CHAPTER 2

REVIEW OF LITERATURE

2.1 Metals, heavy metals and mankind

Glanze (1996) had reported that there have been 35 metals that concern us because of occupational or residential exposure. 23 of these are the heavy metals: antimony, arsenic, bismuth, cadmium, cerium, chromium, cobalt, copper, gallium, gold, iron, lead, manganese, mercury, nickel, platinum, silver, tellurium, thallium, tin, uranium, vanadium, and zinc. Heavy metals and their salts take part in the genetic and cellular functions and also play a vital role in our day to day life. Any alteration in their balance in the environment might cause the health hazards to man and animals.

Ferner (2001) stated that the metals are commercially important substances and hence find use in industries, due to which the workers and environment gets exposed to them. Longer the period of exposure, serious are the ill effects on their health. If unrecognized or inappropriately treated, toxicity can result in significant illness and reduced quality of life.

2.2 Trace elements

Trace elements overlap partially with heavy metals as well as non metals. Shanker (2008) illustrated that all living beings derive nutrition from the earth only that include macro as well micronutrients. Micronutrients consisted of trace elements as well as minerals e.g. iron, cobalt, chromium, copper, iodine, manganese, selenium, zinc and molybdenum. They enter into plant and animal systems due to their distinct chemical properties such as reduction and oxidation reactions under physiological conditions. As they carry mainly a positive charge (cation), their entry is facilitated by the proteins carrying negative charge or anions. Once inside the body, they take part in various chemical reactions making some of these trace metal ions obligatory for life. Although not created by humans, yet several anthropomorphic activities have brought these metals in their greater proximity requiring the need to assess them quantitatively for their effects. According to analytical chemistry, a trace
element should have an average concentration of less than (<) 100 parts per million (ppm) atoms, or less than (<) 100 micrograms per gram of sample. Biochemically, it is needed in minute quantities for the proper growth, development, and physiology of the organism. They become primary cause of toxicity when present in surfeit. Figure 2.1 shows the possible harmful effects encountered upon by animals and plants upon contact with metals.

Figure 2.1 Interaction of toxic metals with living organisms and their possible effects.

Apostoli and Catalani (2011) opined that important mechanisms of action of metal ions on reproductive system have been suggested in more recent decades. These are the endocrine disruption via impact of metal ions on reproductive hormones and the oxidative stress. There is clear cut evidence of effect of many metals in animals, but human data are scant and limited to high levels of a few metal ions, like lead on male fertility. The data is still less for mercury, manganese, chromium, nickel, and arsenic. The demonstration
of effects on female reproduction and on pregnancy is even more complex. Lead, arsenic, cadmium, chromium, and mercury may cause effect in the beginning in fetal life, during early development or maturity. The other effects could be sub fertility, infertility, intrauterine growth retardation, spontaneous abortions, malformations, birth defects, post natal death, learning and behavior deficits, and premature aging. Animal studies suggested the high frequency of infertility or abortions for humans.

Southam (2012) reported about the toxicity of metals encountered by prokaryotes. Prokaryotes can transfer only soluble compounds in and out of their cells. They also use minerals as a source of energy, trace nutrients and electron acceptors. Mineral dissolution exposes microorganisms to a wide range of soluble and potentially toxic metals.

There are numerous reports on first hand exposure as well as laboratory outcomes. A review of literature regarding different reports elaborating the status of metals has discussed here to get useful idea about them and find out their place on genotoxicity scale. A brief summary of their exposure and symptoms is given in Table 2.1.

2.3 Attributes of Mercury

Mercury, a transition metal, is called the mad hatter mineral. It was used in hat-making 150 years ago in America and those who worked with it became somewhat strange, or mad and thus so called mad hatter.

Mercury has extremely widespread applicability today and most people have at least some degree of mercury toxicity. Major sources are silver amalgam dental fillings, eating any fish (larger fish such as tuna and swordfish and Shellfish) or any seafood. Other sources include contact lens solution, fairness crèmes (Al–Saleh and Al–Doush 1997), soaps (Harada et al., 2001) many vaccines including flu shots, and a few other products like tooth whitening toothpaste etc (Per Enghag 2004).
Table 2.1 Exposure symptoms of metals used in present studies.

<table>
<thead>
<tr>
<th>Metal</th>
<th>Acute symptoms</th>
<th>Chronic symptoms</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chromium</td>
<td>GI hemorrhage, hemolysis, acute renal failure (Cr$^{6+}$ ingestion), nephrotoxins, skin allergy, inflammation of respiratory tract</td>
<td>Allergic dermatitis, cancer hazard, bronchogenic, carcinoma</td>
<td>OSHA (2006)</td>
</tr>
<tr>
<td>Lead</td>
<td>Nausea, vomiting, headache, seizure, increased pigmentation of skin, nephrotoxins, neurotoxins, increased blood pressure</td>
<td>Reproductive toxins, hypertension and ostopenia, convulsions, cardiovascular mortality, learning disability, behavioral problem, mental retardation, death, coma, encephalopathy, neuronal degeneration</td>
<td>OSHA (2006); Bagchia and Preussb (2005); Hodgson (2004)</td>
</tr>
<tr>
<td>Mercury</td>
<td>Fever, vomiting, diarrhea, gastroenteritis, increased pigmentation of skin, neurotoxins, nephrotoxin, disturbed vision</td>
<td>Nausea, metallic taste, hypersensitivity (Pink disease), inflammation, tremors, irritation, nephrotoxic</td>
<td>OSHA (2006); Gunnar et al., (2007); Hodgson (2004); Rizvi et al., (2005)</td>
</tr>
<tr>
<td>Lithium</td>
<td>Severe toxicity, giddiness, blurred vision, ringing in ear (tinnitus), severe shakiness, seizures</td>
<td>Mild toxicity, thirst, shakiness, vomiting, increased urination, coordination problems, diarrhea, drowsiness, muscle weakness</td>
<td>Monson and Schoenstadt (2008)</td>
</tr>
</tbody>
</table>
2.3.a Mercury cycle in atmosphere

Zahira et al., (2005) reported that with mercury contaminating the rain-, ground- and sea-water no one is safe. Polluted water leads to mercury laced fish, meat and vegetable. In aquatic environments, inorganic mercury is microbiologically transformed into lipophilic organic compound methyl mercury, from where it enters into clouds in vapor state and eventually water containing mercury come down to sea through rain. So mercury enters atmosphere through land and water. Thus, three forms of mercury are found in atmosphere: inorganic mercury, methyl mercury and mercury vapors. Figure 2.2 depicts mercury cycle operating in atmosphere.

Pirrone and Mason (2009) reported that a total of almost two thousand tonnes of mercury is emitted to the atmosphere each year from anthropogenic sources like fossil fuel burning and small-scale gold mining procedures. Natural sources of mercury such as oceans, rocks, volcanos and forest fires release about five thousand tonnes of mercury. About two third mercury emitted from natural processes has an anthropogenic origin as it is the recycled mercury that has previously been deposited from industrial sources through air or land.
2.3.b Effects on living organisms

1) Epidemiological studies

Chen et al., (2005) reported increased oxidative DNA damage, as assessed by urinary 8-hydroxy-2'-deoxyguanosine concentrations, and serum redox status in persons exposed to mercury.

Jarosińska et al., (2008) found that MCCA workers in Sweden had been highly exposed to mercury, as shown by very high mercury levels in urine (greater than 35μg/gC). Those men suffered early kidney damage. Exposure to low levels of mercury over an extended period of time combined with exposure to other toxic substances, such as lead and cadmium also remains a concern.

In an epidemiological study, Gibicar et al., (2009) observed the impact of mercury in the immediate vicinity of the chlor-alkali plants for mercury cell chlor-alkali (MCCA) process. It was found that the level of mercury in the atmosphere was increased in the range of 8.0–8.7 ng/m³ in populations living near plants. The impact on human health was investigated. Some workers at the plants were at higher risk along with those who consumed contaminated fish or had mercury dental fillings, otherwise the overall health risk was low.

Mutter et al., (2010) on the basis of existing circumstantial evidence that the inorganic mercury might be a part of pathology of Alzheimer’s disease (AD), conducted a systematic review using a comprehensive search strategy. Two reviewers independent of each other scrutinized, and selected 106 studies out of 1041 references fulfilling the inclusion criteria. Out of 40 studies, thirty two were found to have significant memory deficits in individuals exposed to inorganic mercury. Increased mercury levels in brain tissues were found in some autopsy studies of AD patients. Measurements of mercury levels in blood, urine, hair, nails, and cerebrospinal fluid were not correlating. All pathological changes as seen in AD, were reproduced in animal models and in vitro models with inorganic mercury. Inorganic mercury may promote neurodegenerative disorders via disruption of redox regulation due to its high affinity for selenium and selenoproteins. Its role as a cofactor in the
development of AD by inorganic mercury is suggested by increasing the pathological influence of other metals. Potential causal pathways are described in its mechanistic model. As a single most effective public health primary preventive measure the industrial and medical usage of mercury should be eliminated as soon as possible.

Pigatto et al., (2010) observed the three persons enrolled for study with persistent adverse events to the potential long-term immunotoxic effects of inorganic mercury released from mercury-containing dental amalgam fillings had asystemic antibody response to auto-antibodies to nuclear and nucleolar antigen (ANA and/ or ANoA). They also developed abnormal and damaging immune response possibly triggered by the chemical form of mercury vapor (Hg°) from inorganic mercury.

Soto-Rios et al., (2010) collected samples from people living in a mining area with exposure to inorganic mercury and found that having higher mercury levels in urine increases the risk of developing uroepithelial cytogenotoxicity.

2) Experimental studies (Toxic and mutagenic effects)

Benton et al., (2002) studied the genetic effects of mercury contamination on aquatic snail populations for allozyme genotypes and DNA strand breakage. Allozyme data and DNA strand break frequencies were compared among populations of *Pleurocera canaliculatum* from five sites with varying mercury contamination on the North Fork Holston River (NFHR) in southwestern Virginia, USA. There was significant variation in allozyme genotype frequencies for four loci between populations from the three most highly contaminated sites and those from two lesser contaminated sites. In addition, heterozygosity at three of these loci were significantly lower in the populations from the most highly contaminated sites. The DNA strand break frequency was significantly correlated to whole-body total mercury concentration in snails from three sites.

The effect of 4 metal salts on the induction of chromosome aberrations and sister chromatid exchange (SCE) in cultured chinese hamster ovary (CHO) cells was investigated. It was observed that CdCl₂, NiCl₂, CrO₃ and HgCl₂
were effective in causing various types of chromosome aberrations and abnormalities in dose dependant manner. These metal compounds also raised the SCE rates compared to 5-bromo-2'-deoxyuridine controls.

