evidenced by a 91.5% reduction in *mwh* single spots induced by Cr^{6+} between the two strains [i.e. 1 – (7/82) = 0.915] and a similar 85.4% reduction observed for Cr^{4+}-induced spots. Similar results [6] were previously reported for Cr^{6+} in the wing spot assay.

Table 4. Mwh wing spots in inversion-heterozygous and trans-heterozygous flies treated with 40 mM Cr^{6+} and Cr^{4+}.

Strain	Mutagen	No. wings	Small spots	Large spots	Total spots
Inversion-heterozygous	Cr^{6+}	20	7	0	7
(*mwh/TM3, Ser*)	Cr^{4+}	20	5	1	6
Trans-heterozygous	Cr^{6+}	20	16	66	82
(*mwh flr*$^{+}$/*mwh*$^{+}$ *flr*^{3})	Cr^{4+}	20	21	20	41

Table 5. Estimated thermodynamic parameters for activation of chromium: $\Delta E = \Delta H + T\Delta S$; or ΔS = 1–200 cal/mole·degree

	ΔE	ΔH	ΔS
cal/mol	3–4000	660	1–200

Hence, one can infer that the majority (~90%) of single spot induction by Cr^{6+} and Cr^{4+} in trans-heterozygous flies arise from mitotic recombination *as opposed to somatic mutation*.

Thermodynamic parameters estimated from larval ESR spectra [11–13]

The estimated energy changes associated with the reduction mechanism of chromium ions ($Cr^{6+} \Rightarrow Cr^{5+} \Rightarrow Cr^{4+} \Rightarrow Cr^{3+}$) within cells is shown in Table 5.

Eight vials of *Drosophila* larvae exposed to 40 mM K_2CrO_4, 40 mM $CrCl_3$, and H_2O for 1, 3, and 6 h. ESR spectra of the larvae are plotted on premium quality bond paper. Peaks above background tracing are cut out and weighed. Density and thickness of the paper cutouts are assumed to be constant throughout. Therefore, the *weight ratios* of peaks become an appropriate estimate of the ratio of integrated areas under the ESR spectra. Larvae exposed to K_2CrO_4 for 1, 3 or 6 h have, respectively, values of 0.1, 0.07 and 0.04 g. Linear regression of decrease in concentrations of Cr^{5+} in larvae exposed to Cr^{6+} gives the following estimates: a = 0.0296, b = 8.93×10^{-4}, $\gamma = 0.95$. For larvae exposed to $CrCl_3$ for 1 and 3 h, the estimates are 0.06 and 0.08 g. Linear regression analysis yields estimates of: a = 0.0098, b = 9.16×10^{-4}, $\gamma = 0.818$.

An estimate of the ratio of kinetic constants of forward and backward reactions leading to the disappearance of paramagnetic radicals ($Cr^{5+} \Rightarrow Cr^{3+}$, $Cr^{3+} \Rightarrow Cr^{5+}$) is obtained by dividing the constant '**a**' of 2 linear regression coefficients 0.0098/0.0296 = 0.3, or 30.2%/h. This estimate is comparable to *in vivo* rodent data [14], where formation-half-life of $Cr^{6+} \Rightarrow Cr^{5+}$ paramagnetic species was reported as 37 min. In other words, Q_{10} for the elimination of paramagnetic radical Cr^{5+} is between 1–2 in living organisms, assuming a rodent body temperature of 30°C and *Drosophila* body temperature of 20°C.

