# THE MODE-OF-ACTION OF THE CARCINOGEN CHROMIUM(VI): AN INVESTIGATION INTO THE FORMATION OF DNA LESIONS DURING THE REDUCTION OF CHROMATE

by

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A thesis submitted to the University of London for examination
for the degree of Doctor of Philosophy

October 1995

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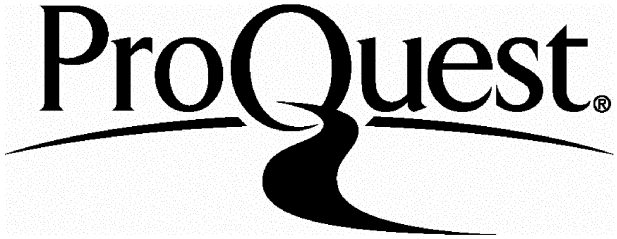


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# ABSTRACT

Chromium(VI) is a well established human carcinogen. Chromate ions are easily taken up by the cells and, once inside, are reduced by a variety of intracellular components including cysteine, ascorbic acid and glutathione. The intracellular reduction of chromium(VI) is a necessary step in the activation to its carcinogenic form and leads to the generation of a variety of DNA lesions. This study has investigated the role of glutathione (GSH) in mediating the formation of apurinic/apyrimidinic sites (AP-sites) by chromate.

The treatment of isolated bacteriophage DNA (PM2) with chromate and GSH caused the formation of AP-sites. The level of AP-sites increased with rising chromate concentrations in the presence of constant amounts of glutathione. An intermediate formed during the reduction of chromate by glutathione was responsible for the damage. AP-sites and single strand breaks (SSB) were formed in similar amounts and their generation followed an almost identical time course. Exclusion of oxygen from the incubation mixtures revealed that molecular oxygen is essential for the process leading to the activation of chromium(VI) to DNA damaging species.

A possible role for molecular oxygen was further investigated by kinetic studies of the reduction of chromate by glutathione. The rate of reduction of Cr(VI) increased under anoxic conditions, suggesting a side reaction involving molecular oxygen during which a species able to damage DNA is formed. Hydroxyl radicals are not generated during the reduction process, as none of the typical DNA base adducts formed by attack of hydroxyl radicals were observed using gas chromatography/mass spectrometry in selective ion mode. The hypothesis that SSB and AP-sites are formed by attack of a reactive species at the sugar moiety of DNA was confirmed by the finding that malondialdehyde-like products were released upon treatment of calf-thymus DNA with chromate and GSH. These studies have provided evidence of a non-Fenton pathway in the formation of DNA lesions during chromate reduction, which may be of importance in the mediation of chromate carcinogenicity.

# ACKNOWLEDGEMENTS

I am greatly indebted to my supervisor Dr. Andreas Kortenkamp for giving me the opportunity to carry out this research and for his invaluable guidance, encouragement and patience throughout the last three years.

I am very grateful to Prof. Paul O'Brien, who first introduced me to the "chromium world", for his interest, for the many stimulating discussions during this work and his patience in teaching me chemical kinetics.

I am indebted to the School of Pharmacy for my scholarship and to Ford Motor Company for their financial support.

My thanks to Dr. S. Faux and Dr. A. Jenner without whom the base-adducts studies would not have been possible; and to Tim and Fatemeh for their interest and effort during the time they were involved in the chromium research.

Finally, many thanks to Paula, with who I have shared the good and bad moments of the "chromium experience", for her help and friendship. And to all my fellow students of the Toxicology Department for all the good moments which brightened the sometimes dull days of research life.

# CONTENTS

# ABBREVIATIONS

| | |
|---|---|
| AP-sites | Apurinic/apyrimidinic sites |
| AsA | Ascorbic acid |
| Cr | Chromium |
| Cys | Cysteine |
| DNA | Deoxyribonucleic acid |
| FAPy-Ade | 4,6-diamino-5-formamidopyrimidine |
| FAPy-Gua | 2,6-diamino-4-hydroxy-5-formamidopyrimidine |
| GC/MS | Gas chromatography/Mass spectrometry |
| GS· | Thiyl radicals |
| GSH | Reduced glutathione |
| HEPES | 4-(2-hydroxy-ethyl)-1-piperazineethane-sulphonic acid |
| HPLC | High performance liquid chromatography |
| Lys-Trp-Lys | Lysine -Tryptophan-Lysine |
| Lys-Tyr-Lys | Lysine-Tyrosine-Lysine |
| MDA | Malondialdehyde |
| NCS | Neocarzinostatin |
| ·OH | Hydroxyl radical |
| 8-OH-Ade | 8-hydroxyadenine |
| 8-OH-dG | 8-hydroxydeoxyguanosine |
| 8-OH-Gua | 8-hydroxyguanine |
| 5-OH-Me-Ura | 5-(hydroxymethyl) uracil |
| 5-OH-Ura | 5-hydroxyuracil |
| SEM | Standard error of the mean (s.d./$\sqrt{n}$) |
| SSB | Single strand breaks |
| TBA | Thiobarbituric acid |
| Tris-HCl | Tris-(hydroxymethyl) aminomethane hydrochloride |

# LIST OF FIGURES

**Figure 2.3-5**: The number of SSB and AP-sites induced in PM2 DNA treated with chromate (0.175 mM) and GSH (5 mM) for different time intervals. AP-sites were revealed by post-incubation with putrescine. All data were corrected for the level of breaks induced in control samples. Results shown are means ± SEM × t (95 %), n = 5 - 10. The null hypothesis that the number of SSB and AP-sites is not significantly different was confirmed using Student's *t*-test with $P < 0.01$.

**Figure 2.3-6**: The level of SSB and AP-sites induced in PM2 DNA after treatment with chromate (0.175 mM) and GSH (5 mM) aged in the absence of DNA for the time intervals indicated. All data were corrected for the level of breaks in the appropriate controls. Results shown are means ± SEM × t (95 %), n = 4. The null hypothesis that the number of SSB and AP-sites is not significantly different was confirmed using Student's *t*- test with $P < 0.01$.

**Figure 2.4-1**: Chromate/GSH-induced SSB and AP-sites are present in different PM2 DNA molecules; one lesion does not evolve from the other. A) The formation of a SSB or the DNA scission at an AP-site upon post-incubation with putrescine or exonuclease III leads to conformational change of the PM2 DNA molecules, from supercoil (form I) to the open circular form (form II). B) However, the presence of an AP-site in the same DNA molecule that has a SSB will remain undetected, as cleavage at an AP-site upon post-incubation will not lead to further conformational changes.

**Figure 2.4-2**: Proposed mechanism of neocarzinostatin-induced DNA damage at C-4', modified from Dedon and Goldberg (1992).

**Figure 3.3-1**: Effect of catalase on the level of SSB and AP-sites induced in PM2 DNA by chromate (0.175 mM) and GSH (5 mM) after 3 hours incubation. Results are means ± SEM × t (95%), n = 4 - 10. The number of SSB and AP-sites did not differ significantly in the presence of catalase (Student's *t*-test with $P < 0.01$). The same was true in the absence of catalase.

**Figure 3.3-2**: The levels of 8-OH-dG in calf-thymus DNA following treatment with chromate (0.4 mM), GSH (5 mM), chromate and GSH, $H_2O_2$ (50 µM) or iron sulphate (42 µM) and $H_2O_2$ for 16 hours. The DNA base-adducts were measured using HPLC with electrochemical detection. Results are means ± s.d. (broken lines) (n = 3).

**Figure 3.3-3**: The chemical · structures of some DNA base adducts formed as consequence of hydroxyl radical attack.

**Figure 4.1-1**: Formation of TBA-MDA adduct, upon reaction of free malondialdehyde or MDA-like products with thiobarbituric acid at 100°C and acidic pH. The pink chromogen has an absorption maximum at 532 nm.

**Figure 4.3-1**: Release of MDA-like products from chromate (0.6 mM)/GSH (5 mM) treated deoxyribose as revealed by the increase in absorbance at 532 nm relative to controls after the TBA assay. Deoxyribose alone (—); controls (- · - · -); Cr(VI)/GSH treated deoxyribose (-----); Cr(VI)/GSH treated deoxyribose in the presence of catalase (·····).

**Figure 4.3-2**: Absorption spectra of MDA at 532 nm after reaction with TBA. A) Standard MDA obtained from TEP hydrolysis; B) Difference spectrum of chromate/GSH (0.8 mM/5 mM) treated deoxyribose and control samples, prepared as described in the experimental section.

**Figure 4.3-3**: The release of MDA-like products from 2-deoxyribose treated with GSH (5 mM) and various Cr (VI) concentrations for 6 hours, as measured by using the TBA assay. All data were corrected for the absorbance in control samples. Results are means ± SEM × t (95 %), n = 5.

**Figure 4.3-4**: The release of MDA-like products from calf-thymus DNA treated with GSH (5 mM) and various Cr (VI) concentrations for 7 hours, as measured by using the TBA assay. All data were corrected for the absorbance in control samples. Results are means ± SEM × t (95 %), n = 5.

**Figure 4.4-1**: Possible pathways for the formation of free MDA or MDA-like products upon attack at C-4' of the deoxyribose moiety.

**Figure 5.1-1**: Possible chemical structures at the 3'terminal end of a DNA SSB: A) 3'phosphorous terminal; B) 3'hydroxy terminal; C) 3'glycolate ester.