Sutton et al., (2002) assessed the cellular and molecular responses of human liver carcinoma cells following exposure to mercury. Cytotoxicity experiment yielded a LD$_{50}$ value of $3.5 \pm 0.6 \mu$g/mL upon 48 hours of exposure, indicating that mercury is highly toxic. Cytotoxicity and gene induction response was dose dependant. These results indicated the potential of mercury to undergo Phase II biotransformation in the liver (GSTYa), and to cause protein damage (HMTIIA, HSP70, and GRP78), cell proliferation (c-fos), metabolic perturbation (CRE), growth arrest, DNA damage (GADD153, GADD45), and apoptosis (p53RE). Thus mercury induced cytotoxicity and transcriptionally activated stress genes in human liver carcinoma (HepG2) cells.

In an experiment by Thier et al., (2003) mercury and lead salts were subjected to micronucleus (MN) assay and the CREST anti-kinetochore antibody analysis (CREST analysis) in V79 chinese hamster fibroblasts. It was found that the compounds were able to disturb the tubulin assembly in vitro. The MN induction (CREST analysis) indicated aneugenic effects of Pb(II) and Hg(II), along with additionally clastogenic effects of Hg(II).

Bonacker et al., (2004) found the interaction of mercury with the motor protein kinetin (mediates cellular transport processes) inhibited and altered microtubule assembly at 10pM concentrations in cell free systems in vitro. The resultant chromosomal genotoxicity and impaired chromosome distribution with increased micronuclei formation was evident in results of micronucleus test.

Stoiber et al., (2004) found V79 (hamster lung fibroblasts) when treated with inorganic mercury acted on microtubules and even amino acetic acid-like chelating agents (EGTA, EDTA, etc.) did not protect and caused micronucleus formation.
According to Silva-Pereira et al., (2005) the biotransformation of mercury chloride (HgCl₂) into methyl mercury chloride (CH₃HgCl) in aquatic environments is well-known and humans are exposed by consumption of contaminated fish, shellfish and algae. Thus methyl mercury affects not only the aquatic organisms but also sea food eaters. They determined the changes induced in vitro by two mercury compounds (HgCl₂ and CH₃HgCl) in cultured human lymphocytes. Genotoxicity was assessed by chromosome aberrations and polyploid cells. Methyl mercury is considered to be a potent mutagen. A significant increase (P<0.05) in the relative frequency of chromosome aberrations and frequency of polyploid cells was observed for all concentrations of CH₃HgCl when compared to control, whether alone or in combination with HgCl₂. Mitotic index was used as a measure of cytotoxicity. CH₃HgCl significantly decreased (P<0.05) the mitotic index at 100 and 1000 µg/l alone, and at 1, 10, 100, and 1000 µg/l when combined with HgCl₂, showing a synergistic cytotoxic effect. It showed that low concentrations of CH₃HgCl might be cytotoxic/genotoxic. Such effects may indicate early cellular changes with possible biological consequences and should be considered in the preliminary evaluation of the risks of populations exposed in vivo to low doses of mercury.

Zahira et al., (2005) also proposed the application of low doses of mercury for experimental purposes to have more relevance.

Rozgaj et al., (2006) found that mercury is a highly toxic element. It absorbs and deposits mainly in the kidney after inhalation or ingestion. The genotoxicity of mercury compounds was investigated with a variety of genetic endpoints in prokaryotic and eukaryotic cells. Results pointed to an inhibition of DNA synthesis, DNA damage, inhibition of spindle microtubule assembly, reduction in the frequency of mitosis, endo reduplication and chromosomal damages. Forty eight hours exposure resulted in increased frequency of micronuclei in micronucleus test of lymphocytes.

Maheswaran et al., (2008) reported that when Clarias batrachus (a fish) was exposed to 0.02-0.10 ppm of HgCl₂ for 35 days, the hemoglobin (Hb) content was decreased but the number of white blood cells (WBC’s) increased.
Carmona et al., (2008) reported that the mercury compounds tested exhibit a lack of genotoxic activity in the wing spot assay of *D. melanogaster*. These results contribute to increase the genotoxicity database on the *in vivo* evaluation of mercury compounds in *Drosophila*.

Arefieva et al., (2010) represented recent data about some properties of mercury, its natural occurrence, the actual mechanisms involved in the transport of mercuric ions inside the cells, its genotoxic, cytotoxic and organotoxic effects. They opined that due to high biological activity towards living organisms, the effect of mercuric compounds on human cells and organism is an object of thorough investigation. Thus the consequences of regular human and animal exposure to mercuric compounds must be investigated.

Durek et al., (2010) indicated that the presence of vitamins C and E at concentrations that are similar to the levels found in plasma could be able to ameliorate or decrease HgCl$_2$-induced oxidative stress by decreasing lipid peroxidation and altering antioxidant defense system in erythrocytes.

Dopp et al., (2011) investigated the cyto and genotoxicity of five volatile metalloid compounds of mercury, tin, arsenic and bismuth in methylated form and found di-methyl mercury to be most toxic in mammalian cells *in vitro*.

Boujbiha et al., (2012) selected erythrocytes as they were a convenient model to understand the subsequent oxidative deterioration of biological macromolecules in metal toxicities. Male rats were subjected to subchronic exposure of mercuric chloride via drinking water for 90 days daily. The possible association of the variation of hematoxic and genotoxic parameters with oxidative stress at 50 ppm (HG1) and 100 ppm (HG2) of mercuric chloride was examined. A significant dose-dependent decrease was observed in red blood cell count, hemoglobin, hematocrit, and mean cell hemoglobin concentration in treated groups (HG1 and HG2) compared with controls. A significant dose-dependent increase was observed in lipid peroxidation; along with significant variation in the antioxidant enzyme activities, such as superoxide dismutase, catalase, and glutathione peroxidase. A significant dose-dependent increase in frequency of total chromosomal aberrations and
aberrant bone marrow metaphases following mercuric chloride treatment was also observed. The major cause for chromosomal aberration may be the oxidative stress induced by mercury treatment as free radicals lead to DNA damage.

Turkez and Dirican (2012) assessed the cytogenetic effects of mercuric chloride (HgCl₂) and the role of aqueous Dermatocarpon intestiniforme lichen extracts in mercury-treated human blood cultures (n=3). The sister chromatid exchange (SCE) and micronucleus (MN) assays were performed to assess DNA damages in lymphocytes. The results clearly revealed that the SCE and MN rates induced by HgCl₂ were alleviated by the presence of D. intestiniforme. In conclusion, the results revealed for the first time that the lichen D. intestiniforme provided increased resistance of DNA against HgCl₂-induced genetic damage on human lymphocytes.

Patra and Sharma (2000) compiled the effect of mercury compounds on seed germination, elongation, growth, development, biochemical disturbances and genetic effects. It concluded that mercury caused multiple effects at low doses.

Mercury, lead and arsenic were effective mitotic poisons (turbagens) at particular concentrations, due to their known affinity for thiol groups and induced various types of spindle disturbances. The availability of cat ions affect the number of aberrations produced quantitatively. Effects of metallic salts were related directly to the dosage and duration of exposure. Plants, following lower exposure, regained normalcy on being allowed to recover. Thus genotoxic effects could be in part responsible for metal phytotoxicity. The most noticeable and consistent effect of mercurials was the induction of c-mitosis resulting in the formation of polyploid, aneuploid cells, and c-tumours. Inorganic salts of lead induced numerous c-mitosis together with strong inhibition of root growth and lowering of mitotic activity.

2.4 Attributes of Chromium

According to Wilson (2007) chromium (III), the transition metal, is called the blood sugar mineral. It is also an energy mineral. A desert rodent called the
sand rat develops diabetes when fed a laboratory diet as it contains no chromium. When returned to the desert, the diabetes goes away. Thus chromium could in some way help prevent diabetes.

Chromium is essential for insulin metabolism. It can also help lower cholesterol. Chromium deficiency is very common, especially in middle-aged and older people. Food sources of chromium are brewer’s yeast, liver, kidney, beef, whole wheat bread, wheat germ, beets, mushrooms and beer. Unfortunately, most of these foods are not recommended for various reasons with ethical reasons attached to it.

2.4.a Effects on living organisms

1) Epidemiological studies

t’Mannetje et al., (2003) in a lung cancer case-control study conducted in Central/Eastern Europe and UK including 2863 cases and 3005 controls recorded the following observations. From all the subjects, detailed smoking information was collected and exposure to 70 occupational agents was also assessed by local expert-teams. Risks for exposure to dust and mist/fumes of chromium, nickel, cadmium, arsenic, inorganic pigment dust and inorganic acid mist were analyzed adjusting for smoking, age, centre, sex and exposure to other occupational agents including metals. It concluded that in above mentioned population 11% were exposed to metals with an attributable risk for lung cancer of 3.7%. Although the strongest risk was observed for arsenic, exposure to chromium dust was most important in terms of attributable risk due to its high occurrence.

Hall et al., (2005) reported that the highest concentration occurred during the welding operations inside large stainless steel pipes (0.26mg/m³ and 0.36mg/m³) and welding fins on a large stainless steel pipes.

Nurminen (2005) reviewed evaluations and studies on the carcinogenicity in humans of metallic chromium and trivalent chromium as exposed in leather tanning, chromium plating and processing units. The evaluations of the potential carcinogenicity of metallic chromium and trivalent chromium by international and national organizations and individual scientists are
unanimous in that the evidence of carcinogenicity is inadequate in humans. For some occupational sources of chromium exposure (e.g. ferrochrome industry and manufacture of chrome pigments) and for some occupations (e.g. leather tanners, painters and chrome platers) there are increased risks, but in most epidemiologic studies the available data does not permit discrimination between simultaneous exposure to trivalent and hexavalent chromium. So far, the type of chromium compound that increases the risk of lung cancer and sinonasal cancer has not been identified but there is general agreement that hexavalent species are responsible for these diseases than the trivalent and metallic species. No consistent pattern of cancer risk has been demonstrated in workers exposed to chromium compounds for cancers other than those of the lungs and sinonasal cavity.

Occupational Safety and Health Administration (OSHA 2006) found occupational exposure to chromium from inhalation of dust, fumes and through dermal contact. The exposure occurs in tanning industries, welding and electroplating units and where pesticides are used.

Epidemiological studies by Halasova et al., (2010) have also indicated that workers occupationally exposed to hexavalent chromium compounds have a high susceptibility to cancers of the respiratory system.

2) Experimental studies (Toxic and genotoxic effects)

Lantzsch and Gebel (1997) reported the genotoxicity of Cr(VI) with tester strain *Escherichia coli* PQ37. A moderate genotoxicity was shown by the two Cr(VI) compounds K₂CrO₄ and K₂Cr₂O₇.

Costa (1997) had proposed the occurrence of liver and kidney cancer than lung cancers in human and animal models because chromium compounds are taken up by the gastrointestinal tract and penetrates to many tissues and organs.