Van't Hoff's equation states: $dlnK/dT = \Delta\mathbf{H}/RT^2$;

$\ln K = -\Delta\mathbf{H}/RT$, where R = 2.3; T = 298, and $\Delta\mathbf{H}$ is estimated to be 656.7 cal/mole

From an earlier estimate [9], the formation of the paramagnetic radical Cr^{+5} from Cr^{+6} is $\Delta E = RT \ln K$, where R = 2.3, T = 303, and $K = 1.5 \times 10^{-2}$, which yields: ΔE = 3–4 Kcal/mole for formation of Cr^{5+} at g = 1.9950, and probably 1.9793. This amount of change in free energy is less than that released from the hydrolysis of high energy bonds in reactions involving ATP/GTP, 7–10 Kcal/mole; GSH, 10 Kcal/mole; or NADH/

NADPH, 12 Kcal/mole. A coupled reaction involving either high energy compounds such as glutathione or NADH would provide sufficient free energy to drive the ion reduction, $Cr^{+6} \Rightarrow Cr^{+4} \Rightarrow Cr^{+5}$ to completion. Now an estimate can be made of how these energy changes relate to the location and reduction mechanisms of chromium ions inside cells, i.e. $Cr^{+6} \Rightarrow Cr^{+5} \Rightarrow Cr^{+4}$. These estimates suggest how xenobiotics such as chromium ions enter cells and become detoxified or activated. The suggested thermodynamic parameters presented here identify the location of such reactions, whether they occur inside the nucleus, an organelle or in the cytoplasm. Our modelling indicates that major proportion of free energy change in mediating chromium ion reduction is provided for by the decrease in *cellular entropy* as chromium is now restricted inside the cell. The decrease in entropy is close to 1–200 cal/mol.deg. The consumption of *free energy* in the chromium reduction is even higher than the 7 kcal/mole of energy used by ATPase in the Na^+-K^+ pump at the plasma membrane. This energy is needed to keep a pentavalent cation Cr^{+5} inside the cell in combination with the coenzyme NAD or a tetravalent Cr^{+4} in combination with glutathione since chromium is no longer in the form of the divalent anion $Cr_2O_4^{2-}$. Moreover, neither our modelling or any evidence from molecular kinetics suggest that a specific enzyme is needed to drive the $Cr^{+6} \Rightarrow Cr^{+5} \Rightarrow Cr^{+4} \Rightarrow Cr^{+3}$ series of reduction reactions. Rather now it appears that activated chromium ions can be cytotoxic in the Cr^{+5} state in the cytoplasm or genotoxic in the Cr^{+4} state.

Discussion

Epidemiology vs. in vitro *experiments*

Evidence from epidemiologic studies of industrial workers strongly suggests chromate is a human carcinogen. Nonetheless, chromate is not mutagenic *in vitro* in the presence of liver P450 nonspecific oxidase preparations and does not form covalent bonds with DNA under physiological conditions [22–24, 26]. This latter idea is relevant since most ambient environmental carcinogens must be activated or metabolized to an ultimate carcinogen [14–18, 20, 21] for carcinogenesis to occur within exposed humans and other organisms.

Hexavalent chromium compounds are, in contrast [30], reduced sequentially and ultimately to a single trivalent chromium compound that has much lower carcinogenic potency. Nevertheless, a cogent theory of chromate-compound-reduction that includes hexavalent chromium Cr^{6+} as a necessary procarcinogen in an activation step leading to carcinogenesis was expounded and elaborated by Wetterhahn *et al.* [20, 25, 27–28, 31–34] and in some of the present our work [11–14, 35–39, 41–46].

ESR of chromium ions of different valencies [21–27]

If chromium is a human carcinogen based on epidemiologic data, which form of chromium Cr^{+6}, Cr^{+5}, Cr^{+4}, or Cr^{+3} is the penultimate or ultimate carcinogen? Cr^{6+} is reduced by ascorbate, glutathione [40] and NADH/NADPH to Cr^{5+} via enzyme complexes such as cytochrome P450, cytochrome b_5, electron transport chains and glutathione reductase. The octahedral Cr^{5+}-NADPH has a vacant site for H_2O_2 to form a long-lived complex, which, in turn, generates ·OH radicals in a Fenton reaction. These ·OH radicals appear to modify either the serum proteins, cell surface lipoproteins or glycoproteins leading to hypersensitivity and cytotoxic reactions. On the other hand, they can also modify specific nucleotides, thus affecting gene expression. Moreover, they can also excise phosophodiester bonds in DNA double helices, thus initiating carcinogenic events in target sites including upper and lower respiratory tract and bladder in humans. NADH (or NADPH) is 2–3 times more effective in forming Cr^{5+} on a molar basis than other potential reducing agents such as ascorbate or glutathione. The experimental evidence underlying this assertion is as follows: hyperfine splitting with 0.095 G spacing was discovered in the 9.6 GHz. X-band EPR showing interactions between protons and oxygen in the diol-Cr^{5+} complex (g = 1.9796). This hyperfine splitting is observed when Cr^{5+} is in the presence of NADH/NADPH or GSH reductase; but not with GSH or ascorbate.