**Figure 5.3-1**: Autoradiograph of a PAGE after treatment of a $^{32}P$ 5'end labelled DNA fragment (150 bp) for four hours with: Lanes 1-2: DNA non treated and non-denatured; lanes 3-4: DNA non treated but denatured; lanes 5-6: DNA treated with Cr(VI) (0.8 mM) and GSH (5 mM); lanes 7-8: DNA treated with Cr(VI) (1.4 mM) and $H_2O_2$ (14 mM); lane 9-10: DNA treated with Fe(II) (160 μM) and $H_2O_2$ (0.5 mM).

**Figure 6.3-1**: Typical UV-visible spectra of solutions of chromium(VI) (0.175 mM) and GSH (20 mM) recorded at different times after initiation of the reaction. I) Chromium(VI) alone; II) Chromium(VI) with GSH after 45 seconds; III) Spectrum after 4.5 minutes. The reduction was completed after 33 minutes.

**Figure 6.3-2**: Variation of absorbance at 372 nm with time in solutions containing chromate (0.175 mM) and GSH at different concentrations under aerobic (grey lines) and anoxic conditions (black lines). The various lines represent independent experiments.

**Figure 6.3-3**: Exposure to air of anoxic mixtures of chromium (0.2 mM) and GSH (5 mM) slows the reduction process. Two anoxic mixtures of Cr(VI) and GSH were prepared and the change in absorbance at 372 nm with time was monitored. After 10 minutes reacting one of the samples was exposed to air.

**Figure 6.3-4**: Variation of absorbance at 433 nm with time in solutions containing chromate (0.175 mM) and GSH at different concentrations under aerobic (grey lines) and anoxic conditions (black lines). The various lines represent independent experiments.

**Figure 6.3-5**: Typical non-linear fits of Abs vs. time. Fitted using equation (**5**) with: A) 372 nm [GSH] = 5 mM $k_{1obs} = 0.63\ min^{-1}$, $k_{2obs} = 0.020\ min^{-1}$; B) 433 nm [GSH] = 10 mM, $k_{1obs} = 0.55\ min^{-1}$, $k_{2obs} = 0.09\ min^{-1}$.

**Figure 6.3-6**: Variation of $k_{2obs}$ with GSH concentration at 372 and 433 nm under anoxic and aerobic conditions. The solid lines are non-linear least square fits to $k_{obs} = a\ b\ [GSH]^2/(1 + b\ [GSH])$.

**Figure 6.3-7**: Variation of $k_{1obs}$ with GSH concentration at 372 and 433 nm under anoxic and aerobic conditions. The solid lines are least square fits to $k_{obs} = k_1\ [GSH] + k_{-1}$.

# LIST OF TABLES

# *CHAPTER 1*

# INTRODUCTION

Chromium and chromium compounds are well established human carcinogens (IARC, 1990). Exposure to chromium chemicals is of concern in a variety of occupational environments and many epidemiological studies have been carried out in order to study its effects. Extensive research is being carried out to clarify at a molecular level the cellular pathways leading to chromium(VI) carcinogenicity. Such studies have important implications for approaches to the biomonitoring of exposed individuals and to the risk assessments in occupational settings. It is the purpose of this work to further contribute to the understanding of the molecular mechanisms mediating chromium genotoxicity.

## *1.1 The Mutagenicity and Carcinogenicity of Chromium Compounds*

### 1.1.1 Uses of Chromium Compounds

Chromium compounds in the three stable oxidation states (0, III and VI) have a very wide range of applications. The production of chromium pigments represents the largest use of chromium in the chemical industry, other uses include the production of stainless steels and alloys, metal plating, leather tanning and anticorrosion agents (for a complete review, see IARC, 1990).

#### Metallic Chromium

The pure chromium metal is important and widely used for the production of alloys. It can be used to confer oxidation and corrosion resistance (e.g. in alloys based in nickel or cobalt) or to control the microstructure of alloys (e.g. in alloys of aluminium or copper). These alloys have diverse uses, ranging from chemical industry equipment to cutting tools and implants.

### Chromium(III) Compounds

Chromic acetate, chloride, hydroxide, sulphate and nitrate are used as textile mordants, in chrome plating, leather tanning and as catalysts for polymerisation and oxidation. Chromic oxide is used mainly as a pigment and as an alloying agent; anhydrous chromic oxide is the most stable green pigment known and is used in applications requiring resistance to heat, light and chemicals.

### Chromium(VI) Compounds

Chromates are used in a variety of forms (lead, zinc, ammonium, barium, sodium etc.) for various applications (pigment production, anticorrosion-coatings, wood and water treatment etc.). Sodium dichromate is the base material for the manufacture of chromium chemicals and is therefore in very high demand (in 1988, 149000 tonnes were required in the USA alone). Lead chromates are widely used in the production of pigments such as chrome-orange and chrome-yellows. The latter is one of the most versatile inorganic pigments and is found in a variety of formulations; they are used in the automobile industry, as a paint in traffic controls and in printing inks.

## 1.1.2 Occupational Exposures and Epidemiological Studies

The exposure of workers to chromium compounds by inhalation, ingestion or skin contact is of concern in a variety of industries. The highest exposures to chromium(VI) occur mainly during the chromate production, pigment manufacture and use, chrome plating and welding. Inhalation is the main route of exposure. Copious metallic fumes are generated during welding; aerosols and mists of chromium compounds are created during chrome plating and spray painting; and dust is produced during the milling, mixing and packaging of chromate pigments (IARC, 1990; Lees, 1991). Elevated levels of chromium in blood, urine, lung tissue and lymph nodes have been observed in workers in these industries. When deposited in the lungs, chromium particles can remain there for very long periods of time, often long after the exposure to chromates has ceased (Cohen *et al.*, 1993). The chromium may then be slowly internalised into cells. The fate of the chromium in the body will depend on various physical properties such as the oxidation state (Cr(VI) compounds, but not Cr(III), can easily cross cell

membranes), the solubility of the initial salt (high solubility may facilitate cell absorption but may also increase the rate of clearance) and the matrix in which the chromium enters the body (Gochfeld and Witmer, 1991).

A great number of epidemiological studies have been performed on workers of chromium-related industries. A link between exposure by inhalation to chromium(VI) compounds and an increased risk of developing lung cancer has been well established in chromate-producing industries, pigment manufacture and chromium plating. Because of multiple exposure to more than one type of chromium(VI) compound, the risk attributable to individual Cr(VI) compounds is unknown. The type of cancer occurring in exposed subjects is dependent on the length of exposure and the nature of the chromium compound. The most frequent type of lung cancer is squamous cell carcinoma, although the occurrence of rare sinonasal cancers has also been reported in workers of the chromium pigment production industry. In spite of many epidemiological studies, lack of adequate worker exposure data has so far prevented establishing a relationship between the level of exposure and the risk of developing lung cancer (for a review see IARC, 1990; Lees, 1991).

Skin contact to chromium compounds can cause severe dermatitis and sensitisation. The potential of chromium compounds to cause intestinal cancer by ingestion has not been assessed. However, it is known that the absorption of chromium from the gastrointestinal tract is much smaller than that from the respiratory tract, probably because of the rapid extracellular reduction of Cr(VI) compounds by components of the saliva and gastric juice (Gochfeld and Witmer, 1991; IARC, 1990; Cohen *et al.*, 1993).

Non-occupational sources of exposure to chromium compounds include food, water from industrial discharges and air from incineration, but the levels are usually much lower than those encountered in occupational situations (IARC, 1990).

### 1.1.3 The Genotoxicity of Chromium Compounds

Chromium compounds are genotoxic and various forms of genetic damage have been observed in bacteria and cultured cells such as chromosomal aberrations, sister chromatid exchanges (SCE), gene mutations and DNA lesions. Cr(VI) and Cr(III) compounds differ in their genotoxic potential as a result of their different abilities to penetrate cell membranes. Whereas the plasma membrane is impermeable to octahedral chromium (III) complexes (in some cases they may be taken up by phagocytosis), tetrahedral chromium(VI) compounds are easily transported into the cell, where they are reduced to chromium(III) (IARC, 1990; Jennette, 1979).

SCE and chromosomal aberrations are induced in mammalian cells upon exposure to chromium(VI) compounds *in vitro*. Elevated levels of both kinds of chromosome mutations have been observed in white blood cells of workers exposed to chromium(VI) compounds in electroplating factories (Bianchi and Levis, 1985; IARC, 1990). These chromium compounds have been shown to be mutagenic in bacterial, yeast and mammalian systems (Bianchi and Levis, 1985). In *Salmonella typhimurium* the mutations induced by potassium dichromate in strain TA102 were totally dependent on the presence of molecular oxygen in contrast to the partial dependence observed for strain TA2638 (Sugden *et al.*, 1990). There are conflicting reports about the specific mutations caused in mammalian genomes by Cr(VI) compounds. Predominance of mutations in A-T-rich gene sequences were observed in studies with HPRT (hypoxanthine guanine phosphoribosyl transferase) mutant Chinese Hamster Ovary (CHO) cells (Yang *et al.*, 1992), whereas three prominent hot spots at G:C base pairs were observed in exon 3 of hprt gene from human lymphoblasts (Chen and Thilly, 1994). Chromium(VI) complexes can induce a variety of DNA lesions including DNA single strand breaks (SSB), DNA-protein cross-links and Cr-DNA binding in *in vivo* and *in vitro* systems (Standeven and Wetterhahn, 1991a).