Proctor et al., (2002) asserted that the weight of scientific evidence supports that Cr(VI) is not carcinogenic in humans via the oral route of exposure at permissible drinking-water concentrations as hexavalent chromium is reduced
to trivalent form in gut. However, Zhitkovich (2011) stated that the chromium that escapes detoxification in gut may elicit cancer.

Blasiak and Kowalik (2000) compared the effects of tri- and hexavalent chromium on the DNA damage and repair in human lymphocytes using the alkaline single cell gel electrophoresis (comet assay). There was increase in comet tail moment with potassium dichromate meaning thereby it induced DNA damage in the lymphocytes. The effect was dose-dependent. Treated cells were able to recover within 120-min incubation. Cr(III) caused greater DNA migration than Cr(VI). The lymphocytes did not show measurable DNA repair. Vitamin C at 50 microM reduced the extent of DNA migration. This was either due to a decrease in DNA strand breaks and/or alkali labile sites induced by Cr(VI) or to the formation of DNA crosslinks by Cr(VI) in the presence of vitamin C. Vitamin C, however, did not modify the effects of Cr(III). Sgs1 helicase were found to be involved in processing of DNA-double strand breaks for recombination repair.

For chromium, the oxidation state is most important for its biochemical activity. Chromium(VI) compounds have been shown to exert genotoxicity both in vivo and in vitro. Lymphocytes of workers exposed to dusts of chromium(VI) compounds showed elevated frequencies of sister-chromatid exchanges (Wu et al., 2001) and DNA strand breaks (Gambelunghe et al., 2003).

Quievryn et al., (2003) examined the mutational spectrum and the importance of different forms of DNA damage in genotoxicity and mutagenicity of Cr(VI) upon activation by physiological concentrations of ascorbate in human and bacterial cells. The yield of replicated plasmids is normalized, if there was disruption of Cr-DNA binding and it abolished mutagenic responses indicating that Cr-DNA adducts were responsible for both mutagenicity and genotoxicity of Cr(VI). Ascorbate Cr(VI) DNA links were much more mutagenic than smaller (III) adducts inhibiting replication.

Paustenbach et al., (2003) found that under all conditions no harmful effects were caused on RBC in vivo at the concentrations of 10 mg /ml. This
concentration was well above the actual concentrations of Cr(VI) found in tap water of U.S.

Xie et al., (2004) found that exposure to particulate lead chromate was clastogenic in a concentration-dependent manner with 0.1, 0.5, and 1 µg/cm² in a human lung cell lines, while complete cell cycle arrest was caused by 5 and 10 µg/cm². They also found concentration-dependent increases in intracellular and extracellular chromium ion levels. Transmission electron microscopy (TEM) was used to investigate particle internalization and found an apparent relative increase with concentration but no apparent particle internalization at the lowest concentration (0.1µg/cm²) even after 24h. There was no lysosomal association with the vacuoles containing particles, suggesting that intracellular dissolution did not occur. Co treating the cells with lead chromate and Vitamin C eliminated both the uptake of ionic chromium and the clastogenic activity of lead chromate but had no effect on particle internalization.

De Flora et al., (2006) found that micronuclei were induced in bone marrow after intraperitoneal injection of chromate(VI) to mice. In comparison to chromate(VI), chromium(III) compounds did not induce genotoxic effects in the majority of studies with intact cells.

Costa and Klein (2006) reported that apart from lung cancer, hexavalent chromium also causes cancer of gastro intestinal and central nervous system. Hexavalent chromium causes increased risk of bone, prostate, lymphomas, leukemia, stomach, renal and bladder cancer. They highlighted the most recent data on the induction of skin tumors in mice by chronic drinking-water exposure to hexavalent chromium in combination with solar ultraviolet light.

Zecevic et al., (2009) examined the importance of WRN helicase in repair of G2-specific double strand break (DSB) caused by abnormal mismatch repair (MMR) of ternary Cr-DNA adducts. They found that Cr(VI) induced rapid dispersal of WRN from nucleolus and as a result it is retained longer in nucleoplasm. WRN is a protein mutated in Werner syndrome containing both helicase and exonuclease activities. WRN-deficient fibroblasts were hypersensitive to (CrVI)-induced clonogenic death with persistant high level of
DNA damage and were saved by the inactivation of mismatch repair protein (MMR). This suggested that MMR-generated DSB were a key substrate for WRN action in Cr(VI) treated cells. It showed the importance of WRN helicase in repair of DNA double-strand breaks.

Figgitt et al., (2010) investigated the comparison between in vitro human fibroblasts chromosomal aberrations at the same concentration as found in the peripheral blood of exposed humans with Cr(III), Cr(VI), Co(II) and Cr in combination with Co. They found that at physiological doses the metals induced predominantly numerical (aneugenic) rather than structural aberrations and concluded that these metal ions could cause chromosome aberrations at physiological concentrations with Cr and Co together producing the maximum effect.

Wise et al., (2010a) conducted study to compare the genotoxicity of zinc chromate with two other particulate Cr(VI) compounds, barium chromate and lead chromate, and one soluble Cr(VI) compound, sodium chromate. The clastogenic effects of barium chromate and zinc chromate were more than that of lead chromate. The three particulate chromium compounds had similar response to the levels of DNA damage measured by gamma-H2A.X foci formation. It was concluded that zinc chromate and barium chromate were the most cytotoxic and lead chromate and sodium chromate were less cytotoxic. Zinc chromate was more clastogenic than all other chromium compounds and lead chromate was the least clastogenic. All compounds induce DNA double strand breaks without any significant difference amongst them. All together, data suggested that the difference in the carcinogenic potency of zinc chromate over the other chromium compounds was not due solely to a difference in chromium ion uptake as chromate ion is equally present in all and the zinc cat ion may in fact have an important role in its carcinogenicity. Epidemiology and animal studies also suggest that zinc chromate is the most potent particulate Cr(VI) compound.

Wise et al., (2010b) compared soluble and particulate chromate cytotoxicity and genotoxicity in human (Homo sapiens) and sea lion (Eumetopias jubatus) lung fibroblasts. It was seen that hexavalent chromium induced increased cell
death and chromosome damage in both human and sea lion cells with increasing intracellular chromium ion levels.

In a study by Mishra et al., (2011) mutagen sensitive strains (mus) in Drosophila that are known for their hypersensitivity to mutagens and environmental carcinogens. These mutants were grouped in pre- and post-replication repair pathways. DNA damage was observed with comet assay. The damage was greater in pre-replication repair mutants after exposure to 5.0μg/ml Cr(VI)), while effects on Oregon R’ and post replication repair mutants were insignificant. Post-replication repair mutants revealed significant DNA damage after exposure to 20.0 μg/ml Cr(VI). Cr(III) (20.0 μg/ml) exposure to these strains did not induce any significant DNA damage in their cells. The study suggested that both pre- and post-replication pathways were affected in Drosophila by Cr(VI). High concentration was required to show effect in post replication repair mutants leading to genotoxicity, which may have consequences for metal-induced carcinogenesis.

Ray and Sarkar (2012) reported that Cr6+ affects erythrocytes by reducing their counts but did not hamper iron absorption in swiss mice. Ahmad et al., (2011) showed that K2Cr2O7 induced oxidative stress (lipid peroxidation, protein oxidation) and alters the antioxidant defense mechanism of human erythrocytes.

It has recently been shown by Stein (2012) that chromium, forms DNA adducts.

Qian (2004) observed the mutagenic effects of chromium trioxide on root tip cells of Vicia faba. The results showed that the effects of CrO3 concentration on the mitotic indices were complicated. CrO3 increased the micronucleus aberration rate of Vicia faba root tip cell but at lower dose only. CrO3 also caused various types of chromosome aberrations at a rate which increased systematically with increased concentration of CrO3 concluding that CrO3 has significant mutagenic effect on Vicia faba root tip cells.
2.5 Attributes of Lead

Lead (Orphan metal) is called the dullness and horror mineral. It may contribute to over 100 human conditions, including neuromuscular and bone diseases, fractures, mental retardation, nervousness and mental depression hyperactivity, anemia, kidney damage and many others. Right from ancient times lead has been used in plumbing systems, utensils, and pottery glazes. Some historians believe the Roman Empire fell because lead water pipes slowly poisoned the people and decreased their intelligence. Sources of lead include old paint, inks, pesticides, a few hair dyes, solder and other metal products.

2.5.a Effects on living organisms

1) Epidemiological studies

Needleman (2003) reported that the availability of more sensitive analytic methods has made it possible to measure lead at much lower concentrations. The removal of lead from gasoline has dramatically reduced the amount of lead in the biosphere. If the remaining lead is also removed, the benefits would be far reaching.

Hengstler et al., (2003) determined co-exposure to cadmium, cobalt, lead and other heavy metals that occur in many occupational settings. The mononuclear blood cells of 78 individuals co-exposed to cadmium, cobalt and lead were tested for DNA single strand break (DNA-SSB) induction and repair capacity for 8-oxoguanine. It was found that even at low concentrations of individual heavy metals (< TRK values) individuals co-exposed to cadmium or lead experienced genotoxic effects.

A review article by Gidlow (2004) reported that lead acted as abortifacient (inducing abortions) in women. He found lead toxicity to be related to the environmental as well as occupational exposure with advances made for their control. The occupational exposure to lead combined with other toxic chemicals can be the reason for different results in the workers. At this stage the effects are seen with demolition and tank cleaning industries. Currently,
the focus of attention is on the subclinical effects of exposure with pressure to reduce it.

Wu et al., (2012) aimed to investigate the association between lead exposure and peripheral white blood cell telomere length (PWBTL) in Chinese battery manufacturing plant workers. Their findings suggested that PWBTL shortening is associated with long-term lead exposure and that PWBTL may be one of the targets damaged by lead toxicity.

2) Experimental studies (Toxic and mutagenic effects)

Devi et al., (2000) studied lead nitrate induced single stranded DNA breakage in mice in vivo using alkaline single cell gel electrophoresis (comet assay). Significant increase in mean tail-length of DNA was observed though it was not dose dependent.

Shaik et al., (2006) took human model and studied the cytotoxic and genotoxic effects of lead nitrate by using lymphocytes from human peripheral blood in vitro. Chromosomal aberration frequency did not show significant aberrations except for some aneuploidy and about 2–4% gaps, breaks (3–4%), and about 5% satellite associations. Pb can induce single-strand DNA breaks, possibly by competing with metal binding sites as significant DNA damage was determined by SCGE (Comet assay). The comet tail length proportionately increased with increasing lead nitrate concentration.

Su Yu et al., (2006) observed that carcinogenic metals reported positive in mammalian system operate by induction of oxidative DNA damage e.g. chromium compounds interact with DNA repair process leading to an enhancement of genotoxicity in combination with a variety of DNA damaging agents. With Pb(II) the repair processes is disturbed at very low doses.