Cr^{6+} enters the cell as CrO_4^{-} via diffusion through the Cl^-, HCO_3^- anionic channel. Cr^{6+} is then reduced intracellularly, sequentially to form Cr^{5+}, Cr^{4+}, Cr^{3+}. Changes in free energy during formation of these paramagnetic chromium radicals are as follows:

> ΔE = 3–4 Kcal/mole for the formation of Cr^{5+} at g = 1.9950, and probably 1.9793.

NADH/NADPH and GSH (reductase) are the cellular reductants [22–25]. It is by forming reactive species, especially ·OH ions through 'Fenton-like' reactions, that transcription and replication of DNA occur, leading to cytotoxicity, hypersensitivity and carcinogenesis at the organismic level [26–35, 47–56].

Similar to glutathione, ascorbate generates Cr^{5+} and Cr^{4+} when incubated with Cr^{6+}. In addition, carbon-centered alkyl radicals and formyl radicals are generated simultaneously. Electrophoretic assays show that ascorbate-derived-radicals induce double-strand-breaks in DNA. In the circulatory system, ascorbate is present in high concentration extracelluarly, while glutathione and glutathione reductase [62] are present intracellularly in higher concentrations especially at the plasma membrane which facilitates uptake of amino acids or xenobiotics like CrO_4^{2-}, or $Cr_2O_7^{2-}$ under the study here.

Cr^{4+} generates Cr^{5+}, ·OH and OH – in the presence of H_2O_2. DNA double strands breaks are also induced by the Cr^{4+}, Cr^{5+}-

mediated Fenton-like reaction [36–52]. ·OH radicals from the reaction of H_2O_2 with Cr^{4+}, Cr^{5+} lead to hydroxylation of 2'-dG to form 8-OH-2'dG. The induction of double strand breaks or modification of the heterocyclic structure of nucleotides change the genomic sequence. These alterations can lead to cell death or genetic expression changes. Experimental chromate treatments have also been shown to inhibit cell growth by specific blockage of the progression of cells through S-phase of the cell cycle, that then affect cell proliferation directly via lipid peroxidation. It is likely that these changes will also induce somatic mutations and or somatic recombinations in exposed organisms leading to cancer development [53–56].

While evidence from epidemiologic data strongly suggests chromium is a human carcinogen, it is not known if all chromium ion species are equally potent in human carcinogenesis. Also unknown is whether Cr^{4+} or Cr^{5+} is the penultimate carcinogen. Also it is not yet established whether either or both paramagnetic radicals Cr^{4+}/Cr^{5+} are responsible for the liver cytotoxicity and or carcinogenicity in other target organs. It is hypothesized here that Cr^{5+} is the hepatoxicant, while Cr^{4+} tetravalent compound forms the penultimate carcinogen in the upper and lower respiratory tract and the bladder.

Carcinogenicity/genotoxicity of Cr^{+4}, Cr^{+5}, Cr^{+6}

Using surrogate prokaryote systems in human carcinogenicity [57] testing ignores genomic differences between bacteria and humans. Employing meiosis and chromosomal pairing in rodent genetic studies also has important drawbacks, since somatic mutation and recombination mechanisms associated with carcinogenesis differ from recombination during meiosis or in mutation of gametes. The best genetic experiments reflecting somatic cellular changes in eukaryotes are somatic recombination and mutation studies such as reported here in *D. melanogaster* [19, 51–61].