Chromium(III) compounds yielded negative results in the majority of genotoxicity tests. Chromosome aberrations have only been observed when using concentrations of chromium two to three orders of magnitude higher than chromium(VI), and SCE are

restricted to cells with active phagocytosis. Some Cr(III) complexes were found to be mutagenic in the Ames test with the *Salmonella* strains TA98, TA100 and TA92 when complexed to certain organic ligands that facilitate their penetration into cells, e.g. 2,2'-bipyridyl (bipy) or 1,10-phenanthroline (Bianchi and Levis, 1985, 1987; IARC, 1990). The mutations induced by Cr(III)-bipy in the oxidation sensitive *Salmonella* strains TA2638 and TA102 were oxygen dependent (Sugden *et al.*, 1990). Chromium(III) complexes can interact with isolated DNA forming DNA lesions such as Cr-DNA binding and DNA-protein cross-links *in vitro* (Tsapakos and Wetterhahn, 1983; Salnikow *et al.* 1992). Low concentrations of Cr(III) bound to DNA have been shown to increase DNA polymerase activity and decrease DNA replication fidelity (Snow, 1994).

### 1.1.4 The Formation of DNA Lesions by Chromium Compounds

The genotoxicity and carcinogenicity of chromates appears to be mediated by the formation of DNA lesions. A wide range of DNA damage has been observed upon treatment of animals, cells or isolated DNA with chromium compounds, including DNA single strand breaks (SSB), Cr-DNA adducts, protein cross-links and alkaline labile sites.

*In vivo* treatment of chick embryos with Cr(VI) induced persistent SSB in the DNA of red blood cells (Hamilton and Wetterhahn, 1986). However, administration of Cr(VI) to chick embryo or rats produced no detectable SSB in chick embryo liver, or in rat lung, liver and kidney (Standeven and Wetterhahn, 1991a). In contrast to the *in vivo* situation, treatment of cultured cells with chromate led to extensive breakage of the DNA. However, this DNA lesion is repaired very efficiently (Fornace, 1982; Cupo and Wetterhahn, 1984; Snyder, 1988). DNA breakage was observed in normal human fibroblasts and in excision-deficient *Xeroderma Pigmentosum* cells after treatment for four hours with 0.5 μM potassium chromate, indicating that the breaks observed were not caused by the action of the DNA repair mechanism (Fornace, 1982). SSB induced in human diploid fibroblasts and in cultured chick embryo hepatocytes exposed to non-toxic doses of Cr(VI) were no longer observed two hours after the removal of the metal

from the media (Cupo and Wetterhahn, 1984; Snyder, 1988). Although the incubation of human diploid fibroblasts with catalase inhibited the formation of SSB by Cr(VI), the hydroxyl radical scavengers potassium iodide and mannitol had no effect on the level of DNA breakage. Simultaneous treatment with glutathione (GSH) and Cr(VI) led to an increase in the amount of SSB (Snyder, 1988).

Chromium-DNA adducts were detected in liver cells of chick embryos upon *in vivo* treatment with chromate. These adducts have been proposed to mediate the formation of interstrand cross-links and protein cross-links in liver cells of chick embryos. No DNA adducts or cross-links were detected in red blood cells of chick embryos upon *in vivo* exposure to Cr(VI) (Hamilton and Wetterhahn, 1986; Misra *et al.*, 1994). Extensive Cr-DNA binding was observed upon treatment of cultured mammalian cells with chromate for two hours (Salnikow *et al.*, 1992; Xu *et al.*, 1994; Zhitkovich *et al.*, 1995). The observation that bound chromium was extractable using the chelator ethylenediaminetetraacetic acid (EDTA) suggested that chromium was bound to DNA mainly as chromium(III). Chromium in other oxidation states ((VI),(V) or (IV)) cannot be chelated by EDTA (Salnikow *et al.*, 1992). Nearly 50 % of the Cr(III) bound to the DNA of CHO cells upon treatment with Cr(VI) was found to be cross-linked to free amino acids or GSH, increasing the stability of the binding (Zhitkovich *et al.*, 1995). The majority of the Cr-adducts in CHO treated cells were detected in the nuclear matrix subfraction of the chromatin, in which a number of essential nuclear processes, including replication and transcription take place. These adducts were very persistent, and they were observed even 48 hours after the Cr(VI) treatment (Xu *et al.*, 1994).

*In vivo* treatment of chick embryos with chromium(VI) led to the formation of protein cross-links and interstrand cross-links in liver cells but not in red blood cells (Hamilton and Wetterhahn, 1986). Cultured chick embryo hepatocytes exposed to sodium chromate for two hours presented persistent DNA-protein cross links, which were detectable even 40 hours after the removal of chromate. In contrast, the DNA interstrand cross-links and SSB were completely repaired after 12 and 3 hours respectively (Cupo and Wetterhahn, 1984). Pre-treatment of Chinese hamster V-79 cells with ascorbic acid led to an increase in the level of protein cross-links detected after Cr(VI) treatment

(Sugiyama *et al.*, 1991). Actin was described as one of the proteins complexed with the DNA from CHO cells exposed to chromate; no histone proteins were cross-linked. EDTA dissociated the Cr-protein adduct, indicating that Cr(III) was mediating the binding (Costa, 1991).

Evidence for the formation of alkaline labile sites was obtained from alkaline elution studies using cultured mammalian cells exposed to chromate. Christie *et al.* (1984) and Cantoni and Costa, (1984) observed that DNA from treated cells eluted with increasing rates at alkaline pH. The shape of the resulting elution curves differed markedly from those usually found with agents known to cause what Cantoni and Costa (1984) have termed "frank" single strand breaks. This elution pattern was attributed to the formation of "alkaline labile sites", a kind of lesion that under the conditions applied during alkaline elution ultimately results in the induction of single strand breaks. The alkali-labile sites observed in these experiments could have been the result of an apurinic/apyrimidinic site (AP-site, loss of a base from DNA) or a phosphotriester adduct. Both DNA lesions are known to cause an increased lability of the phosphodiester linkages in the DNA, thereby making them vulnerable to alkaline hydrolysis. The formation of AP-sites is potentially mutagenic (Loeb and Bradley, 1986).

## *1.2 The Mechanisms Underlying Chromium Genotoxicity*

The genotoxicity and carcinogenicity of chromium compounds has been extensively studied in the last 15 years (for reviews see Standeven and Wetterhahn, 1991a; Cohen *et al.*, 1993). One of the major goals is to elucidate the mechanism by which DNA lesions are induced at a molecular level, in order to establish a link between the formation of DNA lesions, the induction of mutations and causation of carcinogenicity. Moreover, an understanding of the effects of chromium compounds at the cellular and subcellular levels can provide the basis for a development of physiologically based risks assessments, which aim at describing the relationship between external exposure to the carcinogen, dose at target tissue and levels of DNA lesions.

### 1.2.1 The Uptake-Reduction Model

The uptake-reduction model proposed by Jennette (1979) is now widely accepted to describe in general and simple terms the processes leading to chromium genotoxicity. Cr(VI) compounds are easily transported inside cells via non-specific anion channels, in the same way as other tetrahedral physiological anions, such as $SO_4^{2-}$ and $PO_4^{3-}$. Due to their octahedral structure trivalent chromium complexes cannot easily enter the cell unless they are taken up by endocytosis or pinocytosis.

Because of the rapid reduction of chromium(VI) complexes by cellular constituents, chromium, now in the trivalent state, becomes effectively trapped inside the cell; the reduction further enhances the uptake of chromate anions because an equilibrium between extracellular and intracellular chromium(VI) cannot be established. In Chinese hamster V-79 cells exposed to solutions of sodium chromate the uptake of chromate increased linearly with the chromate concentration applied, no plateau was reached even at intracellular concentrations 5-fold over the ones applied extracellularly. In the nuclei a 60-fold accumulation over the extracellular chromate concentrations (0.1 mM) was observed within three hours, indicating that a millimolar concentration of chromium in the nuclei can be easily reached (Sehlmeyer *et al.*, 1990).

The intracellular reduction of chromate is essential in the steps leading to the formation of DNA lesions *in vivo* and *in vitro*. It has been shown in *in vitro* studies that chromium(VI) itself is not able to induce DNA lesions (Tsapakos and Wetterhahn, 1983; Kortenkamp *et al.*, 1989; Salnikow *et al.*, 1992). Therefore, the reduced chromium(III) and/or a reactive intermediate species generated during the reduction process are thought to be responsible for the induction of the DNA damage.

The amount of chromium(VI) being able to enter the cells will be determined by the effectiveness of extracellular reductants; considerable amounts of ascorbic acid exist in the alveolar lining fluid of the rat and guinea pig and high levels of glutathione have been reported in the epithelial lining fluid of the human lower respiratory tract. Because it lowers the amount of chromium(VI) available to cross the cell membranes, the extracellular reduction, unlike the intracellular, can be considered a detoxification process (Suzuki, 1990; Cohen *et al.*, 1993).

In order to understand the mechanism by which chromium(VI) compounds induce the formation of DNA lesions, which are important in the mediation of mutagenicity and carcinogenicity, it is necessary to address the following problems:

- to establish the contribution of the different intracellular reductants in the reduction of chromate anions and the generation of DNA damaging species,
- to define the nature of the DNA damaging species,
- to delineate the importance of the various DNA lesions in the mediation of mutagenicity.

### 1.2.2 The Intracellular Reductive Conversion of Chromium(VI)

Once chromium(VI) compounds cross the cell membranes, the reduction by different intracellular components can occur at different cellular sites, such as the cytoplasm, mitochondria, endoplasmatic reticulum or nucleus (Connett and Wetterhahn, 1983). *In vitro* studies have shown that a variety of enzymatic and non-enzymatic factors present

in the cell are able to reduce Cr(VI) to Cr(III) at neutral pH (Standeven and Wetterhahn, 1991a; Cohen *et al.*, 1993).