Leandro dos Santos et al., (2010) tested the drugs used to treat sickle cell anemia containing lead. The results were positive for mutagenicity with Ames test.

Haq et al., (2011) reported the adverse effects of lead acetate on the morphological changes of larvae and adult flies of Drosophila melanogaster.
Carmona et al., (2011) performed wing-spot test and the comet assay on *Drosophila melanogaster* by taking two inorganic lead compounds namely lead chloride and lead nitrate. It was found that they were not able to induce significant increases in the frequency of mutant spots. It also seemed that the lead compounds tested do not interact with the repair of the genetic damage even when induced by ionizing radiations. *in vivo* comet assay reported significant increases of DNA damage with a direct dose-response pattern.

Ahmed et al., (2012) investigated the toxic effect of prolonged lead acetate exposure on the chromosomal and testicular tissue of twenty male rabbits. There was statistically significant (p<0.01) increase in the number of abnormal sperms, and chromosomal abnormalities. The histopathological examination of the testicular tissues showed that spermatogonia and spermatocytes had degenerated. Spermatogonia and sertoli cells also showed advanced degeneration and vacuolation with pyknotisis and necrosis. In conclusion, lead acetate had genotoxic and cytotoxic effect in male rabbit and may cause infertility.

Review articles by García-Lestón et al., (2010, 2012) stated that exposure to lead, both in environmental and occupational settings, is frequently associated with an increase in genotoxic damage in humans.

Malinowskaa et al., (2004) found lead to be accumulated in fruiting bodies of mushrooms bay bolete, *Xerocomus badius*.

Kumar and Tripathi (2008) tested lead nitrate on the meiotic cells of grass pea (*Lathyrus sativus*) and found to be causing abnormalities like condensed bivalents, secondary association, laggards, bridges, cytomixis and stickiness etc.

Gichner et al., (2008) treated Tobacco (*Nicotiana tabacum* L. var, *xanthi*) seedlings with aqueous solutions of lead nitrate (Pb$^{2+}$) at varying concentrations. The DNA damage measured by the comet assay was high in the root nuclei. In tobacco plants, growing for six weeks in soil polluted with Pb$^{2+}$ had severe toxic effects, expressed by the decrease in leaf area and a slight but significant increase in DNA damage was observed. The plants were
severely injured and showed stunted growth, distorted leaves and brown root tips. But mutations in tobacco plants growing in the Pb$^{2+}$ polluted soil did not significantly increase. Analytical studies found the accumulation of the heavy metal was forty fold higher in the roots than in the above ground biomass explained the lower levels or the absence of Pb$^{2+}$ induced DNA damage in leaves.

Shahid et al., (2011) found that when Vicia faba seedlings were exposed for 6 h in controlled hydroponic conditions to 51$^{1/4}$ M of lead nitrate alone and chelated to varying degrees by different organic ligands. The genotoxicity decreased in the presence of chelated agent like EDTA as shown with micronucleus test even with increase in the measure of lead uptake by plants.

Pourrut et al., (2011) reported that lead has many interesting physio-chemical properties that make it a very useful heavy metal. Despite its lack of essential function in plants, lead is absorbed mainly through the roots from soil solution and thereby may enter the food chain. After uptake, lead primarily accumulates in root cells and trapped by the negative charges that exist on roots cell walls. Excessive lead accumulation causes phytotoxicity by changing cell membrane permeability, reacting with active groups of different enzymes involved in plant metabolism, phosphate groups of ADP or ATP (adenosine di-phosphate and tri-phosphate and by replacing essential ions. Lead toxicity causes inhibition of ATP production, lipid peroxidation and DNA damage by the over production of reactive oxygen species (ROS). In addition, stomatal closure by lead strongly inhibits seed germination, root elongation, seedling development plant growth, transpiration, chlorophyll production, and water and protein content due to distortion of chloroplast ultrastructure, obstructed electron transport, inhibition of Calvin cycle enzymes, impaired uptake of essential elements such as Mg and Fe and induced deficiency of CO$_2$.

Truta et al., (2011) observed the changes induced in cytogenetic parameters from root meristems of Triticum aestivum cv. Maruca seedlings after treatment with lead acetate and lead nitrate solutions, at four concentrations (10-100μM) containing 2.07, 5.18, 10.36, respectively 20.72μg ml$^{-1}$ Pb$^{2+}$. Lead induced
mitotic disturbances in root meristematic cells of wheat seedlings. There was decrease of mitotic index and changes in prevalence of division phases. This heavy metal has genotoxic effects as in all Pb²⁺ treated variants, many chromosomal aberrations were expressed. Pb²⁺ nitrate shows a more distinct genotoxic potential than lead acetate trihydrate.

2.6 Attributes of Lithium

Lithium, the alkali metal, is the brain protection mineral. It has a calming, balancing and protective effect on the brain and the entire nervous system. It is found in many innate foods so it does not require any supplementation. A natural lithium supplement such as lithium orotate is for the benefit of anyone who is taking an anti-depressant or any brain-altering drug, or suffering from any brain-related problem. The lithium used by medical doctors for bipolar disorder is quite toxic and must be avoided. The natural product is far less potent and is better absorbed and assimilated and totally non-toxic. Lithium is not a dietary mineral for plants but it does stimulate plant growth.

2.6.a Effects on living organisms

1) Epidemiological studies

Chmielnicka and Nasiadek (2003) found that lithium salts induced renal toxicological symptoms.

A review by Aral and Vecchio-Sadus (2008) has indicated that lithium battery disposal sites (NEMA 2001) and lithium rich brines and minerals (Moore 2007) along with mining sites (Aral 2007) obviously contained higher than normal concentration of lithium. Lithium is not expected to bioaccumulate in human and also the environmental toxicity is low. Information on teratogenic (affecting fetus) effect is contradictory. The reproductive effects of lithium are highly unlikely in an occupational setting but those being treated for manic-depressive disorders may be at risk. There is no worthwhile report on mutagenic potential for the last 15 years or so. Neither lithium intake from food and water nor from occupational exposure presents a toxicological hazard. It rather stimulates plant growth. Material safety data sheets (MSDS) released by Chemwatch (2004) do not have an upper exposure limit.
However, CDC (2007) and EPA (2005) have quoted some upper exposure limits as there is growing concern of the safety and health of workers in battery manufacturing units. Australian Inventory of Chemical Substances (AICS 2007) has classified lithium as health, physiochemical and ecotoxicological hazard. It attacks central nervous system and kidneys.

2) Experimental studies (Toxic and mutagenic effects)

Huang and Jianfu (2000) took lithium chloride test substance in human peripheral blood lymphocytes and Kunming mice as subjects. They performed lymphocyte sister chromatid exchange (SCE) test, Kunming mice somatic cell genotoxicity test and tissue LPO, GSH-Px and SOD determination. The results showed lithium chloride exposure in vitro cultured human peripheral blood lymphocytes increased the SCE rate (P <0.01), oral gavage mouse bone marrow cell chromosome aberration rate, the bone marrow micronucleus rate, fetal liver metastases and micronucleus rate. However, the mouse heart and ovarian SOD activity decreased (P <0.01). They, therefore, concluded that certain dose of lithium chloride on human peripheral blood lymphocyte chromosome and somatic cells in mice with a genetic injury and suggested that it may stimulate free radicals generated on the cell damage.

Repetto et al., (2001) observed that lithium at low toxicity was able to cause alterations in different tissues. The toxic effects of lithium were assessed in mouse neuroblastoma cell cultures (Neuro-2a) using two inorganic chemical species: lithium chloride and nickel(II)chloride. The cytotoxic effects evaluated were cell proliferation by quantification of total protein content, cytoplasmic membrane integrity to cytosolic lactate dehydrogenase leakage, and lysosomal hexosaminidase release. Lithium stimulated nearly all the indicators studied, particularly lactate dehydrogenase, mitochondrial succinate dehydrogenase and acetylcholinesterase activities, as well as hexosaminidase release. Lithium also showed that functional metabolic alterations are more important than cytoplasmic damage.

Phiel and Klein (2001) reviewed the cause and effect of lithium and showed that at therapeutic levels of lithium, the functioning of multiple enzymes in the body is clearly inhibited. Lithium, as a medicine, has multiple effects on
embryonic development, glycogen synthesis, hematopoiesis (the formation and development of blood cells involving both proliferation and differentiation from stem cells), and other physiological processes.

Allagui et al., (2006) in their experiments with rats found toxic changes in growth rate and thyroid- and sex-hormones blood levels under sub-chronic lithium treatment.

Freeman and Freeman (2006) found that lithium is excreted primarily through the kidneys after approximately 24h of administration. The effective dose for most of the patients causes toxicity. The therapeutic serum concentrations (Jaeger 2003) are normally about 5.6–8.4 mg/L, mild toxicity is usually seen at about 10.5–17.5 mg/L, moderate toxicity at about 17.5–24.5 mg/L and severe symptoms are seen at >24.5 mg/L.

Large doses of lithium (up to 10 mg/L in serum) are given to patients with bipolar disorder, lithium poisoning occurs at 10 mg/L of blood. Adverse effects like confusion and speech impairment occur at 15 mg/L and at 20 mg/L dose poses risk of death. A provisional recommended daily intake of 14.3 μg/kg body weight lithium for an adult has been suggested by Aral and Vecchio-Sadus (2008). Lithium compounds are not significantly clastogenic (capable of causing breakage of chromosomes) and, mutagenic activity is doubtful based on studies on microorganisms.

Pastor et al., (2009) observed the cytotoxicity and mitotic alterations induced by non-genotoxic lithium salts in chinese hamster ovary (CHO) cells in vitro.

In a study by Nciri et al., (2009) male mice were taken to study the effects of lithium carbonate administration on some biochemical parameters. Lithium carbonate (20, 40, or 80 mg/kg body weight corresponding to 3.77, 7.54, or 15.08 mg Li element/kg body weight, respectively) was injected daily for 14 or 28 days. They recorded: drinking water consumption, body weight, lithium and testosterone serum concentrations, activities of catalase (CAT), superoxide-dismutase (SOD), and glutathione-peroxidase (GPX). The level of lipid peroxidation (expressed as TBARS) in liver was determined. Lithium treatment, especially at the highest dose for 28 days, was found to induce
weight gain and polydipsia and a significant decrease of plasma testosterone level. Lipid peroxidation level and activities of SOD and GPX were increased in liver, which suggested a perturbation of the antioxidative status. The results indicated that weight gain, polydipsia under experimental conditions, damage to the male reproductive system and triggered oxidative stress in the liver were all due to sub chronic exposure to lithium to male mice.