From epidemiologic studies of chromate production workers, there is evidence for a carcinogenic effect but demonstrated only after a prolonged latency from time of first exposure . This latter finding is likely relevant to information from the ESR data that show Cr^{+6} in the presence of lysosomeal phosphate is converted in cells to form Cr^{+4}, Cr^{+5} and ultimately into relatively insoluble Cr^{+3}. Our interpretative results from cell physiology [62] indicate that Cr^{+6} likely enters cells as $CrO_4^{-2}/Cr_2O_7^{-2}$ via an anion channel, whereas Cr^{+3} enters cells following phagocytosis [63] and is stored in lysosomes [64] in combination with the phosphate ion. In comparing these mechanisms, Cr^{+3} is at least 2–3 orders of magnitude lower in bioavailability than Cr^{+6} [65].

Thus, potent genotoxic effects of $Cr^{4+,\ 5+}$ observed in *D. melanogaster* somatic wing spot tests suggest that Cr^{+4} is the penultimate (and ·OH the ultimate carcinogen) in Cr^{6+} carcinogenesis. Moreover, this mechanism is an initiation event. Since genetic alterations induced by Cr^{6+} and Cr^{4+} appear to arise from induced mitotic recombination, it is likely the initiation event in chromium carcinogenesis involves the double strand excision of the DNA helix leading to a loss of heterozygosity in various target sites in the nuclear genome.

References

1. Sengstag C: The role of mitotic recombination in carcinogenesis. Crit Rev Toxicol 24: 323–353, 1994
2. Shammas MA, Reis RJS: Recombination and its role in DNA repair, cellular immortalization and cancer. Age 22: 71–88, 1999
3. Cavenee WK, White RL: The genetic basis of cancer. Sci Am 272: 72–79, 1995
4. Tischfield JA: Loss of heterozygosity or: How I learned to stop worrying and love mitotic recombination. Am J Hum Genet 61: 995–999, 1997
5. Shao C, Deng L, Henegariu O, Liang L, Stambrook PJ, Tischfield JA: Chromosomal instability contributes to loss-of-heterozygosity in mice lacking p53. Proc Natl Acad Sci USA 97: 7405–7410, 2000
6. Graf U, Würgler FE, Katz AJ, Frei H, Juon H, Hall CB, Kale PG: Somatic mutation and recombination test in *Drosophila melanogaster.* Environ Mutagen 6: 153–188, 1984
7. Graf U, Frei H, Kagi A, Katz AJ, Würgler FE: Thirty compounds tested in the *Drosophila* wing spot test. Mutat Res 22: 359–373, 1989
8. Frei H, Würgler FE: Statistical methods to decide whether mutagenicity test data from *Drosophila* assays indicate a positive, negative or inconclusive result. Mutat Res 203: 297–308, 1988
9. Tischfield JA: Loss of heterozygosity or: How I learned to stop worrying and love mitotic recombination. Am J Hum Genet 61: 995–999, 1997
10. Gupta PK, Sahota A, Boyadjiev SA, Bye S, Shao C, O'Neill JP, Hunter TC, Albertini RJ, Stambrook PJ, Tischfield JA: High frequency *in vivo* loss of heterozygosity is primarily a consequence of mitotic recombination. Cancer Res 57: 1188–1193, 1997
11. Liu KJ, Jiang JJ, Shi XL, Gabrys H, Wakczaj T, Swartz HM: Low frequency EPR study of Cr (V) formation from Cr (VI) in living plants. Biochem Biophys Res Comm 206: 829–834, 1995
12. Liu KJ, Jiang JJ, Swartz HM, Shi XL: Low frequency EPR detection of Chromium (V) formation by Chromium (VI) reduction in whole live mice. Arch Biochem Biophys 313: 248–255, 1995
13. Liu KJ, Shi XL, Dalal NS: Synthesis of Cr^{4+}-GSH, its identification and its free hydroxyl radical generation: A model compound for Cr^{6+} carcinogenicity. Biochem Biophys Res Comm 235: 54–58, 1997
14. Chiu A, Chiu N, Shi X, Beaubier J, Dalal N: Activation of a procarcinogen by reduction: $Cr^{6+} \rightarrow Cr^{5+} \rightarrow Cr^{4+} \rightarrow Cr^{3+}$: A case study by electron spin resonance spectroscopy (ESR/PMR). J Env Health Part C. Env Carc Ecotox Rev 16: 135–148, 1998
15. Vogel EW, Nivard JM: Performance of 181 chemicals in *Drosophila* assay predominantly monitoring interchromosomal mitotic recombination. Mutagenesis 8: 57–81, 1993
16. Vogel EW, Zijlstra JA: Mechanistic and methodological aspects of chemically-induced somatic mutation and recombination in *D. melanogaster*. Mutat Res 182: 243–264, 1987
17. Vogel EW, Zijlstra JA: Somatic cell mutagenesis in *D. melanogaster* in comparison with genetic damage in early germ-cell stages. Mutat Res 180: 189–200, 1987
18. Würgler FE, Vogel EW: *In vivo* mutagenicity testing using somatic cells of *D. melanogaster*. In: F. de Serres (ed). Chemical Mutagens, Vol. 10. Plenum Press, New York, 1986, pp 1–59