The reduction of chromate by small molecules was shown to be kinetically controlled and only the thiols, glutathione and cysteine (Cys), and ascorbic acid (AsA) reacted at significant rates at pH 7.4 and were likely to contribute substantially to the Cr(VI) reduction in the cell (Connett and Wetterhahn, 1985). Enzymatic factors reported to be able to reduce chromium(VI) include cytochrome P-450, electron transport complexes of the mitochondrial membrane, glutathione reductase, aldehyde oxidase, and DT-diaphorase (Connett and Wetterhahn, 1983; Standeven and Wetterhahn, 1991a). Among those, the ones found in intact mitochondria seem to have the most relevant effect on Cr(VI) reduction. Inside the mitochondria, Cr(VI) is reduced to Cr(V) causing inhibition of respiration and decrease of cellular levels of adenosine triphosphate (ATP) and guanosine triphosphate. The impairment of oxygen metabolism may have an effect on the oxygen concentration elsewhere in the cell, which may facilitate the formation of reactive species (Cohen *et al.*, 1993).

Because of their high concentrations in cells and their ability to reduce Cr(VI) the low molecular weight thiols and ascorbic acid are thought to be the main contributors to the chromium(VI) reduction, and their influence on the formation of DNA lesions has been extensively studied (Connett and Wetterhahn, 1983; Kortenkamp *et al.*, 1989; Suzuki, 1990; Standeven and Wetterhahn, 1991a, b; Cohen *et al.*, 1993).

### Glutathione

Intracellular levels of GSH (γ-glutamyl-cysteinyl-glycine) are thought to be in the millimolar range in most cells; cytoplasmic levels of GSH in the rat lung have been shown to be around 2 mM (Suzuki, 1990), and in liver and red blood cells from chick embryos the GSH concentration was 6 mM and 2 mM respectively (Misra *et al.*, 1994). One of the physiological roles of GSH is to protect cells from external injuries such as oxidative stress, however, in the case of chromium(VI), the involvement of GSH may have serious consequences for the cell.

The importance of GSH in the reduction of chromate *in vivo* is supported by various observations. A correlation between the level of DNA SSB induced in chick embryo hepatocytes treated with chromate and the level of GSH was established. A depletion of GSH levels with buthionine sulfoximine led to a decrease in DNA breakage, while increasing GSH levels with N-acetylcysteine resulted in higher number of DNA SSB (Hamilton and Wetterhahn, 1986). Rats treated with chromate excreted chromium in the bile as Cr(III), however when the levels of GSH were chemically depleted the chromium was excreted exclusively as Cr(VI), indicating that depletion of GSH prevented liver cells from normal reduction of Cr(VI) (Standeven and Wetterhahn, 1991a). In other studies no depletion of GSH has been observed upon treatment of animals with Cr(VI) (Standeven and Wetterhahn, 1991a, b; Misra *et al.*, 1994). These observations can be explained in various ways. One possibility is that GSH does not have a main role in the intracellular reduction of Cr(VI). Alternatively the changes in GSH levels may have been too small to be detected or the cells rapidly increased the biosynthesis of GSH to counteract its depletion.

The reduction of Cr(VI) by GSH leads to the formation of DNA lesions in *in vitro* systems, such as DNA SSB (Kortenkamp *et al.*, 1989, 1990; Kortenkamp and O'Brien, 1994), Cr-DNA adducts (Aiyar *et al.*, 1989; Borges *et al.*, 1991) and DNA-GSH cross links (Borges and Wetterhahn, 1989).

A variety of reactive intermediates, which have the potential to damage DNA are formed during the reduction of chromate by GSH including Cr(V) (O'Brien *et al.*, 1985; Goodgame and Joy, 1986; Kitagawa *et al.*, 1988; Aiyar *et al.*, 1989; Borges *et al.*, 1991), Cr(IV) (Bose *et al.*, 1992; Kortenkamp *et al.*, 1995), Cr(II) (Perez-Benito *et al.*, 1994a) and thiyl (GS·) radicals (Aiyar *et al.*, 1989).

### Ascorbic acid (AsA)

The importance of AsA in the intracellular reduction of chromate has recently received much attention. Although the intracellular levels of AsA are thought to be lower than GSH, e.g. approximately 1 mM in liver and red blood cells from chick embryos (Misra

*et al.*, 1994), the reduction of Cr(VI) by AsA is kinetically favoured (Connett and Wetterhahn, 1985; Suzuki, 1990).

Upon treatment of male Sprague-Dawley rats with Cr(VI) (134 μmol/kg) by intraperitoneal injection, a depletion of renal, but not of hepatic, AsA levels was observed. No decrease in hepatic or renal non-protein sulphydryl groups was noticed (Standeven and Wetterhahn, 1991b). A reduction of AsA level but not GSH was also observed in rat lung after intratracheal injection of Cr(VI) (Suzuki and Fukuda, 1990); however, no changes in liver or red blood cells AsA levels of chick embryos treated with sodium chromate were detected (Misra *et al.*, 1994). Ascorbic acid was found to be the main chromium(VI) reductant in rat liver and kidney ultrafiltrates (30,000 MW). When AsA was specifically depleted using the enzyme ascorbate oxidase there was an 80 % loss on the Cr(VI) reductive potential of the ultrafiltrates. However, when non-protein sulphydryl groups were blocked just 20 % inhibition on the reduction activity was observed (Standeven and Wetterhahn, 1991b). These data suggest that AsA may have an important role in the intracellular reduction of chromate.

Kinetic studies of the reduction of chromate by mixtures of AsA and GSH suggest that the two reductants may act synergistically in the reduction of chromium(VI) (Suzuki, 1990).

DNA SSB and Cr-DNA binding are induced in isolated DNA during the reduction of chromate by AsA (Stearns and Wetterhahn, 1994; Stearns *et al.*, 1995; da Cruz Fresco and Kortenkamp, 1994; Kortenkamp and O'Brien, 1994). The reactive intermediates generated during the reduction process include Cr(V), ascorbyl radicals (Goodgame and Joy, 1987; Lefebvre and Pézerat, 1992; Stearns and Wetterhahn, 1994), Cr(IV) and carbon centred radicals (Stearns and Wetterhahn, 1994).

### Cysteine

Although cysteine reduces chromate more readily than GSH (Connett and Wetterhahn, 1985), its concentration in various tissues is low, around 0.2 - 0.5 mM or even lower,

and its contribution to Cr(VI) reduction has not been properly assessed (Standeven and Wetterhahn, 1991a).

Short-lived Cr(V) species have been detected during the reduction of chromate by cysteine (Kitagawa *et al.*, 1988). Borges and Wetterhahn (1989) showed that cysteine was cross-linked to calf-thymus DNA upon incubation with chromate and Cys *in vitro*, although at much lower levels than GSH.

## 1.2.3 DNA Lesions Induced upon Reduction of Cr(VI) *In Vitro*

Despite the variety of DNA damage observed in *in vivo* systems and in cultured cells upon treatment with chromate, the relative importance of the different DNA lesions in the mediation of chromium mutagenicity and carcinogenicity is not known. *In vitro* systems have been extensively studied to address this question.

### DNA Single Strand Breaks (SSB)

The formation of SSB in isolated DNA upon reduction of chromate by a variety of intracellular reductants is well documented. Kawanishi *et al.* (1986) and Aiyar *et al.* (1989) observed the formation of SSB in plasmid DNA and in a 110 base pair (bp) DNA fragment during the reduction of chromate by hydrogen peroxide. Because of the random distribution of breaks along the radiolabelled DNA fragment, it was suggested that hydroxyl radicals (·OH radicals) were the cleaving species (Kawanishi *et al.*, 1986). However the concentrations of $H_2O_2$ used were extremely high (18 mM), which questions the relevance of these observations in an *in vivo* system.

The formation of SSB during the reduction of chromate by GSH has been the subject of some controversy for several years. Whereas Kortenkamp and co-workers were able to observe SSB in PM2 DNA (Kortenkamp *et al.*, 1989, 1990; Kortenkamp and O'Brien, 1994), Wetterhahn and co-workers were unable to detect this DNA lesion during the reduction of chromate by GSH (Aiyar *et al.*, 1989; Borges *et al.*, 1991). The involvement of reactive oxygen species generated by residual metal ions such as iron

and copper that are present in the reaction solutions, was suggested to be the reason behind the formation of SSB (Borges *et al.*, 1991). However, Kortenkamp and O'Brien (1994) showed that the level of DNA breakage induced during the reduction of chromate by GSH remained largely unchanged upon removal of catalytic metal ions from the solutions. Kortenkamp *et al.* (1995) showed that the reason for the apparent contradiction lay in the different experimental conditions used by both groups. Kortenkamp and co-workers used physiologically relevant concentrations, 5 mM GSH, up to 0.3 mM Cr(VI) and approximately three hours incubation; whereas Wetterhahn and co-workers used higher concentrations, 1.8 mM Cr(VI)/18 mM GSH, and shorter incubation times, *ca.* 30 minutes. Glutathione at concentrations exceeding 15 mM is an extremely efficient scavenger of the reactive species arising from chromate reduction, consequently solutions of chromate and GSH lose their ability to cause SSB at high concentrations. A reactive intermediate formed during the reduction of Cr(VI) by GSH was responsible for the formation of the SSB. The formation of this DNA lesion was inhibited by the presence of catalase, but was unaffected by glucose, a strong ·OH scavenger (Kortenkamp *et al.*, 1990; Kortenkamp and O'Brien, 1994).

