the cell membrane concerned exclusively the Cr(VI) species. However, very recent models also considered the uptake of reduced Cr species generated by extracellular redox mechanisms [155]. Certain extracellulary generated Cr(V) and Cr(III) complexes also have high permeabilities through the cell membrane and therefore such species have to be taken into account when describing the complex model of chromium carcinogenicity [156].

Chromium, Biological Reductants and Free Radicals and Activation of Transcription Factors

Chromium(VI) alone does not react with DNA *in vitro*, or isolated nuclei. However, once inside the cell, in the presence of cellular reductants, it causes a wide variety of DNA lesions including Cr–DNA adducts, DNA–protein crosslinks, DNA–DNA crosslinks, and oxidative damage [157]. Within the cell glutathione rapidly forms a complex with Cr(VI), followed by a slow reduction of Cr(VI) to yield Cr(V) (reaction 1, Fig. (3)) [156]. Thus, glutathione can act as an intramolecular stabiliser of Cr(VI) *via* the formation of a thiolate ester. Once formed, Cr(V) species were found to alter the DNA conformation. In addition to GSH, a number of *in vitro* studies have confirmed that various other substances were capable of reducing Cr(VI). These include ascorbate, cystein [158, 159], lipoic acid, NAD(P)H, fructose, ribose and others [160, 161]. It may be more appropriate to think of the small-molecule Cr(VI) reducing agents, such as glutathione and ascorbate, as detoxifying agents, where the ultimate genotoxic agents are species formed between Cr(V) and diolato or sugar-like molecules. Some Cr(V)–sugar species are very stable at physiological pH values, with an EPR signal detectable after 48 h of the initiation of the reaction [162]. Among the most probable candidates for *in vivo* reduction of Cr(VI) substances is GSH and also ascorbate, especially because of their occurrence within cells. Kinetically, ascorbate is reported to be even more favored in reduction of Cr(VI) compared to GSH in the rat lung [163]. *In vivo* reduction of Cr(VI) was also reported by Liu *et al.* [164]. Using a specially designed EPR spectrometer for *in vivo* detection of free radicals, this group was able to show that Cr(V) intermediates, generated as a result of one-electron reduction, were detected in liver and also in the blood. Liver autopsies confirmed the *in vivo* findings. While pretreatment of the animals with ascorbate and GSH decreased formation of Cr(V), pretreatment with NAD(P)H augmented it. Based on these studies it was suggested that the *in vivo* one-electron reductant of Cr(VI) is most probably NAD(P)H flavoenzymes (reaction 2, Fig. (3)).

Several *in vitro* studies of the reaction of Cr(VI) with GSH were conducted. Using an EPR spin trapping technique it was possible to demonstrate the formation of Cr(V) species (most probably the Cr(V)-glutathione complex) and the glutathione-derived thiyl radical (GS•) (reaction 1, Fig. (3)) [165]. Once formed Cr(V) can react *via* Fenton reaction (reaction 3, Fig. (3)) with H_2O_2 forming the hydroxyl radical capable of causing DNA damage [159]. In addition to the cellular damaging effect of the GS• radical, it can further react with other thiol molecules in oxygenated tissues to give the superoxide radical (reactions 4 and 5, Fig. (3)). Superoxide can further reduce Cr(VI) to Cr(V) (reaction 6) which can then catalyze the decomposition of H_2O_2 thus creating the DNA damaging hydroxyl radical (reaction 7, Fig. (3)). Cr(V) can also be reduced by cellular reductants (e.g. ascorbate, GSH) to Cr(IV) (reaction 8, Fig. (3)), again participating in Fenton chemistry generating hydroxyl radical (reaction 9, Fig. (3)).