The fasting of Muslims during the month of Ramadan was observed by Farooq et al., (2010) for possible lithium requirements. They found that the mood changes in patients suffering from bipolar affective disorder is related to serum lithium levels, along with side effects and toxicity.

Overall the effect of lithium for patients undergoing lithium therapy is hazardous and not at occupational or environmental level.

2.7 Literature based classification of metals under various agencies:

Under ICMM (2007 a) directives, mutagens are classified and kept in one of three categories and decisions leading to inclusion into these categories can be based upon the demonstration of genotoxic and mutagenic changes.

**Category 1** mutagens are substances known to be mutagenic to man. To place a substance in category 1, positive evidence from human epidemiology studies documenting mutagenic effects is needed. Till date, no substance has been classified as a Category 1 mutagen.

**Category 2** mutagens are substances that should be regarded as if they are mutagenic to man and are shown to be mutagenic to animals. Substances are placed into this category based upon evidence that supports a strong presumption that human exposure to the substance may result in the development of heritable genetic damage. For them, *in vivo* germ cell mutagenesis assays can be applied. Alternatively, *in vivo* assays like sister chromatid exchanges (SCE’s), unscheduled DNA synthesis (UDS), covalent binding of a substance to DNA or assaying other types of DNA damage (e.g. DNA strand breaks) can predict the results.
**Category 3** mutagens are substances that cause concern for man owing to possible mutagenic effects. Evidence will be available from mutagenicity studies, but is insufficient for placement in category 2. Such substances are identified based upon positive results from *in vivo* assays that demonstrate either mutagenic effects in somatic cells or other cellular interactions relevant to mutagenicity in mammals. The effect can be detected with *in vivo* somatic cell mutagenicity assays that include the bone marrow micronucleus test, metaphase analysis of peripheral lymphocytes, or the mouse coat colour spot test. To determine whether a substance is capable of interacting with the DNA of somatic cells is provided by genotoxicity tests for SCE’s, UDS, covalent binding to DNA, or other forms of DNA damage. Supporting data from *in vitro* mutagenicity assays assist in assigning this classification.

Based on these directives and a thorough review of literature regarding information on the tests conducted, it can be said that

a) Mercuric compounds, chromium compounds and lead compounds may be placed in Category 2 and

b) Lithium compounds in Category 3

According to International Agency for Research on Cancer (IARC 2009) knowing the causes of human cancer and its prevention is one of the priority areas today. The global burden of cancer is high and continues to increase. The annual number of new cases was estimated at 10.1 million in 2000 and is expected to reach 15 million by 2020 (Stewart and Kleihues 2003). The monographs published by the agency emphasized hazard identification, along with dose–response assessment which is possible in epidemiological and experimental studies to evaluate a cancer hazard. These monographs are used by national and international authorities to make risk assessments, formulate decisions concerning preventive measures, provide effective cancer control programmes and providing other relevant information.

### 2.8 Guidelines for selecting test systems

According to Environmental Protection Agency (EPA 2005) guidelines, recommendations for standard tests for gene mutations in bacteria and
mammalian cells and for structural chromosomal aberrations both in vitro and in vivo are important examples of relevant methods. New molecular approaches, such as mouse mutations and cancer transgenic models, are providing a means to examine mutation at tissue sites where the tumor response is observed.

2.9.a Test systems commonly applied in the study of metals and metal compounds (ICMM 2007 a)

It is actually the metal compounds with high water solubility for which the majority of test data is generated and thus more convenient to use in the conduct of testing. They need not be the substances of commercial importance and if inferences are to be drawn regarding the properties of poorly soluble substances or the metal itself than extrapolation is required. Assessments of the mutagenic properties of metals and their compounds are typically first based upon in vitro testing, with follow-up work using in vivo testing as appropriate. A variety of endpoints can be assessed in vivo or in vitro, but are not equivalent with respect to assessment of mutagenic potential meaning thereby that the results are to be considered separately. The sensitivity and specificity of individual assays varies with different classes of compounds and should be considered when evaluating the significance of data from each assay type.

**Mutagenicity Assays**

1) Gene mutation assays

2) Germ cell alterations

3) *in vitro* mutagenicity tests are more commonly applied in the initial assessment of potential mutagenic properties. The most common test systems studies include cultured bacteria or mammalian cells. Properly conducted assays employ both positive and negative controls, include metabolic activation preparations, evaluate the impact of multiple concentrations of the test substance, and seek to define dose-dependent increases in mutation frequency in the absence of high levels of cytotoxicity.
Dose dependent induction of mammalian cell mutations in the absence of high levels of cytotoxicity is regarded to provide the most relevant indication of mutagenic potential that can be obtained from in vitro studies. The acceptable level of cytotoxicity associated with mutagenic response varies with the assay employed but is typically in the order of 50% or less.

The most commonly used strains of Salmonella have poor sensitivity to genetic changes associated with the action of oxygen radicals which are in turn suspected to be the indirect mediators of genotoxicity for some metals. False negative responses will thus result if the reverse mutation assay has limited sensitivity to the spectrum of genetic changes that occur as a result of exposure to metal compounds.

Mutagenicity studies with mammalian cells are usually forward mutation assays that assess the ability of a substance to inactivate a cell enzyme such as thymidine kinase (TK) or hypoxanthine phosphoribosyl transferase (HPRT).

Genetically modified test systems are sometimes used to assess the mutagenic potential of substances suspected to primarily cause large deletions in DNA. The cells used in such test systems contain a bacterial gene (e.g. guanine-hypoxanthine phosphoribosyl transferase or gpt). While genetically modified cells may have sensitivity to a broader subset of genetic alterations, assays using such cells have been only recently developed and have not been as extensively validated as traditional forward mutation assays and assay specificity, sensitivity remains to be determined.

**Chromosomal Changes**

1) Alterations in DNA sequence also result from changes in the structure of chromosomes and alterations in the amount of DNA (breaks) from changes in the number of chromosomes (aneuploidy). Using cells exposed in vitro, or cells (e.g., bone marrow from animals or peripheral blood lymphocytes from humans) harvested and placed in cell culture after in vivo exposure, metaphase chromosomes can be routinely screened for numerical and structural chromosome aberrations (CA). They include:-
a) Chromatid-type aberrations, which affect only one chromatid and

b) Chromosome-type aberrations

Chromosome-type aberrations represent unrepaired or incompletely repaired double strand breaks. Agents that produce other DNA lesions (adducts, crosslinks, pyrimidine dimers and single strand breaks) produce chromatid-type aberrations and these are DNA replication-dependant and form strands in vitro during S-phase. Lesions can also result from cellular events such as topoisomerase action or repair of endogenous lesions. Although they are induced by different mechanisms, both types are reported to be associated with increased cancer risk although the correlation with chromosome-type lesions may be stronger (Hagmar et al., 2004).

2) The in vitro micronucleus (MN) assay was developed as a short-term screening assay for animal and human genotoxic exposures and cytogenetic effects.

Genotoxicity tests providing indications of potential mutagenicity

1) Sister Chromatid Exchange

2) Single Cell Electrophoresis (COMET assay)

2.9.b Recommendations for a mutagenicity testing strategy for metals and metal compounds (ICMM 2007b)

Current base set strategies for genotoxicity testing start with assays for bacterial mutagenesis.

1) Bacterial mutagenicity tests like Ames Salmonella mutagenicity assay appear to have little utility for the testing of metals (test results are almost always negative) and should be replaced with mammalian cell test systems. However, the indirect mechanisms suspected to mediate metal genotoxicity may induce large deletions for which the sensitivity of gene mutation assays such or forward mutation at the HPRT locus may be limited.

Although Onnis-Hayden et al., (2009) recommended prokaryotic real-time gene expression profiling for toxicity assessment using Escherichia coli for
environmental pollutants in water samples. It uses a cell-array library of 93 *E. coli* K12 strains with transcriptional green fluorescent protein (GFP) fusions covering most known stress response genes.

2) The mouse lymphoma test system, assaying mutations at the thymidine kinase gene, has been suggested to be sensitive to both types of lesions.

3) Genetically engineered cell lines with bacterial gene inserts (such as the gpt gene) appear capable of detecting both large and small scale mutagenic events that inactivate the gpt gene. These newer test systems are not, however, yet validated or extensively characterized with respect to assay sensitivity or specificity. Tests for chromosome aberrations or micronucleus induction are currently recommended as part of base set testing and appear to be sensitive to the effects of metals.

4) Assays for micronucleus induction

The proposed scheme would conduct *in vitro* testing for gene mutations and chromosomal changes/micronucleus induction. There is high prevalence of positive *in vitro* data for metals, with the theoretical premise that metal ions will bind to multiple cellular targets and induce genetic changes via indirect mechanisms. Based upon both assumptions an *in vivo* follow-up strategy is thus defined which can, on a case by case basis, be used to assess the relevance of mutagenic potential that may be suggested by *in vitro* testing. Consideration may also be given to the prioritization of follow-up in accordance with the relevance of the concentrations required to produce effects *in vitro* to the concentrations of metal that can be tolerated by *in vivo* systems. For example, if μM concentrations of a metal are the maximum that can be tolerated *in vivo* then priority might be assigned to evaluated effects induced by μM (as opposed to mM) concentrations *in vitro*.

The above recommendations with regard to testing chemicals discourage the use of bacterial assays particularly the Ames assay as it can detect only specific type of mutagens.
Therefore we selected Rec assay as well as SOS Chromotest for their ability to test wide range of chemicals among the prokaryotic and lipid peroxidation, DNA adduct and alkaline unwinding among the eukaryotic assays.

The following section contains a separate review on reports containing an important and meaningful data regarding principles, mechanisms and applications of test assays. Results with different types of chemicals, organic or inorganic along with metal compounds tested in present studies with these systems are analyzed. All the assays reported sufficient efficacy and output in dealing with the test chemicals.

2.10 Rec assay

2.10.a Principle of the Rec assay

According to Mathews et al., (2000) bacteria carrying mutations in the recA gene are defective in general recombination and DNA repair. Thus these bacteria are deficient in Rec A protein and have a complex phenotype. Two important properties of RecA are as follows:

1) It catalyzes strand pairing, or strand assimilation-the joining of two different DNAs by homologous base pairing with each other.

2) It is a genetic regulator, activating the synthesis of many proteins (including DNA repair proteins) that help a bacterium adapt to a variety of metabolic stresses. This adaptation is called the SOS response.

Rec assay is based on the principle that when inhibition of cellular growth by a chemical is more pronounced with recombination repair deficient (Rec-) than with wild bacteria (Rec+), it is supposed that this chemical is damaging cellular DNA (Kanematsu et al., 1980) (Figure 2.3). Mazza and Gallizzi (1978) reported the isolation of mutants selected for increased sensitivity (or resistance) to DNA damaging agents and in some instances, the responsible gene products was also identified. Rec A protein (38 kDa) monomer binds more co-operatively to ssDNA than to dsDNA with half life of 30 min. The association is more favourable at low pH.
According to Cooper (2000) recombination repair is a post replication repair process in bacteria which involves the recombination of daughter strands of DNA to reconstruct the correct genome. This process is error free, but errors can occur if the repair requires denovo synthesis. This has been established in bacteria but has not been demonstrated conclusively in mammalian cells.