19. Davis PS, Shen MW, Judd BH: Asymmetric pairings of transposons in and proximal to the white locus of *Drosophila* account for four classes of regularly occurring exchange products. Proc Natl Acad Sci USA 84: 174–178, 1987
20. Miller EC: Some current perspectives on chemical carcinogenesis in humans and experimental animals: A presidential address. Cancer Res 38: 1479–1496, 1978
21. Miller EC, Miller JA: Searches for ultimate chemical carcinogens and their reactions with cellular macromolecules. Cancer 47: 2327–2345, 1981
22. Petrilli FL, DeFlora S: Toxicity and mutagenicity of hexavalent chromium in *Salmonella typhimurium*. Appl Environ Microbiol 33: 805–809, 1977
23. Petrilli FL, DeFlora S: Oxidation of inactive trivalent chromium to the mutagenic hexavalent form. Mutat Res 58: 167–178, 1978
24. Petrilli FL, DeFlora S: Metabolic deactivation of hexavalent chromium mutagenicity. Mutat Res 54: 139–147, 1978
25. Liu KJ, Shi X, Jiang JJ, Goda F, Dalal NS, Swartz HM: Chromate-induced chromium (V) formation in live mice and its control by cellular antioxidants: An L-band EPR study. Arch Biochem Biophys 323: 33–39, 1995
26. Bianchi V, Celott L, Lanfranchi G, Majone F, Marin G, Montaldi A, Sponza G, Tamino G, Venier P, Zantedeschi A, Levis, AG: Genetic effects of chromium compounds. Mutat Res 117: 279–300, 1983
27. Cupo DY, Wetterhahn KE: Modification of Cr (VI)-induced DNA damage by glutathione and cytochrome P-450 in chicken embryo hepatocytes. Proc Natl Acad Sci USA 82: 6755–6759, 1985
28. Hamilton JW, Wetterhahn KE: Differential effects of chromium (VI) on constitutive and inducible gene expression in chick embryo liver *in vivo* and correlation with chromium (VI) induced DNA damage. Mol Carcinogen 2: 274–286, 1989
29. Kasprzak KS: The role of oxidative damage in metal carcinogenesis. Chem Res Toxicol 4: 604–615, 1991
30. Klein CB, Frenkel K, Costa M: The role of oxidative processes in metal carcinogenesis. Chem Res Toxicol 4: 592–604, 1991
31. Aiyar J, Berkovits HJ, Floyd RA, Wetterhahn KE: Reaction of chromium (V) with hydrogen peroxide in the presence of glutathione; reactive intermediates and resulting DNA damage. Chem Res Toxicol 3: 595–603, 1990
32. Aiyar J, Borges KM, Floyd RA, Wetterhahn KE: Role of chromium (V) glutathione thiyl radical and hydroxyl radical intermediates in chromium (VI)-induced DNA damage. Toxicol Environ Chem 22: 135–148, 1990
33. Jennette KW: Microsomal reduction of the carcinogen chromate produces chromium (V). J Am Chem Soc 108: 874–875, 1982
34. Tsapakos MJ, Wetterhahn KE: The interaction of chromium with nucleic acid. Chem Biol Interact 46: 265–277, 1983
35. Chen F, Ye JP, Zhang XY, Rojanasakul Y, Shi XL: One-electron reduction of Cr^{6+} by α-lipoic acid and related hydroxyl radical generation, dG hydroxylation and nuclear transcription factor-κB activation. Arch Biochem Biophys 338: 165–172, 1997
36. Craf E, Mahoney JR, Bryant RG, Eaton JW: Iron catalyzed hydroxyl radical formation: Stringent requirement for free iron coordination site. J Biol Chem 259: 3620–3624, 1984
37. Liu KJ, Shi XL, Jiang JJ, Goda F, Dalal N, Swartz HM: Chromate induced Cr (V) formation in live mice and its control by cellular antioxidants; an L-band EPR study. Arch Biochem Biophys 323: 33–39, 1995
38. Liu KJ, Shi XL, Jiang JJ, Goda F, Dalal N, Swartz HM: Low frequency electron paramagnetic resonance investigation on metabolism of $Cr^{6+}$by whole live mice. Ann Clin Lab Sci 26: 176–184, 1996
39. Furono K; Suetsuga T, Sugihara N: Effects of metal ions on lipid peroxidation in cultured rat hepatocytes loaded with α-linolenic acid. J Toxicol Environ Health 48: 121–129, 1996
40. Meister A, Anderson ME: Glutathione. Ann Rev Biochem 52: 711–760, 1983