The reduction of Cr(VI) by AsA leads to the induction of SSB in isolated DNA with a maximum level observed at ratios of Cr(VI):AsA of 1:1 (Kortenkamp and O'Brien, 1994; da Cruz Fresco and Kortenkamp, 1994; Stearns *et al.*, 1995). The DNA SSB are generated by a reactive intermediate and they are dependent on the presence of molecular oxygen (da Cruz Fresco and Kortenkamp, 1994). In light of spectroscopic studies, Stearns *et al.* (1995) suggested that carbon-centred radicals were responsible for the induction of SSB; however these radicals have been reported to be formed by an oxygen independent pathway (Stearns and Wetterhahn, 1994).

Although it is clear that chromium(VI) upon reduction has the potential of inducing DNA SSB, it is not very likely that this DNA lesion will contribute significantly to the mediation of chromium genotoxicity, in view of their efficient repair (see section 1.1.4).

### Cr - DNA Adducts

Cr(III) complexes can slowly bind to isolated DNA and to cell nuclei (Tsapakos and Wetterhahn, 1983; Kortenkamp *et al.*, 1991; Hneihen *et al.*, 1993). The ability of these complexes to interact with the DNA is highly dependent on the nature of the ligands (Kortenkamp *et al.*, 1992; Hneihen *et al.*, 1993). Hneihen *et al.* (1993) observed high levels of chromium binding in isolated calf-thymus DNA and salmon sperm nuclei with aquo-chromium(III) complexes, but the reactivity was greatly diminished when Cr(III) was complexed to tridentate peptides or amino acid ligands. In previous work, Kortenkamp *et al.* (1992) had already shown the importance of the nature of the ligands and charge of the Cr(III) complex in mediating DNA interactions; they observed that cationic Cr(III) induced DNA condensations. In view of their results and the lack of evidence suggesting that cationic chromium species are formed upon Cr(VI) reduction *in vivo*, they argued that the use of such chromium(III) species *in vitro* to model effects *in vivo* was inappropriate. It is therefore clear that the reactivity of the reduced Cr(III) *in vivo* will be affected by its co-ordination to various intracellular ligands.

Cr(VI) does not bind to isolated DNA or nuclei unless a reducing agent is present. Binding has been observed using a variety of intracellular reductants, including thiols (GSH, cysteine) (Aiyar *et al.*, 1989; Borges and Wetterhahn, 1989; Borges *et al.*, 1991), microsomes/NADPH (Tsapakos and Wetterhahn, 1983), AsA (Stearns and Wetterhahn, 1994; Bridgewater *et al.*, 1994) and rat lung cytosol (Hneihen *et al.*, 1993). Considering that chromium binding is observed in some systems after only 30 minutes of incubation (Borges and Wetterhahn, 1989; Borges *et al.*, 1991) it is likely that the binding is mediated by chromium species in one of the intermediate oxidation states rather than by the final product of the reduction, Cr(III). However, this question has yet to be addressed experimentally.

Chromium seems to be able to bind to the DNA bases and to the phosphate backbone. Tsapakos and Wetterhahn (1983) suggested that chromium was bound to the DNA bases because higher binding was observed in rich G polynucleotides. The specific polymerase arrest observed at bases preceding guanine residues in a double stranded pSV2*neo*TS plasmid DNA treated with chromate and AsA also suggested base binding,

as no specificity would be expected from binding to the DNA phosphate backbone (Bridgewater *et al.*, 1994). However, Salnikow *et al.* (1992) observed that chromium was not bound to any specific base during analysis by high performance liquid chromatography (HPLC) of DNA from human osteosarcoma cells treated with $^{51}Cr(VI)$. Therefore they concluded that the chromium was bound to the phosphate backbone of the DNA. $^{31}P$ NMR studies using ATP showed that Cr(III) complexes were capable of interacting via and outer-sphere complex with one of the phosphates of the ATP (Kortenkamp and O'Brien, 1991).

The formation of Cr-DNA adducts during the reduction of chromate by intracellular reductants is likely to have longer lasting effects than the formation of DNA SSB. This type of DNA lesion is repaired very slowly and can affect the replication and transcription of DNA. Snow (1994) showed that Cr(III) bound to M13 *mp2* single stranded DNA increased polymerase activity with a subsequent decrease in nucleotide incorporation fidelity and thus a rise in mutagenicity. Moreover, the bound chromium can act as a bridge linking DNA to protein or DNA to DNA. The formation of interstrand cross-links on a double stranded pSV2*neo*TS plasmid DNA upon incubation with chromate and AsA or Cr(III) complexes have been shown to cause DNA polymerase arrests during DNA replication *in vitro* (Bridgewater *et al.*, 1994).

### DNA - Protein Cross-Links

The formation of DNA-protein cross links represents another type of DNA lesion which is not readily repaired and may therefore affect the DNA replication process (Costa, 1991; Xu *et al.*, 1994).

Aqueous Cr(III) complexes can directly induce the formation of DNA-protein cross-links in plasmid DNA in the presence of bovine serum albumin (BSA), actin and histones, the efficiency of the cross-linking depending on the content of cysteine/histidine in the proteins, BSA > actin > histone (Salnikow *et al.*, 1992).

Cr(VI) does not induce protein cross-links unless a reducing agent is present in the system such as microsomes/NADPH or ascorbic acid (Tsapakos and Wetterhahn, 1983, Salnikow *et al.*, 1992). Borges and Wetterhahn (1989) observed that incubation of calf-thymus DNA in the presence of Cr(VI) and GSH or Cys for 30 minutes resulted in the cross-linking of the thiols to DNA via Cr(III), the levels of Cys cross-linked were ten times lower than with GSH.

Recently, a new type of DNA cross-link was identified in CHO cell exposed to Cr(VI). Chromium was cross-linked to GSH and free amino acids, mainly cysteine, glutamic acid and histidine. These type of adducts represent a major group of DNA adducts as it involved 50 % of the bound chromium. The biological significance of these type of cross-links is currently under investigation (Zhitkovich *et al.*, 1995).

### 1.2.4 The Nature of the Damaging Species

The nature of the reactive species responsible for the formation of DNA damage is far from clear. *In vitro* studies have shown that Cr(VI) cannot induce the formation of lesions in isolated DNA unless a reducing agent is present (Tsapakos and Wetterhahn, 1983). Reactive intermediates formed during the reduction of chromate by various reducing agents are thought to be responsible for the induction of DNA lesions such as SSB and Cr-DNA adducts (Kortenkamp *et al.*, 1989; Salnikow *et al.*, 1992). However, the redox reactions of Cr(VI) with GSH and AsA are complex and the DNA damaging species are not easily identified (O'Brien *et al.*, 1992; Stearns and Wetterhahn, 1994). In addition, the formation of reactive intermediates in *in vitro* systems is highly dependent on the experimental conditions of the study (buffer, pH, concentrations of the reactants), which makes extrapolations to the *in vivo* situation extremely difficult. Cr(III) complexes, despite their kinetic inertness, have been shown to be able to react slowly with isolated DNA leading to the formation of Cr-DNA adducts and protein cross-links, although their ability to attack DNA *in vivo* is unknown (Tsapakos and Wetterhahn, 1983; Salnikow *et al.*, 1992). *In vivo*, the contribution of the different species in the mediation of chromium genotoxicity will depend on the amount of unreduced Cr(VI) or

reactive intermediates able to reach the cell nuclei. The following is a discussion of the various reactive intermediates formed during the reduction of chromium(VI) and their potential to cause DNA lesions.

## Chromium Species

### Chromium(V)

Chromium(V) is a common intermediate in the reduction of Cr(VI), its formation is easily detected by electron spin resonance (ESR) because these compounds contain one unpaired electron ($d^1$).

Upon treatment of chick embryos *in vivo* with sodium dichromate the formation of two transient Cr(V) complexes was observed in the red blood cells, while a single more stable Cr(V) species was formed in the liver (Liebross and Wetterhahn, 1992). The formation of a Cr(V) complex was observed upon incubation of cultured Chinese hamster V-79 cells with sodium chromate (200 μM) for two hours. Pre-treatment of the cells with AsA resulted in a dramatic decrease in the amount of Cr(V) formed (Sugiyama *et al.*, 1991). On the other hand, depletion of cellular GSH using buthionine sulfoximine led to 170 % increase in the Cr(V) signal when the GSH depletion exceeded 85 % (Sugiyama and Tsuzuki, 1994).

In *in vitro* systems, Cr(V) complexes are formed upon reduction of Cr(VI) by several thiols (GSH, Cys, dithiothreitol and β-mercaptoethanol), the amount and half-life of the Cr(V) signal being dependent on the nature of the thiol and that of the buffering agent (O'Brien *et al.*, 1985; Goodgame and Joy, 1986; Kitagawa *et al.*, 1988; Aiyar *et al.*, 1989; Borges *et al.*, 1991). The Cr(V) complex was greatly stabilised when GSH was used as reductant, whereas it disappeared rapidly in the presence of Cys. In a mixture of 10 mM Cr(VI)/30 mM GSH or Cys at pH 7, the half-lives of Cr(V) species were 23 - 30 minutes and 3 minutes respectively. The stability of the Cr(V) in GSH solutions may have important consequences in the mediation of chromium(VI) toxicity (Kitagawa *et al.*, 1988). In fact, it was possible to isolate a Cr(V) species formed during the reduction of chromate by GSH with a formula $Na_4Cr(GSH)_4 \cdot 8H_2O$ (O'Brien *et al.*, 1990). The formation of Cr(V) during the reduction by GSH is unaffected by the removal of molecular oxygen (Kortenkamp *et al.*, 1995).