*Bacillus subtilis* BD 224 mutant strain used here for rec assay seem to lack DNA polymerase I and Rec A proteins because the cells show reduced survival after UV exposure for different intervals of time.

### 2.10.b Methodology of Rec assay

Spores of *Bacillus subtilis* BD 170 and BD 224 mutant strains were used. The exposure of chemicals at the time when spores are germinating into vegetative state (Figure 2.4) ensures highest sensitivity (Mazza 1982).
Figure 2.4 Germination of spores of *Bacillus subtilis* to vegetative state.

Each strain has some specificities as to its membrane permeability, metabolic modifications of metals etc, so repeated trials were conducted to detect mutagenic compounds. The role of cellular metabolic capacities that modify the valency of metal by oxidation or reduction is also studied by using S9 mix preparations. When using S9 mix, the system operates differently. Below are listed steps taken into care for comprehending the result.

1) Hodgson, (2004) described that S9 preparations include both cytosolic as well as microsomal fractions. Enzymes found in the microsomal fraction include Phase I enzymes cytochrome P<sub>450</sub> monooxygenase (CYP), flavin monooxygenase (FMO), cyclooxygenase, membrane bound enzymes including necessary co-enzymes, such as NADPH cytochrome P<sub>450</sub> reductase for CYP. Enzymes found in the cytosolic fraction includes hydrolases, conjugating enzymes such as glutathione transefase, glucuronidases, sulfotransferases, methyl transferase, and acetylases. Monooxygenations are oxidations in which one atom of a molecule of oxygen is incorporated into substrate while the other is reduced to water. Because the electrons involved in reduction of CYPs or FMO are derived from NADPH, the reaction is written as

$$\text{RH} + \text{O}_2 + \text{NADPH} + \text{H}^+ \rightarrow \text{NADP} + \text{ROH} + \text{H}_2\text{O}$$
The xenobiotics induce CYP, and then CYP’s oxidize xenobiotics. NADPH is included in S9 to help in this process. S9 also contains phase II enzymes such as hydroxylases, alcohol dehydrogenase, aldehyde dehydrogenase and esterases. Some of them cause reduction reactions also for organic substances. Alcohol dehydrogenase requires NADP for its activity and thus added along with G-6-P as cofactor solution. S9 is added to mimic the metabolic system as if happening inside the body. It is assumed that S9 works outside bacterial cells and it is only the detoxified or toxified products that enter the bacterial cells.

2) For bacterial assays, (Figure 7.1) S9 fraction containing both phase I and II enzymes is used as activating system. Phase I enzymes add polar group and Phase II enzymes add bulky sugar group, sulphates or amino acids to make it water soluble so that it can be excreted out of the body, which is a detoxification sequence. But the reactive intermediates formed in between are much more toxic than the parent compound.

3) When metals undergo reduction or they gain electrons e.g. Cr(VI) changing to Cr(III), next they lose electrons and get oxidized (redox reactions). The lost electrons get transferred to already present molecular oxygen changing it into oxygen radical, which cause damage to DNA as they enter inside bacterial cells.

4) It is now the function of reductases like glutathione to increase the detoxifying effect if the concentration of glutathione is enough to detoxify the compound.

5) If a compound is mutagenic at lower doses that means cytochrome P<sub>450</sub> system is activated. Thus, cofactors added for conjugation reactions to an S9 supplemented with G-6-P, NADP have detoxifying effect on compound if they supply reducing equivalents to glutathione. They have an activating effect on compound if they supply reducing equivalents to cytochrome P<sub>450</sub> reductase. Depending upon the type of substrate and complex interplay of enzymes of S9 that the compound is activated or deactivated.
2.10.c Applications of Rec assay

Takigami et al., (2002) found rec assay to be very powerful for the detection of mutagens in aquatic systems as it has higher sensitivity.

Liwasrsa-Bizukojca et al., (2005) studied the toxicity of alkyl sulphates (AS), alkylbenzene sulphonates (ALS) and alkylpolyoxyethylene sulphates (AES) as well as nonionic surfactants: polyoxyethylene alkyl ethers (AE) and polyoxylethylene alkylphenyl ethers (APE). Three different toxicity assays to aquatic organisms: Physa acuta Draparnaud, Artemia salina and Raphidocelis subscapitata were applied along with Bacillus subtilis M45 Rec- and H17Rec+. The obtained result showed that all the assays gave the similar results.

Grabinska-Sota and Kalka (2006) evaluated the toxicity of 5 new cationic surface active substances (CSAS)–imidazolium chlorides against fresh-water organisms. For genotoxic effect evaluation, Bacillus subtilis rec-assay was performed. It was shown that none of the examined surfactants possessed genotoxic properties.

Mc Carroll et al., (2006) found that 61 additional compounds along with B(a)P, 3–AP, DMBA, 3-MC and 4-NBP some of them direct and promutagenic agents were tested effectively with rec assay using microsuspension method.

Oksuzoglu et al., (2007) analyzed benzoxazoles chemicals with rec assay. They were tested positive showing the DNA damaging activities. Previous genotoxicity tests of aqueous fullerene C60 suspension yielded both positive and negative results. In the present study, aqu-C60 elicited positive responses in two bacterial genotoxicity tests, the Bacillus subtilis Rec-assay and the umu test, based on the activity of umu gene, were observed giving confirming results.

National Industrial Chemicals Notification and Assessment Scheme (NICN & AS) in its full public report in 2009 reported that test substance CHA tested in rec assay along with other mutation assay can be used for initial screening of chemicals.
Alad et al., (2009) tested Trichosetin, a novel tetramic acid antibiotic using rec and micronucleus assay and found to be negative with both the tests. Cherdshewasart et al., (2009) found Pueraria mirifica, a Thai phytoestrogen-rich herb traditionally used for the treatment of menopausal symptoms has shown the absence of mutagenic and the presence of anti-mutagenic activities in rec assay and further supported by a micronucleus test. Tekiner-Gulbas et al., (2010) studied the response of benzoxazole and benezimidazole derivatives in rec assay and found its mutagenicity equal to 4-Nitroquinoline 1-oxide (4-NQO).

Aquino et al., (2012) found rec assay, a rapid screening tool for detection of aflatoxins using microbial metabolizing system.

2.10.d Rec assay with metal salts

Kanematsu et al., (1980) carried out rec assays on 127 metal compounds with Bacillus subtilis to check their DNA-damaging capacity and mutagenicity. Certain compounds of beryllium, cobalt, cesium, iridium, osmium, platinum, rhodium, antimony, tellurium, thallium and vanadium were newly found to be positive in addition to those of known positive metals such as arsenic, cadmium, chromium, mercury, molybdenum and selenium.

Gentile et al., (1981) reported that metal ions have a high charge/radius ratio and hence a strong tendency to hydrolyze. Thus conclusions as to the mutagenicity and carcinogenicity of metal salts can be ambiguous and misleading. Using the rec-assay, it was determined whether the mutagenicity of chromium salts was reduced by complexation, as in the case of Cr(VI), or induced in the case of Cr(III). They found that several chelants like EDTA, salicylate (SA), and tiron (disodium 1,2-dihydroxybenzene-3,5-disulfonate), in proportion to concentration, reduce or eliminate the mutagenicity of CrO32-. Cr(III) was rendered slightly mutagenic by salicylate and citrate. None of the chelating agents or their combinations were mutagenic.

The genotoxicity of beryllium, gallium and antimony compounds was studied with the Rec, Salmonella mutagenicity and SCE assays by Kuroda et al., (1991) to arrive at conclusive results. In the rec assay, all the salts of the metals, BeCl2, Be(NO3)2, GaCl3, Ga(NO3)3, SbCl5, SbCl3, and an oxide, Sb2O3, had DNA-damaging activity whereas none of the compounds was
mutagenic to *Salmonella*. In the SCE assays using V79 cells, 2 antimony(III) compounds, SbCl₃ and Sb₂O₃, and two beryllium compounds, BeCl₂ and Be(NO₃)₂, induced SCEs significantly. Sb₂O₃, slightly soluble in water, was positive in both the rec assay and the SCE assay at very low doses. Sobti *et al.*, (1997) reported a better quantitative analysis lacking so far. As bacterial systems are subjected to cellular repair of different types, a recombination (rec) repair test applied to chemicals/carcinogens produced positive results in the rec assay. Two chromium compounds, chromium trioxide (CrO₃) and potassium dichromate (K₂Cr₂O₇) had been subjected to rec assay. The rec effect was greater with K₂Cr₂O₇ than with CrO₃. The S9 metabolic activation mixture had a detoxifying effect for both the compounds.

2.11 SOS Chromotest

2.11.a Principle of the SOS Chromotest

It is a simple colorimetric assay for genotoxicity. SOS response as well as damage inducible (din) genes is induced when DNA damaging agent act upon it. In this assay, lacZ, the structural gene for β-galactosidase is placed under the control of the sfiA gene, an SOS function involved in cell-division inhibition. The strain E. coli PQ37 carries a sfiA::lacZ fusion and has a deletion for the normal lac region so that β-galactosidase activity is strictly dependant on sfiA expression. (Figure 2.5) It is the β-galactosidase activity which is measured and quantitated (Quillardet and Hofnung 1985).

![Figure 2.5 Genetic events leading to the transformation of uninduced cell to induced cell in *E. coli* PQ 37 tester strain.](image-url)
2.11.b Applications of SOS chromotest

Mankiewicz et al., (2002) considered the fact that the toxicity of cyanobacterial blooms is well documented, but investigation into their genotoxicity has been insufficient relative to the study of their overall toxicity and carried out the estimation and comparison of the genotoxicity using the SOS chromotest (bacterial test) with *E. coli* PQ37 and the comet assay with human lymphocytes was done that was found to be correlating.

Petta et al., (2004) tested the genotoxic potential of saponified coconut oil surfactant with SOS chromotest and observed a negative result along with other prokaryotic mutant strains *E. coli* CC104 and CC104mutMmutY.

Mansour et al., (2011) evaluated the genotoxicity of textile effluent, before and after biodegradation with *P. putide* mt-2, *in vitro* using the SOS chromotest and *in vivo* in mouse bone marrow, by assessing the percentage of cells bearing different chromosome aberrations compared with untreated mice. It found that the results of both the tests were correlating.

Kakasi et al., (2012) studied the genotoxic potential of three fluorochromes, SYBR-14, Propidium iodide, and Hoechst 33342, using the colorimetric SOS Chromo Test™.