41. Shi XL, Dalal NS: On the mechanism of chromate reduction by glutathione: ESR evidence for the glutathionyl radical and an insoluble Cr (V) intermediate. Biochem Biophys Res Comm 156: 137–142, 1988
42. Shi XL, Dalal NS: Chromium (V) and hydroxyl radical formation during the glutathione reductase catalyzed reduction of chromium (VI). Biochem Biophys Res Comm 163: 627–634, 1989
43. Shi XL, Dalal NS: On the hydroxyl radical formation in the reaction between hydrogen peroxide and biologically generated Cr (V) species. Arch Biochem Biophys 277: 342–350; 281-90-5, 1990
44. Shi XL, Dalal NS: Evidence for a Fenton-type mechanism for the generation of ·OH radicals in the reduction of Cr (VI) in cellular media. Arch Biochem Biophys 281: 90–95, 1990
45. Shi XL, Dalal NS: NADPH dependent flavoenzyme catalyzed one electron reduction of metal ions, molecular oxygen and generate hydroxyl radicals. FEBS Lett 276: 189–191, 1990
46. Shi XL, Dalal NS: ESR spin trapping detection of hydroxyl radicals in the reactions of Cr (V) complexes with hydrogen peroxide. Free Radical Res Comm 10: 17–26, 1990
47. Szakmary A, Huang S-M, Chang DT, Beachy PA, Sander M: Overexpression of a Rrp1 transgene reduces the somatic mutation and recombination frequency induced by oxidative DNA damage in *Drosophila melanogaster*. Proc Natl Acad Sci USA 93: 1607–1612, 1996
48. Shi, XL, Dalal N: Generation of hydroxyl radical by chromate in biologically relevant systems: Role of Cr(V) complexes versus Tetraperoxochromate(V). Environ Health Persp 102: 231–236, 1994
49. Shi XL, Dalal NS, Kasprzak KS: Generation of free radicals from hydrogen peroxide and lipid peroxides in the presence of Cr^{3+}. Arch Biochem Biophys 302: 294–299, 1993
50. Shi XL, Dong ZA, Dalal NS, Gannett PM: Chromate mediated free radical generation from cysteine, penicillamine, hydrogen peroxide, and lipid peroxides. Biochim Biophys Acta 1226: 65–74, 1994
51. Shi XL, Mao Y, Knaptor AD, Ding M, Rojanasakul Y, Gannett PM, Dalal N, Liu KJ: Reaction of Cr^{6+} with ascorbate and hydrogen peroxide generates hydroxyl radicals and causes DNA damage: Role of a Cr^{4+} mediated Fenton-like reaction. Carcinogenesis 15: 2475–2478, 1994
52. Shi XL, Sun XO, Gannett PM, Dalal NS: Deferoxamine inhibition of Cr^{5+}mediated radical generation and deoxyguanine hydroxylation: ESR and HPLC evidence. Arch Biochem Biophys 293: 281–286, 1992
53. Tsuda H, Kato K: Chromosomal aberrations and morphological transformation in hamster embryonic cells treated with potassium dichromate *in vitro*. Mutat Res 46: 87–94, 1977
54. Wedrychowski A, Ward WS, Schmidt WN, Hnilica LS: Chromium-induced cross-linking of nuclear proteins and DNA. J Biol Chem 260: 7150–7155, 1985
55. Xu J, Bubley GJ, Detrick B, Blankenship LJ, Patierno SR: Chromium (VI) treatment of normal human lung cells results in guanine-specific DNA polymerase arrest, DNA–DNA cross-links and S-phase blockade of cell cycle. Carcinogenesis 17: 1511–1517, 1996
56. Ye J P, Zhang XY, Young HA, Mao Y, Shi XL: Cr^{6+} induced nuclear factor-B activation in intact cells via free radical reactions. Carcinogenesis 16: 2401–2405, 1995
57. Ames, BN, McCann J, Yamasaki E: Methods for detecting carcinogens and mutagens with the Salmonella/mammalian-microsome mutagenicity test. Mutat Res 31: 347–364, 1975
58. Guzman-Rinconl J, Graf U: *Drosophila melanogaster* somatic mutation and recombination test as a biomonitor. In: F.M. Butterworth (ed). Biomonitor and Biomarkers as Indicators of Environmental Change. Plenum Press, New York, 1995
59. Spano MA, Rincon JG, Abraham SK, Andrade HH: The wing somatic mutation and recombination test (SMART) in *Drosophila melanogaster*: An efficient tool for the detection of genotoxic activity of pure