The formation of chromium(V) during the reduction of chromate by AsA is well documented in unbuffered solutions and in the presence of 4-(2-hydroxy-ethyl)-1-piperazineethane-sulphonic acid (HEPES) buffer. The intensity of the ESR signal has been shown to be dependent on the ratio of both reactants, the optimal formation of Cr(V) was observed at AsA:Cr(VI) ratio of 0.5:1, with a half life of 10 - 15 minutes at room temperature. The Cr(V) signal decreased with increasing AsA concentration, and at ratios 1:2, 1:3 and 1:5 it was no longer detected. No Cr(V) signal was observed at any ratio in Tris-(hydroxymethyl) aminomethane hydrochloride (Tris-HCl) buffer (Goodgame and Joy, 1987; Stearns and Wetterhahn, 1994). The formation of Cr(V) is independent of the presence or absence of oxygen (Lefebvre and Pézerat, 1992; Stearns and Wetterhahn, 1994).

Changes in the pH of the solutions led to dramatic effects on the spectra observed when using either GSH or AsA as reductants (Kitagawa *et al.*, 1988; Goodgame and Joy, 1987). The species formed are clearly affected by the experimental conditions such as nature of the buffering agent, pH and concentrations of the reactants.

### *Induction of DNA Lesions*

Cr(V) complexes are able to induce SSB in isolated DNA. $Na_4Cr(GSH)_4 \cdot 8H_2O$, isolated from Cr(VI)-GSH reaction (O'Brien *et al.*, 1990), induced SSB in PM2 DNA in a concentration dependent manner in HEPES buffer, pH 7 (Kortenkamp *et al.*, 1989). It is however unclear whether Cr(V) itself is the causative agent under these conditions.

The ability of certain Cr(V) complexes to cause SSB is correlated with the lability of their ligands. At acidic pH, $[Cr(V)(ehba)_2O]^-$ and $[Cr(V)(hmba)_2\ O]^-$ caused nicks in plasmid DNA, but not the very stable $Cr(V)[(mampa\text{-}dcb)(O)]^-$ (ehba = 2-hydroxy-2-ethylbutanoato (2-); hmba = 2-hydroxy-2-methylbutanoato (2-); mampa-dcb = 5,6-(4,5,-dichlorobenzo)-3,8,11,13-tetraoxo-2,2,9,9,-tetramethyl-12,12-diethyl-1,4,7,10-tetraazacyclotridecane). Nevertheless, the three Cr(V) compounds were mutagenic when assayed with *Salmonella typhimurium* TA100 (Farrell *et al.*, 1989; Dillon *et al.*, 1993).

Potassium tetraperoxochromium(V) was able to cause not only SSB but also alkaline labile sites in a 110-bp DNA fragment (Kawanishi *et al.*, 1986).

It has been proposed by Wetterhahn and co-workers (Borges *et al.*, 1991; Misra *et al.*, 1994; Stearns *et al.*, 1995) that Cr(V) complexes mediate the binding of chromium to DNA. Such suggestion is based on the fact that the levels of Cr(V) produced in a particular system correlate with the level of Cr-adducts observed. During the reduction of chromate by various thiols, such as dithiothreitol, β-mercaptoethanol, GSH and Cys, the highest levels of Cr-binding were observed for dithiothreitol and β-mercaptoethanol which also produced the highest amount of Cr(V) (Borges *et al.*, 1991). In chick embryos treated with Cr(VI), the formation of Cr-DNA adducts occurred in the liver but not in red blood cells. In the liver, Cr(V) species with longer half-lives than the ones in red blood cells are formed (Misra *et al.*, 1994). Upon treatment of DNA with ascorbic acid/Cr(VI) *in vitro,* the highest level of chromium-DNA binding was observed at low AsA concentrations when maximum Cr(V) was produced (Stearns *et al.*, 1995).

### Chromium(IV)

Bose *et al.* (1992) observed the formation of a long-lived Cr(IV) intermediate during the reduction of chromate by GSH at acidic pH, its presence was inferred from magnetic susceptibility measurements, as Cr(IV) complexes are ESR silent. It was found that Cr(IV) was more predominant than Cr(V).

Recently, Stearns and Wetterhahn (1994) monitored the formation of Cr(IV) indirectly by using Mn(II) as a probe in solutions containing Cr(VI) and AsA. The decrease in the ESR signal of Mn(II) was recorded (Mn(II) + Cr(IV) → Cr(III) + Mn(III)), and the level of Cr(IV) was observed to increase with rising AsA levels. Kortenkamp *et al.* (1995) have extended this approach to Cr(VI) and GSH solutions at neutral pH, and observed a similar decrease in the ESR signal of Mn(II) which was taken as evidence for the generation of Cr(IV) during the reduction process. These species are highly oxidising and extremely reactive, and could therefore lead to the formation of DNA lesions (Scott *et al.*, 1992).

### Chromium(II)

Recently, Perez-Benito *et al.* (1994a) observed the formation of a superoxo-chromium(III) species during the reduction of chromate by GSH, by following the appearance of two peaks in the 200 - 300 nm region of the UV-Visible spectra. This observation was taken as indirect evidence that Cr(II) had been formed during the reduction process. The only known route for the formation of the superoxo-chromium(III) is by the reaction of Cr(II) with oxygen, $Cr(II) + O_2 \rightarrow Cr(III)(O_2)^{\cdot}$. They proposed that Cr(II) species, identified for the first time, could be important in the mediation of chromium(VI) mutagenicity (Perez-Benito *et al.*, 1994a). It is unlikely that Cr(II) species would be able to attack directly DNA, as these complexes are readily oxidised to Cr(III) in the presence of air. However, because of their reactivity towards oxygen, they could lead to the formation of other reactive species capable of inducing DNA lesions.

### Chromium(III)

Cr(III) species are the final product of the reduction of chromium(VI). Although the octahedral complexes are quite inert, certain Cr(III) complexes bind to isolated DNA and cell nuclei (Tsapakos and Wetterhahn, 1983; Hneihen *et al.*, 1993; Kortenkamp *et al.*, 1991), and can cross-link proteins such as BSA, actin or histones to plasmid DNA *in vitro* (Salnikow *et al.*, 1992).

### Reactive Oxygen Species:

Due to their high reactivity, oxygen reactive species such as hydroxyl radicals can only be monitored using indirect methods. One possible approach is ESR spectroscopy in combination with spin-traps which react with ·OH to form more stable radicals. Another possibility is the screening for DNA lesions, such as certain DNA base-adducts e.g. 8-hydroxydeoxyguanosine (8-OH-dG), which are thought to arise from attack by oxygen reactive species.

The involvement of such species in the induction of DNA lesions by chromate has been suggested by several workers (Kawanishi *et al.*, 1986; Aiyar *et al.*, 1989; Kortenkamp *et*

*al.*, 1989; Shi and Dalal, 1992; Shi *et al.*, 1994). Kawanishi *et al.* (1986) detected the formation of ·OH during the reduction of chromate by $H_2O_2$ in the presence of the spin-trap 5,5-dimethyl-1-pyrroline-N-oxide (DMPO). These results were confirmed by Aiyar *et al.* (1989), who not only observed the formation of the DMPO-OH adduct during the reaction of Cr(VI) with $H_2O_2$, but also the formation of 8-OH-dG when calf-thymus DNA was present in the reaction mixture. Reaction of Cr(VI) in the presence of GSH did not lead to the formation of ·OH radicals unless $H_2O_2$ was added to the mixture (Aiyar *et al.*, 1989). In similar studies Shi *et al.* (1994) detected the formation of ·OH radicals in mixtures of Cr(VI)/AsA/$H_2O_2$. It has been proposed that the generation of such reactive species can occur via Fenton chemistry by reaction of chromium in an intermediate oxidation state with $H_2O_2$ ($M^{(n-1)+} + H_2O_2 \rightarrow M^{n+} + \cdot OH + OH^-$). The presence of a reductant would ensure a continuous supply of catalytic metal ions (Kawanishi *et al.*, 1986; Shi *et al.*, 1994). However, the relevance of these observations to the *in vivo* situation is not clear. The concentrations of $H_2O_2$ used in the above studies were in the millimolar range (1 - 18 mM) which are in vast excess of the expected intracellular levels (*ca.* $10^{-7}$-$10^{-9}$ M) (Standeven and Wetterhahn, 1991a).

Jones *et al.* (1991), in spin trapping experiments with 3,5-dibromo-4-nitrosobenzene (DBNBS) and dimethyl sulfoxide (DMSO), detected the formation of the DBNBS-$CH_3$· radical in the presence of the Cr(V)-GSH complex, $Na_4Cr(GSH)_2(GSSG)\cdot 8H_2O$. A strongly oxidising species was able to cleave the C-S bond of DMSO to form $CH_3$·, which was then trapped by DBNBS. At the time it was thought that these species were hydroxyl radicals. A mechanism involving thiyl radicals and Fenton chemistry was proposed as a route for the formation of ·OH. However, the same authors later disproved that ·OH radicals were generated during the reduction of chromate by GSH by competition kinetic experiments (Kortenkamp and O'Brien, 1994). They concluded that the formation of ·OH radicals was unlikely, and suggested that a highly oxidising species which is able to abstract a $CH_3$· from DMSO was generated.