A reaction of Cr(VI) with ascorbate generated a mixture of both Cr(V) and Cr(IV) intermediates; addition of H_2O_2 led to competitive Fenton reactions with both Cr(V) and Cr(IV) species generating hydroxyl radicals [166]. Fenton chemistry and the redox couple Cr(III)/Cr(II) has been studied by Ozawa and Hanaki [167] who demonstrated that Cr(III) can be reduced to Cr(II) by the biological reductants L-cystein and NAD(P)H. In turn, the newly formed Cr(II) reacts with hydrogen peroxide to produce hydroxyl radical, detected by both EPR spectroscopy and HPLC. Shi *et al.* [168] investigated free radical generation from hydrogen peroxide and lipid hydroperoxides in the presence of Cr(III) using spin trapping EPR spectroscopy. At physiological pH, incubation of Cr(III) with H_2O_2 resulted in generation of hydroxyl radical. Diethylenetriamine pentaacetic acid (DPTA) substantially reduced generation of hydroxyl radicals; L-cystein, glutathione, and NADPH exhibited no significant effect. These results indicate that Cr(III) is capable of producing free radicals from both hydrogen peroxide and lipid peroxides. Sugden *et al.* [169] also reported mutagenic properties of Cr(III) complexes consistent with the ability of Cr(III) complexes to serve as cyclic electron donors in Fenton chemistry. These data are in agreement with the

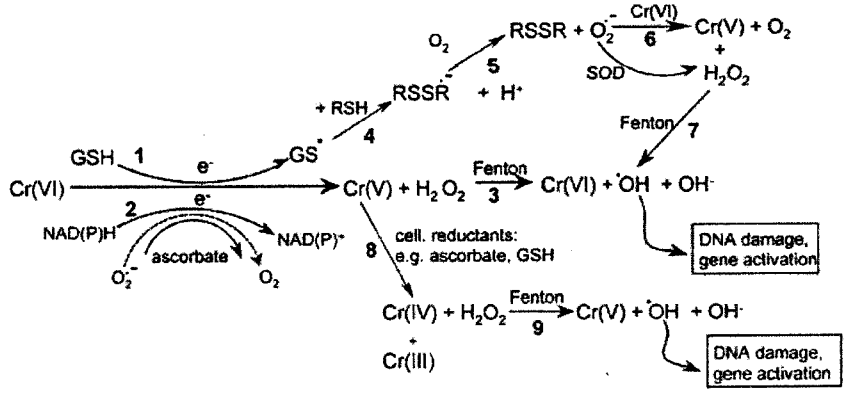

Fig. (3). Biological reductants of Cr(VI) and its reactions (For more details see the text).

studies of Cr(III)-picolinate toxicity (see above), indicating that the bioavailability *vs.* toxicity of Cr(III) compounds strongly depends on fine tuning by the ligand.

There has been much effort devoted to identification of the intermediates responsible for the induction of strand breaks during reactions of chromium(VI) with biological reductants. The results obtained by Stearns and Wetterhahn [166] suggest that it is not a single type of species that universally produces the DNA strand breaks observed in different chromium(VI) systems and that the reactivity of intermediates will depend on the experimental conditions, e.g. the concentration of chromium(VI), the presence of biological reductants, pH and others.

A mixture of Cr(VI) and ascorbate, both with and without H_2O_2, was tested for DNA strand breaking [170]. As detected by agarose cell electrophoresis, DNA strand breaks occurred when the DNA was incubated with Cr(VI) and ascorbate. The amount of damage was concentration-dependent with respect to both reactants and was significantly increased when H_2O_2 was introduced into the reaction mixture. The $^{\bullet}OH$ generated is able to react with guanine residues creating a variety of radical adducts, the most studied being 8-hydroxy-deoxyguanosine (8-OH-dG). It has been well established that the 8-OH-dG adduct is a good marker of oxidative damage [33] implicated in ROS-induced toxicity and carcinogenicity.