compounds or complex mixtures as well as for studies on antigenotoxicity. Afr Newslett Occup Health Safety 6: 9–13, 1995
60. Davis PS, Shen MW, Judd BH: Asymmetric pairings of transposons in and proximal to the white locus of *Drosophila* account for 4 classes of regularly occurring exchange products. Proc Natl Acad Sci USA 84: 174–178, 1987
61. Szakmary A, Huang Shu-Mei, Chang DT, Beachy PA, Sander M: Overexpression of a Rrp1 transgene reduces the somatic mutation and recombination frequency induced by oxidative DNA damage in *Drosophila melanogaster*. Proc Natl Acad Sci USA 93: 1607–1612, 1996
62. Standeven AM, Wetterhahn KE: J Am Coll Toxicol 8: 1275, 1989
62a. Kitagawa S, Seki H, Kametani F, Sakurai H: Uptake of hexavalent chromium by bovine erythrocytes and its interaction with cytoplasmic components: The role of glutathione. Chem Biol Interact 40: 265–274, 1982
63. Buttner B, Beyersmann D: Modification of the erythrocyte anion carrier by chromate. Xenobiotica 15: 735–741, 1985
64. Berry JP: The role of lysosomes in the selective concentration of mineral elements. A microanalytical study. Cell Mol Biol 42: 395–411, 1996
65. Kerger BD, Paustenbach DJ, Corbett GE, Finley BH: Absorption and elimination of trivalent and hexavalent chromium in humans following ingestion of a bolus dose in drinking water. Tox Appl Pharm 141: 145–58, 1996