Kaur et al., (2012) reported successful screening by SOS Chromotest for antimutagenic derivatives of *Glycyrrhiza glabra* L.

Deng et al., (2012) prepared a report where the major phytochemical components of noni blossoms were studied for their toxicological properties and antioxidant activities. A primary DNA damage test in *E. coli* PQ37 (SOS-chromotest) and a twenty-four hour brine shrimp toxicity test did not reveal any genotoxic or cytotoxic activity.

2.11.c SOS Chromotest with metals

According to Couture et al., (1989) zircon (Zr) is an environmental hazard. The conformation to this was arrived at by means of bioassays on bacteria, microscopic algae and fish. It was found that Zr has low toxicity. Mutagenicity
(Fluctuation test) and genotoxicity (SOS Chromotest) assays failed to show any effects on DNA due to this metal.

The SOS Chromotest was reviewed (Quillardet and Hofnung 1993) through over 100 publications corresponding to the testing of 751 chemicals. 404 (54%) of these chemicals present a genotoxic activity detectable in the SOS Chromotest. Their SOS inducing potencies span more than 8 orders of magnitude. The results of six metal compounds, generally chromium compounds namely chromium acetate, chromium oxide, potassium dichromate, chromium oxide, chromium chloride, and mercuric chloride. The results were compared with results obtained from Ames test. Chromium chloride and mercuric chloride were tested negative equivocally. The status of chromium acetate was controversial as both negative and positive reports were received.

Gebel et al., (1997) compared and evaluated the genotoxic potential of platinum and palladium metal salts in mammalian and bacterial cells using the cytokinesis-block micronucleus test (MNT) with human lymphocytes and the bacterial SOS Chromotest. Results showed low genotoxicity in mammalian and bacterial cells for palladium metal salts.

Rabbow et al., (2002) used novel bacterial tests, the SOS-LUX- and LAC-FLUORO-TEST, which is a combination of two bioassays, that simultaneously measure the genotoxicity (SOS-LUX-TEST) and the cytotoxicity (LAC-FLUORO-TEST) of substances and mixtures of substances.

Simultaneously, a set of recombinant S. typhimurium strains carrying either the SOS-LUX plasmid or the fluorescence-mediating lac-GFP uv plasmid was used to determine in parallel on one microplate the genotoxic and the cytotoxic potential of heavy metal salts like K$_2$Cr$_2$O$_7$, CrCl$_3$, ZnSO$_4$, CuSO$_4$, NiSO$_4$, KH$_2$AsO$_4$ and As$_2$O$_3$. Microplate luminometer–fluorometer–photometer combination was used to measure the light and fluorescence emission of untreated and chemical-treated cells and the genotoxic and/or cytotoxic potential of the heavy metal salts was determined using luminescence induction as well as the fluorescence reduction.
2.12 Lipid peroxidation

Historically it all started when Kohn and Liversedge (1944) found that on attack of free radicals, polyunsaturated lipids with three or more double bonds gives aldehyde mainly malonaldehyde which reacts with thiobarbituric acid in a colorimetric reaction.

The main process involved in peroxidation is the xenobiotic induced proliferation of the cytoplasmic organelle, the peroxisome in mammalian liver cells (Lock et al., 1989). It is a stemming factor as it gives rise to peroxisomal enzyme which induces hydrogen peroxide (Figure 2.6).

2.12.a Principle of Lipid Peroxidation (Beyersmann and Hartwig 2008; Becker 2001)

1) It is also known by now that the one possible mode of action of soluble metal compound to cause DNA damage is due to the following factors:-

![Figure 2.6 Precursors to the events leading to carcinogenesis.](image)

Figure 2.6 Precursors to the events leading to carcinogenesis.

a. Oxidative stress (undergoing redox cycling and generation of oxygen radical) or /and

b. Forming adduct (Cr-DNA adduct) or compound-protein adduct or

c. By inducing the Fenton Haber Weiss reaction, where metal ions like iron, mediated by hydroxyl radicals are generated from hydrogen peroxides. The reaction is as follows:-

\[ \text{Fe}^{3+} + \text{O}_2 \rightarrow \text{Fe}^{2+} + \text{O}_2 \]
\[ 2\text{O}_2 + 2\text{H}^+ \rightarrow \text{H}_2\text{O}_2 + \text{O}_2 \]
\[ \text{Fe}^{2+} + \text{H}_2\text{O}_2 + \text{H}^+ \rightarrow \text{Fe}^{3+} + \text{HO} + \text{H}_2\text{O} \]
2) In second mode, input of a metal e.g. chromium also results in increased iron level and they act as catalyst by initiating lipid peroxidation as it generates peroxides and hydroxyl radicals, also from fenton reaction.

3) The metal ion e.g. chromium may itself get stabilized by chelation and catalyze peroxide formation through Fenton reaction. These peroxides and hydroxyl radicals take part in lipid peroxidation as shown in Figure 2.7.

![Diagram of lipid peroxidation process]

**Figure 2.7 Principle and measurement of lipid production.**

### 2.12.b Validation studies on lipid peroxidation

Li Huang (1999) investigated whether exposure to hexavalent chromium induces lipid peroxidation in humans. He took 25 chrome-plating factory workers and a reference group of 28 control subjects. The whole-blood and urinary chromium concentrations were determined by graphite furnace atomic absorption spectrophotometry. Malondialdehyde (MDA), the product of lipid peroxidation, was determined by high-performance liquid chromatography. The results suggested that concentrations of both chromium and MDA in...
blood and urine were significantly higher in the chromium-exposed workers. The activities of superoxide dismutase (SOD), glutathione peroxidase (GPX), and catalase (CAT) were not markedly different between control and exposed workers. Data indicated that MDA may be used as a biomarker for occupational chromium exposure. Antioxidant enzymatic activities are not a suitable marker for chromium exposure.

Obesity is a well-known risk factor of atherosclerosis. Studies by Yesilbursa et al., (2005) showed that obesity is associated with enhanced lipid peroxidation. It was found that weight reduction with orlistat treatment decreased lipid peroxidation that was observed by measuring the decreased concentration of plasma malondialdehyde (MDA).

Hung Chuang et al., (2009) reported the efficacy of DNA strand breakage and lipid peroxidation in detecting the carcinogenic agents in welding fumes in vivo.

Manimaran and Rajneesh (2009) applied lipid peroxidation test for detecting free radical activity inducing oxidative stress in ovarian cancer patients and found it to be useful in explaining the mechanism.

El-Adawi et al., (2012) evaluated the safety degree of lactic acid bacterial (LAB) usage on mammalian cells and anti-oxidative ability of their intact cells, and both extra-and intra-cellular extracts. The antioxidant properties of LAB strains were evaluated using \( \cdot \text{OH} \) scavenging assay, superoxide scavenging assay, hydrogen peroxide scavenging assay, DPPH radical scavenging assay, reducing ability assay and inhibition of lipid peroxidation assay.

2.12.c Lipid peroxidation assay with metals

Kadiiska et al., (1990) in experiments on female albino rats studied the effect of 30-day treatment with salts of Co, Cd, Ni, Zn and Hg on the liver monooxygenase system. It was found that \( \text{CdCl}_2 \) and \( \text{HgCl}_2 \) significantly decreased the activity of aniline hydroxylase whereas the activity of ethylmorphine-N-demethylase tended to remain almost constant and no significant changes were observed. Co, Cd and Hg decreased the NADPH-dependent lipid peroxidation whereas Ni and Zn did not change it. There was
no marked alterations in microsomal membrane fluidity in the female rat liver caused by all the metal salts.

Ahmed et al., (2008) studied the possible involvement of activated oxygen species in the mechanism of damage by NaCl stress in leaves of two varieties of pea (Pisum sativum L.) cv. EC 33866 and Puget. The level of lipid peroxidation, enzyme activity of superoxide dismutase (SOD, EC 1.15.1.1), ascorbate peroxidase (APX, EC 1.11.1.11), glutathione reductase (GR, EC 1.6.4.2), dihydro ascorbate reductase (DHAR, 1.8.5.1) were measured. They found that salt stress increased the rate of lipid peroxidation in both the varieties of pea.

Scibior et al., (2009) studied the effect of Va(5+) and Mg treatment on spontaneous and stimulated lipid peroxidation (LPO) in liver supernatants obtained from outbred 5-month-old, albino male wistar rats.

Bhardawaj et al., (2009) tested many parameters in their study on Phaseolus vulgaris L. plants that were grown in soil supplemented with different Pb and Cd concentrations (2, 4, 6, 8 gKg-1 for lead and 1.5, 2.0, 2.5, 3.0 gKg-1 for cadmium). They found that % germination remained unaffected at only low concentrations. Total free amino acid content and lipid peroxidation were increased with increasing concentration of heavy metals. In electrophoretic studies the acid phosphatase, peoxidase and esterase isoenzyme activities were found to increase with increasing concentration of heavy metals. The result of all these experiments suggested that the metal ions do result in oxidation which can be measured by lipid peroxidation.

Hossain and Fujita (2010) observed during biochemical analysis in mung bean seedlings, that salt stress (300 mM NaCl) caused a significant increase in reduced glutathione (GSH) content within 24 h of treatment as compared to control whereas a slight increase was observed after 48 hr of treatment. Proline and glycinebetaine provided protective action against salt induced oxidative damage by reducing H2O2 and lipid peroxidation level and by enhancing antioxidant defense and MG detoxification systems.
Dubey (2010) studied the oxidative stress generated through ROS in plants due to exposure of heavy metals like lead and mercury.

Upadhyay and Panda (2010) investigated the changing effect of different concentrations (0, 0.01, 0.1, 1, 10mM) of hexavalent and trivalent chromium on different biochemical parameters along with antioxidant enzymes on water lettuce (*Pistia stratiotes* L.) to know the possible involvement and effect of this metal in oxidative injury. The activities of antioxidant enzymes was also monitored. The effect of elevated concentrations of metal salts on biochemical and oxidative process was also monitored. This pointed to the possibility in induction of oxidative stress, with the increasing lipid peroxidation, followed by a differential pattern in antioxidant metabolisms by chromium ions in *P. stratiotes* L.

Mohamed *et al.*, (2010) targeted physiological and biochemical evaluation of transgenic potato plantlets under salt stress conditions on transformed and non-transformed clones. It was investigated for *in vitro* evaluation of salinity effects for one month. Different biochemical parameters such as proline accumulation, lipid peroxidation, pigments content, ascorbate, and glutathione contents were tested in order to put forward the relative tolerance of both clones to salinity at NaCl (0, 30, 60 and 90 mM).