In *in vivo* studies, no significant increase in the levels of 8-OH-dG in liver or kidney DNA from rats treated with sodium chromate were observed (Standeven and

Wetterhahn, 1991a). In chick embryos treated with sodium chromate, an increase in the levels of 8-oxo-dG was observed in red blood cells but not in liver cells. The authors hypothesised that the high levels of haemoglobin present in red blood cells may have induced the formation of ·OH (Misra *et al.*, 1994). More *in vivo* studies are required in order to establish the formation of reactive oxygen species during the reduction of Cr(VI). Moreover, the 8-OH-dG assay needs validation, because it is not known whether it is sensitive enough to detect the low levels of reactive oxygen species generated *in vivo* (Standeven and Wetterhahn, 1991a).

### Thiyl, Ascorbyl and Carbon Centred Radicals

Thiyl radicals are formed *in vitro* during the reduction of chromate by GSH as detected by ESR/spin-trap studies (Aiyar *et al.*, 1989). Although GS· is not likely to cause DNA damage directly, it can start a series of radical reactions which could lead to the production of reactive species such as superoxide, hydrogen peroxide and even hydroxyl radicals (Saez *et al.*, 1982).

Ascorbyl radicals and carbon centred radicals are two of the intermediates detected during the reduction of chromate by AsA, their ability to cause DNA lesions is unknown (Stearns and Wetterhahn, 1994). Stearns *et al.* (1995) have suggested that carbon centred radicals could be responsible for the formation of SSB in supercoiled DNA pBR322 during the reduction of chromate by AsA in HEPES or Tris buffers (pH 7). This idea is based on the fact that the highest levels of SSB were generated under conditions which also led to the highest level of carbon radicals being formed.

A broad spectrum of reactive intermediates is generated during the reduction of chromate, which makes difficult to discern the ones that are responsible for the induction of DNA lesions. However, it is becoming clear, from *in vivo* and *in vitro* studies, that molecular oxygen has an important role in the mediation of chromate carcinogenicity (Snyder, 1988; Sugden *et al.*, 1990; Kortenkamp *et al.*, 1990; Lefebvre

and Pézerat, 1992). It is not known if reactive oxygen species such as hydroxyl radicals, which are formed under certain experimental conditions *in vitro*, are of any importance in the *in vivo* situation.

The oxidative nature of Cr(V) and (IV) complexes formed during the reduction process, makes them likely candidates to interact with DNA. However, it is not firmly established if these compounds can directly attack DNA or they require previous activation by oxygen species (Farrell *et al.*, 1989; Lefebvre and Pézerat, 1992; Kortenkamp *et al.*, 1995). Further *in vitro* studies, mimicking the intracellular environment that chromate may encounter, are necessary to answer these questions and to elucidate the species capable of inducing DNA lesions.

## *1.3 Apurinic/Apyrimidinic-Sites (AP-Sites)*

The formation of alkaline labile sites was observed upon exposure of cultured mammalian cells to chromate (Cantoni and Costa, 1984). However the nature of those sites has never been investigated. The alkaline lability of the DNA phosphate backbone can be due to the presence of phosphotriester adducts or AP-sites. Considering the potential mutagenicity of the latter it is important to establish if AP-sites are formed in DNA upon exposure to chromates. The following is a review of the processes leading to the formation of AP-sites, their chemical structures, their repair and potential mutagenicity.

### 1.3.1 Formation and Structure of AP-Sites

The formation of DNA apurinic/apyrimidinic sites results from the cleavage of the N-glycosidic bond that connects the deoxyribose sugar with the purine or pyrimidine base. The base is released leaving the DNA phosphodiester backbone intact.

Depurination and depyrimidation occur at significant rates under physiological conditions (Lindahl and Andersson, 1972). The rate of base release increases with low pH and high temperature, purines being released more readily than pyrimidines (Loeb and Bradley, 1986; Povirk and Steighner, 1989). Chemical modification of either the DNA base or the deoxyribose sugar can further increase the rate of AP-sites formation.

Electrophilic mutagens that readily react with the nucleophilic sites of the DNA bases (N-7 for G; N-3 for A and O-2 for C and T) to form base-adducts can decrease the stability of the N-glycosidic bond rendering it prone to hydrolysis. The formation of AP-sites by such agents is well documented in different studies (Drinkwater *et al.*, 1980; Gamper *et al.*, 1980; Loeb and Bradley, 1986; Prakash and Gibson, 1992). Using AP-endonucleases in combination with electrophoretic techniques, Drinkwater *et al.* (1980) detected the formation of AP-sites upon treatment of isolated SV40 DNA with a variety of electrophilic compounds, ranging from simple alkylating agents, such as methyl

methanesulfonate and N-methyl-N-nitrosourea, to aralkylating agents like benzo(a) pyrene-diol-epoxide and aromatic amines such as N-acetoxy-2-acetylaminofluorene.

AP-sites are also generated as intermediates of the DNA repair process. DNA glycosylases catalyse the hydrolysis of N-glycosidic bonds of damaged or modified DNA bases, thereby forming an abasic site. The repair is completed by the action of AP-endonucleases, DNA polymerase and ligases (Lindahl, 1982). As shown in **Figure 1.3-1**, the AP-sites formed by hydrolysis of the N-glycosidic bond exist in equilibrium between the furanose and the open aldehyde form, the latter representing less than 1 % of the total abasic sites (Wilde *et al.*, 1989).

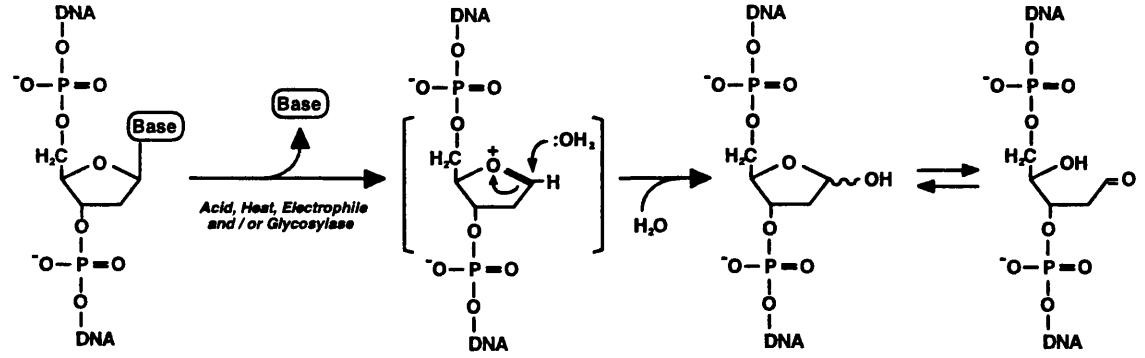



**Figure 1.3-1**: Formation and structure of AP-sites induced by hydrolysis of the N-glycosidic bond. Adapted from Loeb and Bradley (1986).

Hydrogen abstraction from C-1', C-2' or C-4' of the deoxyribose moiety is another route by which AP-sites can be formed (see Povirk and Steighner (1989) for a review). The formation of these oxidised AP-sites requires the presence of a very powerful oxidant such as radical or ferryl species; the position from which the hydrogen is removed will determine the structure of the AP-site (**Figure 1.3-2**). The natural antitumour antibiotics, bleomycin and neocarzinostatin (NCS), are two of the best studied examples and their mechanism of action is known in great detail (for reviews see Stubbe and Kozarich, 1987; Dedon and Goldberg, 1992a). Whereas bleomycin

exclusively induces 4'-hydroxylated AP-sites by oxidation of C-4' (**Figure 1.3-2 A**), NCS not only generates 4'-hydroxylated AP-sites but also lactone type AP-sites via C-1' oxidation (Kappen *et al.*, 1988; Kappen and Goldberg, 1989) (**Figure 1.3-2 B**). These two types of AP-sites have also been detected in DNA treated with Cu-phenanthroline or ionising radiation (Sigman, 1986; von Sonntag, 1987). The formation of AP-sites via C-2' hydrogen abstraction has only been observed upon γ-irradiation of aqueous DNA solution (Povirk and Steighner, 1989) and not much is known about its chemistry. They contain an erythrose group (**Figure 1.3-2 C**). Recently, Sugiyama *et al.* (1993) developed a method for their detection. Abstraction of the hydrogens from C-3' or C-5' results in immediate strand breakage, and although the bases may be released in the process, they are not classified as AP-sites.

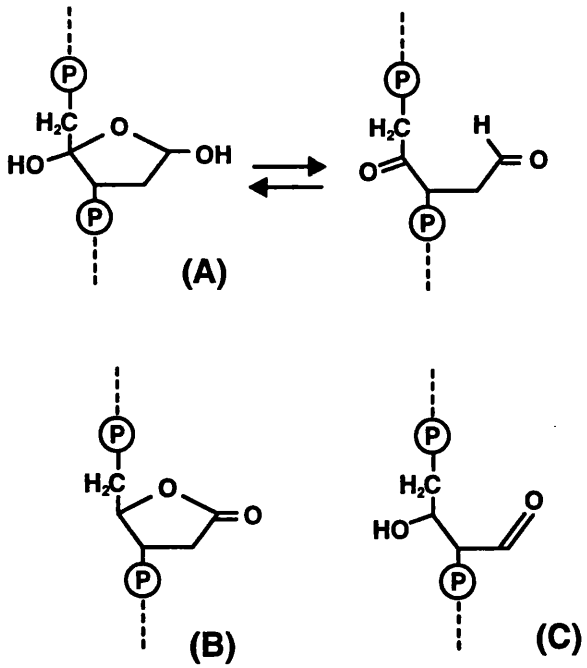



**Figure 1.3-2**: Structure of AP-sites formed by oxidation of the different carbons of the deoxyribose moiety. A) 4'-Hydroxylated AP-site, induced by oxidation at C-4'; B) Lactone-type AP-sites, involving C-1' oxidation; C) Erythrose-containing AP-site, formed by C-2' oxidation.