The effect of Cr(VI) on the growth, survival, and mode of cell death in normal human lung fibroblasts (HLF cells) in the presence of ascorbate and tocopherol was examined [171]. The predominant cellular response to Cr(VI) was growth arrest. It was found that Cr(VI) caused up to 20% of HLF cells to undergo apoptosis, and documented apoptotic morphology and the phagocytosis of apoptotic bodies by neighbouring cells. p53 levels increased 4- to 6-fold in chromium-treated cells. Pretreatment with vitamin C did not affect the p53 induction observed after chromium treatment and neither vitamin had any effect on Cr-DNA adduct formation.

An increase in lipid peroxidation of 1.8 and 2.2-fold occurred in rat hepatic mitochondria and microsomes, respectively, 48 h after oral administration of 25 mg sodium dichromate, Cr(VI), while increases of 1.2 and 1.4-fold, respectively, were observed after 895 mg chromium chloride hexahydrate, Cr(III). The urinary excretion of malondialdehyde, formaldehyde, acetaldehyde, and acetone were determined at 0-96 h after Cr administration. In Cr(VI)-treated rats, the excretion of all four lipid metabolites were 1.7- and 3.0-fold greater than for Cr(III)-treated animals [172]. The protective effect of vitamin E on chromium(VI)-induced cytotoxicity and lipid peroxidation in primary cultures of rat hepatocytes was investigated by Susa *et al.* [173]. Pretreatment of primary cultures of rat hepatocytes with α-tocopherol succinate (vitamin E) for 20 h prior to exposure to $K_2Cr_2O_7$ resulted in a marked decrease of chromium (VI)-induced cytotoxicity. These results indicate that the protective effect of vitamin E against chromium(VI)-induced cytotoxicity as well as lipid peroxidation (monitored by malondialdehyde formation) may be associated rather with the level of nonenzymatic antioxidants than the activity of enzymatic antioxidants (including glutathione reductase, superoxide dismutase, and catalase).

A series of detailed studies advocating a Cr(III)-dependent pathway in Cr(VI) carcinogenicity and mutagenicity was presented by Zhitkovich and his group who presented the evidence that Cr(III)-DNA adducts play the dominant role in the mutagenicity caused by the metabolism of Cr(VI) by a biological reducing agent [174]. Several further studies from the same laboratory disproved the existence and genotoxic/mutagenic effect of the Cr(V) species and the hydroxyl radical. Reduction of carcinogenic Cr(VI) by physiological concentrations of vitamin C has been shown to generate ascorbate-Cr(III)-DNA crosslinks, binary Cr(III)-DNA adducts, both potential sources of oxidative DNA damage by intermediate reaction products [175]. The results show that Cr-DNA adducts are responsible for both the mutagenicity and genotoxicity of Cr(VI). A lack of any significant production of the hydroxyl radical and Cr(V)-peroxo complexes in Cr(VI)-ascorbate reactions was confirmed. While ascorbate-Cr(III)-DNA cross-links were much more mutagenic, the Cr(VI)-induced DNA adducts were only highly genotoxic, but not mutagenic under either normal or SOS-induced conditions. The Cr(VI)-induced mutational spectrum consisted of an approximately equal number of deletions and G/C-targeted point mutations (51% G/C → T/A and 30% G/C → A/T). Lower toxicity and high mutagenicity of ascorbate-Cr(III) -DNA adducts in human cells may result from the recruitment of an error-prone bypass DNA polymerase(s) to the stalled replication forks. Further experiments [176] from the same laboratory demonstrated that in addition to reduction of Cr(VI) to DNA-reactive Cr(III), vitamin C contributes to the genotoxicity of Cr(VI) *via* a direct chemical modification of DNA. The absence of ascorbate in A549 and other human cultured cells indicates that cells maintained under the usual *in vitro* conditions lack the most important reducing agent for Cr(VI) and would primarily display slow thiol-dependent activation of Cr(VI). Again, similar to previous study, no evidence was found for the involvement of Cr(V) or Cr(IV) intermediates in the formation of either binary or ternary DNA adducts. These findings are in agreement with studies exploring the toxicity of Cr(III)-picolinate (see above) indicating that the toxicity of chromium(III) compounds is largely dependent on the type of ligand.