### 2.13 Alkaline unwinding assay

#### 2.13.a Principle of alkaline unwinding

Thyagarajan *et al.*, (2007) reported that alkaline unwinding assay is based on the principle that upon DNA damage, the strand breaks in double-stranded DNA yield single-stranded DNA with the number of strand breaks being proportional to the amount of DNA damage. Alkaline unwinding assay helps to measure the damage as well as repair to evaluate the possible mutagenic effects of radiations and chemicals by measuring the amount of excision repair.
2.13.b Mechanism of alkaline unwinding

According to Poirier (2004) chemical mutagens acts directly as electrophilic reagents or undergo metabolic activation to become electrophilic reagents for reaction with nucleophilic sites in DNA, RNA and proteins (Figure 2.8). DNA damage can be classified into three (3) main categories.

1) Base modification without associated strand breaks.

2) Strand breaks with or without associated base damage.

3) Cross links between strands or between DNA and protein.

A highly accurate process of excision repair where repair of strands breaks does not require endonuclease action, but may require exonuclease for the repair of damaged termini adjacent to breaks. The methods for studying excision repair for detection of damaged strands and / or bases include chromatographic method, enzymatic method, use of specific antibodies etc.

2.13.c Methodology of alkaline unwinding

Shugart (1988) showed if cells exposed to radiations or chemical mutagens are briefly exposed to an alkaline pH and then neutralized, denaturation of DNA will proceed to a certain extent from each single strand break. The DNA is extracted as shown in Figure 2.9. The amount of DNA that becomes single stranded will depend on the frequency of single strand breaks and the length of exposure to alkali. Thus the fraction of DNA that becomes single stranded during exposure to alkali for a fixed period of time will be a measure of the number of single strand breaks. The method has considerable value in detecting single strand breaks at low doses but the precise experimental conditions must be adjusted for each cell type and each mutagen to achieve reproducible results. Interpretation of the results can be complicated if the mutagens alter the rate of DNA denaturation by forming DNA–DNA or DNA–protein crosslinks or by other obscure mechanisms.
2.13.d Applications of alkaline unwinding

Hartmann et al., (2003) described that the in vivo alkaline single cell gel electrophoresis assay, now the comet assay, can be used to investigate the genotoxicity of industrial chemicals, biocides, agrochemicals and pharmaceuticals. Advantages of using alkaline unwinding assay are the relative ease of application to any tissue of interest, the detection of multiple classes of DNA damage and the generation of data at the level of the single cell are few of the many advantages. These features give the comet assay potential advantages over other in vivo test methods, which are limited largely to proliferating cells and/or a single tissue. The comet assay has demonstrated its reliability in many testing circumstances and is, in general, considered to be acceptable for regulatory purposes.

Faust et al., (2004) reviewed the data of 45 alkaline comet assay studies with lymphocytes published during the last three years with the objective of monitoring human exposure to genotoxic agents as a result of occupation, drug treatment, diseases or environmental pollution. They found that a lot of data could be obtained in short period of time in cost effective manner as the experiment takes less time than the conventional culture method.

Khan et al., (2005) took soy isoflavones that possess potential chemopreventive properties against a wide variety of chronic diseases. They
evaluated the antimutagenic potential of soy isoflavones against benzo(a)pyrene (B[a]P) (125 mg/ kg) induced genotoxicity in Swiss albino mice. The DNA strand breaks were monitored using an alkaline unwinding assay. The pretreatment of soy isoflavone showed gradual reduction in strand breaks significantly (P<0.001) and dose dependently.

Thyagarajan et al., (2007) has found the usefulness of alkaline unwinding flow cytometry assay to measure nucleotide excision repair (NER), one of the DNA repair pathways, and is primary mechanism for repair of bulky adducts caused by physical and chemical agents, such as UV radiation, cisplatin and 4-nitroquinolones.

Desai et al., (2010) studied the DNA damage caused by fraction of petroleum hydrocarbons (WAF-P) towards diatoms namely Chaetoceros tenuissimus and Skeletonema costatium. The exposure was of chronic nature in water. Alkaline unwinding assay was used to assess genotoxic potential. It was found that genotoxicity increased with increasing level of WAF-P.

Webb and Gagnon (2012) used alkaline unwinding to determine the impact of human activity at the Swan-Canning Estuary, in the south-west of Australia. Adult black bream (Acanthopagrus butcheri) were collected from the estuary and maintained in clean water (S24) for 3 months and the results were compared with field-captured black bream from three sites within the estuary. Biomarker levels were up to 3.8 times higher in field captured fish compared with depurated fish, while DNA integrity was lower. From the results obtained, field-captured black bream depurated for 3 months are suitable to determine reference/baseline levels for biomarker of health studies in estuarine environments.

2.13.e Alkaline unwinding with metals

Schwerdtle et al., (2003) in their study compared the induction of oxidative DNA damage by arsenite and its methylated metabolites MMA(III) and DMA(V) generated DNA strand breaks (alkaline unwinding) in a concentration-dependent manner. Results showed that very low
physiologically relevant doses of arsenite and the methylated metabolites induce high levels of oxidative DNA damage in cultured human cells.

2.14 DNA adduct

2.14.a Principle of DNA adduct

Plastaras and Marnett (1999) understood that the malondialdehyde is a carcinogenic and mutagenic electrophile that is endogenously produced during peroxidation of polyunsaturated fatty acids. It is also known that carcinogens attack integrity of DNA and hence determining the levels of various DNA adducts could become an essential tool in understanding the toxicology of carcinogens. They correlated the direct measurement of DNA adduct levels, the true biologically effective dose of a mutagen, and biological outcomes with convincing results.

2.14.b Mechanism of DNA adduct

Hadjiliadis and Sletten (2009) explained the mechanism of DNA adduct formation in the most comprehensive way for a variety of chemicals especially metal salts. It involves the following steps:-

1) In case with chromium(VI), binding appears only when the chromium is being reduced as the intermediate chromium(V) and chromium(IV). The oxidation states of chromium play a role in binding mechanism.

2) Chromium in any oxidation state can be attracted to negatively charged DNA-phosphate backbone forming transient metal ligand complex with phosphate oxygens in a covalent manner.

3) Chromium-DNA adducts are ternary adducts that involve bidentate chelation between the DNA phosphate backbone and N7 group of a guanine residue.

4) Covalent bonding allows DNA-DNA and DNA-protein crosslinks and eventually leading to single strand (ss) breaks which are manifested into double strand (ds) breaks following a round of replication.
5) Replication fork repair is used to mend ds breaks. However, SCE can arise if the leading strand template becomes covalently attached to daughter lagging strand.

2.14.c Applications of DNA adduct

Cutts et al., (2005) separately studied the drug doxorubicin under the trade name Adriamycin, widely used anticancer agent which exhibits good activity against a wide range of tumors through DNA adduct formation only. Although the major mode of action appears to be normally as a topoisomerase II poison, it also exhibits a number of other cellular responses, one of which is the ability to form adducts with DNA. Watzek (2012) measured the levels of an acrylamide-derived DNA adduct in DNA extracted from several tissues of rats sixteen hours following the administration of a wide range of acrylamide doses. Acrylamide is a suspect carcinogen present in fried food. The results provided valuable insights into dose-response relationships for acrylamide-induced DNA damage and suggested that, at the levels associated with normal human consumption of relevant foodstuffs, tissue levels of acrylamide-derived DNA adducts are at the distinctly low end of 'steady state' levels of background DNA damage caused by diverse endogenous and environmental substances.

In a study by Hua et al., (2009) three different soils of contrasting features, were incubated with 300 mg benzo[a]pyrene (BaP)/kg dry soil, at 20 degrees C and 60% water holding capacity for 540 days. BaP and DNA were extracted and quantified at different time points. DNA adducts were quantified by (32)P-postlabelling. Some amount of initial BaP added remained in Cruden Bay, Boyndie and Insch soils. A significantly different amount of DNA-BaP adducts were found in the three soils exposed to BaP over time. This led to the concept that DNA adducts can be detected on DNA extracted from soil. Thus this new method provided a potential way to detect mutagenic compounds in contaminated soil.

According to Farmer (2011) suggested that the presence of an adduct (DNA covalently linked to foreign compound) indicate prior exposure of DNA to a
potential carcinogen, but does not by itself indicate the presence of cancer in the subject animal.

Balbo et al., (2012) developed the technology to quantify acetaldehyde-DNA adducts in human tissues and kinetics of DNA adduct formation in the oral cavity after drinking alcohol. Alcohol consumption is one of the top ten risks for the worldwide burden of disease and an established cause of head and neck cancer, as well as cancer at other sites. Acetaldehyde, the major metabolite of ethanol, reacts with DNA to produce adducts, which are critical in the carcinogenic process and can serve as biomarkers of exposure and, possibly, of disease risk. Acetaldehyde associated with alcohol consumption is considered "carcinogenic" to humans.

Forrest et al., (2012) studied the enhanced cytotoxicity of doxorubicin, a clinically used anti-neoplastic drug, by formaldehyde (either endogenous or exogenous) to promote the formation of doxorubicin-DNA adducts.

2.14.d DNA-adduct with metals

Hang (2010) highlighted that DNA adducts play a central role in chemical carcinogenesis. He analyzed the formation and repair of smoking-related DNA adducts that usually have complex structure. It also recorded the recent progress made in the areas concerning formation and repair of bulky DNA adducts in the context of tobacco carcinogen-associated genotoxic and carcinogenic effects and concluded that cellular DNA repair pathways have been known to operate in human cells on specific types of bulky DNA adducts.

Choudhary et al., (2012) found chromium can damage DNA in several ways, including DNA double strand breaks (dsbs) which generate chromosomal aberrations, micronucleus formation, sister chromatid exchange, formation of DNA adducts and alterations in DNA replication and transcription.

McDaniel et al., (2012) studied the formation of DNA adducts and mutations induced by aristolochic acid (AA) in rat spleen. It was found that over the dose range studied, there were strong linear dose-responses for AA-DNA adduct formation in the treated rat spleens. Isolated mutants when sequenced...
indicated significant difference between the pattern of mutation in the 10 mg/kg AA-treated and the vehicle control rats.


A critical review by Sava et al., (2012) examined the possibilities for the development of metal-based anticancer drugs and their synthesis with innovative strategies. It is focused on adding appropriate leaving and non-leaving groups to a transition metal in order to get more favorable DNA binding properties. The focus is on the nature of the ligands which are added to a metal forming adducts depending on the selected tumor cells and on their molecular targets.

A careful analysis of all the research reports presented in this review sum up that some metal compounds are extremely harmful, many are moderately harmful and few are not so harmful in different test systems under different conditions. Whatever the approach, it has become certain from the all existing data that to have a clear picture and wider applicability two requirements viz sensitive assay system and an essential animal system in vivo studies are required. Simultaneously, a quantitative study, so far lacking of the effect of metal compounds has to be done covering all the aspects of physiological, anatomical as well as genetical effects to impart convincing status.