The effect of trivalent chromium Cr(III) was estimated through DNA-protein crosslinks (DPC) – a promising biomarker of exposure to chromium. DPC quantification was carried out in lymphocytes of a group of tannery workers exposed to trivalent chromium, a small group of manual metal arc stainless steel welders exposed to hexavalent chromium and a control group [177]. The results indicate a significant increase in the formation of DPC in tannery workers compared with controls. Urinary chromium was increased in both groups, with a greater increase observed in tanners compared with controls. It was concluded that chronic occupational exposure to trivalent chromium can lead to a detectable increase in lymphocyte DNA damage which correlates with a significant exposure of the cells to the metal. The same group in another study speculated that bulky DPC could also have a significant promutagenic effect [178]. Another study on Cr(III)-mutagenicity has shown that not only cysteine, but also glutamic acid and histidine were the major amino acids crosslinked to DNA in chromate-treated cells [179].

Carcinogenic Cr(VI) compounds were previously found to induce amino acidiglutathion-Cr(III)-DNA crosslinks with the site of adduction on the phosphate backbone [180]. Utilizing the pSP189 shuttle vector plasmid it was found that these ternary DNA adducts were mutagenic in human fibroblasts. The Cr(III)-glutathione adduct was the most potent in this assay, followed by Cr(III)-His and Cr(III)-Cys adducts. Single base substitutions at the G:C base pairs were the predominant type of mutations for all Cr(III) adducts. Cr(III), Cr(III)-Cys and Cr(III)-His adducts induced G:C → A:T transitions and G:C → T:A transversions with almost equal frequency, whereas the Cr(III)-glutathione mutational spectrum was dominated by G:C → T:A transversions. These studies of Cr(III)-induced phosphotriesters demonstrate the importance of a Cr(III)-dependent pathway in Cr(VI) carcinogenicity.

Cr(VI) was also found to induce activation of NF-κB in Jurkat cells [181]. Activation of NF-κB was attributed to hydroxyl radicals generated by the Cr(V)/Cr(IV)-mediated Fenton reaction. In addition, activity of another AP-1 transcription factor is stimulated by the Cr(VI) species. The induction of AP-1 by Cr(VI) is associated with phosphorylation of MAP kinase p38 and JNK, but not extracellular-signal-regulated kinase (ERK). Interestingly, aspirin was found to inhibit the activation of both AP-1 and NF-κB induced by Cr(VI). The induction of AP-1 and NF-κB by Cr(VI) was attenuated by inhibition of p38 and IkB kinase (IKK), respectively. These results suggest that Cr(VI)-mediated generation of radicals as a result of the Fenton reaction may serve as upstream signal initiating the activation of both AP-1 and NF-κB, whereas p38 and JNK act as a downstream executive kinase for the activation of AP-1 and NF-κB respectively.

The protein p53 is tumour suppressor protein which plays an important role in protecting cells from tumourigenetic alterations. For the majority of cancers it was found that cells contained mutations in the p53 gene. This transcription factor can be activated by a variety of stimuli, involving oxidative stress, radiation and others. While SOD, which converts $O_2^{\bullet-}$ to H_2O_2, was found to increase p53 activity; catalase, a scavenger of H_2O_2, inhibited p53 activation. In addition aspirin, a scavenger of $^{\bullet}OH$, suppressed activation of p53. Increased formation of $^{\bullet}OH$ enhanced p53 activation in A549 cells through Cr(VI) reduction by NAD(P)H followed by Cr(V) catalyzed decomposition of H_2O_2. Wang and Shi [182] also studied the mechanism of Cr(VI)-induced p53 activation. They found that the activation of p53 was at the protein level instead of the transcriptional level. In response to Cr(VI) treatment, protein p53 becomes phosphorylated and acetylated at Ser15 and Lys383, respectively.