## 1.3.2 Repair and Recognition of Abasic Sites

AP-sites render the adjacent DNA phosphate backbone susceptible to hydrolysis. Under physiological conditions, the average lifetime of the DNA chain at an abasic site is *ca.* 190 hours; however at high pH values, the DNA phosphate backbone is readily hydrolysed via a β-elimination reaction forming a DNA SSB (Lindahl and Nyberg, 1972; Mazumder *et al.*, 1991). Abasic sites induced under normal physiological conditions, as a consequence of DNA-base adducts or by the action of DNA glycosylases (**Figure 1.3-1**), are repaired efficiently by AP-endonucleases which catalyse the excision of the DNA phosphate backbone and the removal of the abasic nucleotide. These enzymes, which are ubiquitous in both prokaryotes and eukaryotes cells are classified into two major types: Type I enzymes cleave at the 3' side of an AP-site, and type II cleave at the 5'side. Exonuclease III, the major AP-endonuclease of *Escherichia coli* (*E. coli*), belongs to the latter group (Lindahl, 1982; Loeb and Bradley, 1986). Oxidised AP-sites (**Figure 1.3-2**) represent modified substrates which may not be recognised by these enzymes (Loeb and Bradley, 1986; Povirk and Steigner, 1989). It has been demonstrated *in vitro* that AP-sites induced by the antitumour agents NCS and bleomycin are far less sensitive to recognition by exonuclease III and endonuclease III, than those induce by acid-heat depurination, which have the same chemical structure as AP-sites induced under normal physiological conditions (Povirk and Goldberg, 1985; Povirk and Houlgrave, 1988).

Hydrolysis of the DNA backbone at AP-sites is also catalysed by a variety of aliphatic and aromatic amines, such as putrescine, spermidine, the tripeptides Lysine-Tryptophan-Lysine (Lys-Trp-Lys) and Lysine-Tyrosine-Lysine (Lys-Tyr-Lys), 9-$NH_2$ ellipticine and chromatin components, especially histones. The cleavage occurs via β-elimination upon formation of a Schiff base between the C-1' aldehyde group of the hydrolytic induced AP-sites and an amino group of these agents (see **Figure 2.3-4**) (Lindahl and Nyberg, 1972; Pierre and Laval, 1981, Malvy *et al.*, 1986; Bailly and Verly, 1988; Mazumder *et al.*, 1991). The capacity of AP-sites to react via their aldehyde group with amino groups could have important physiological consequences, as it may provide a route for the

formation of DNA interstrand-cross-links and protein cross-links. The former may arise from reaction of the AP-sites with the amino groups of the bases on the opposite DNA strand, whereas the latter DNA lesion could be formed by the interaction between the lysine residues of histones and the AP-sites (Freese and Cashel, 1964; Prakash and Gibson, 1992; Mirzabekov *et al.*, 1978).

### 1.3.3 Mutagenicity of AP-Sites

There is a growing evidence that AP-sites are highly mutagenic in both prokaryotic and eukaryotic cells (see Loeb and Bradley, 1986 for a review). DNA polymerases have been shown to be able to bypass these DNA lesions *in vitro* by inserting a non-complementary nucleotide opposite to an AP-site. The misincorporations induced are generally single-base substitutions with preferential incorporation of adenine.

The mutagenic potential of AP-sites in bacteria was studied by transfecting *E. coli* with viral DNA (ΦX174 *am3*, M13 *mp2*) containing various amounts of AP-sites induced by acid/heat treatment. An increase in the mutagenicity was observed, which was SOS-repair dependent and could be abolished by pre-incubating the DNA with AP-endonucleases. Base substitutions were the most frequent cause of mutations, adenine having the higher frequency of misincorporation relative to the other bases. AP-sites have been suggested as the causative lesion of mutagenesis for a variety of carcinogens, including aflatoxin B1, benzo[a]pyrene-diolepoxide, bleomycin and NCS. This proposition is mainly based on the finding that the mutations induced by these agents present characteristics similar to those induced by acid/heat depurinated DNA, as well as similar mutational spectra (predominantly G:C-T:A, A:T-T:A transversions and G:C-A:T transitions). Moreover, the mutations are SOS-repair dependent. In the case of bleomycin and NCS, using a combination of mutation screening and sequencing techniques, it was possible to establish a correlation between mutation hot-spots and sequence specifically induced AP-sites (Povirk, 1987; Povirk and Steighner, 1989), adding further support to the suggestion that the mutagenicity caused by bleomycin and NCS is mediated by AP-sites.

## 1.4 Scope of This Thesis

Considerable progress has been made in trying to elucidate the mechanisms by which chromium compounds exert their mutagenicity and ultimately their carcinogenicity. Much is now known about the different DNA lesions induced *in vivo* and *in vitro* by chromium compounds, about the importance of the different intracellular reductants and about the nature of the various intermediate reactive species which may cause DNA damage.

In order to further advance the elucidation of the mechanisms of chromium genotoxicity, it is necessary to identify and describe the DNA lesions which are likely to contribute to the mutagenicity of chromate. SSB induced by chromium are easily repaired by cells and are therefore unlikely to lead to mutations. On the other hand, protein cross-links and chromium-DNA adducts may represent a challenge for the DNA repair machinery and may affect replication and transcription of the DNA. The mutagenic potential of alkaline labile sites had not been assessed, because the nature of the lesion was unknown. Alkaline labile sites may be caused by either loss of a DNA base, i.e., the generation of AP-sites, or by the formation of an adduct with the DNA phosphodiester bond i.e., forming a phosphotriester adduct.

The aim of the present studies was to investigate if the alkaline labile sites observed in mammalian cells upon exposure to chromate (Cantoni and Costa, 1984) were caused by AP-sites. These lesions are potentially mutagenic (Loeb and Bradley, 1986) and could be important in the mediation of chromium carcinogenicity.

It was our intention not only to establish the possible formation of AP-sites during the reduction of chromate, but also to investigate the nature of the reactive species responsible for the damage and its mechanism of action. Therefore, a simple well defined *in vitro* system was selected to carry out the investigations. *In vivo* systems and cultured cells present too many variables and are far too complex for mechanistic studies.

We decided to investigate the possible formation of AP-sites in isolated PM2 DNA during the reduction of chromate by GSH. The tripeptide glutathione was chosen as a reductant because extensive work had already been carried out on the effects of chromate and GSH mixtures in the formation of DNA lesions (Kortenkamp *et al.*, 1989; Borges *et al.*, 1991).

The following questions were addressed in the present investigation:

- Possible formation of AP-sites in isolated DNA during the reduction of chromate by GSH (Chapter 2).

- Possible link between the formation of AP-sites and other DNA lesions induced by chromate and GSH (Chapter 2).

- The nature of the reactive species responsible for the DNA damage. The role of molecular oxygen and the possible formation of hydroxyl radicals were investigated (Chapters 3, 6).

- Mechanistic pathways leading to the formation of SSB and AP-sites during the reduction of chromate by GSH (Chapters 2, 4, 5).

- Reduction of chromate by GSH under anoxic and aerobic conditions (Chapter 6).

# *CHAPTER 2*

# THE FORMATION OF SSB AND AP-SITES IN ISOLATED DNA DURING THE REDUCTION OF CHROMATE BY GSH

## *2.1 Introduction*

Alkaline elution studies using cultured mammalian cells exposed to chromate provided evidence of the formation of alkaline labile sites (Christie *et al.*, 1984; Cantoni and Costa, 1984). These sites can be a consequence of the formation of phosphotriester adducts or AP-sites. Both DNA lesions cause an increased lability of the phosphodiester backbone, rendering it vulnerable to alkaline hydrolysis.

The potential mutagenicity of AP-sites (Loeb and Bradley, 1986) prompted us to investigate the possible formation of this DNA lesion by chromate. A simple, well defined *in vitro* system was chosen to carry out the studies: supercoiled DNA from *Pseudomonas* bacteriophage PM2, potassium chromate and an intracellular reductant, at physiologically relevant concentrations and neutral pH. The tripeptide GSH was selected as the reductant. Present at millimolar concentrations in the cytosol of mammalian cells, it is one of the intracellular reductants thought to be involved in the mediation of chromate carcinogenicity (Connett and Wetterhahn, 1983). DNA lesions known to be induced during the reduction of chromate by GSH include SSB, Cr-DNA binding and DNA protein cross-links (Kortenkamp *et al.*, 1989; Borges *et al.*, 1991; Standeven and Wetterhahn, 1991a).

The presence of AP-sites can be unambiguously identified by the use of agents that recognise the absence of a DNA base and cleave the DNA phosphate backbone, such as amino-containing agents (putrescine, spermidine, Lys-Trp-Lys and 9-$NH_2$-ellipticine

among others) and AP-endonucleases (endonuclease III, exonuclease III and endonuclease IV from *E. coli* among others) (Pierre and Laval, 1981; Behmoaras *et al.*, 1981; Male *et al.*, 1982; Loeb and Bradley, 1986). The induction of one break in one of the strands of the supercoiled PM2 DNA will result in the relaxation of the molecule to the open circular form. The two forms can be easily separated by gel electrophoresis and the level of breakage quantified as described in Appendix I.

The use of an *in vitro* system in which the conditions can be carefully controlled provides the means to gain information on the nature of the reactive species responsible for the DNA damage, as well as the possibility to elucidate the mechanism by which DNA lesions are being formed.

## 2.2 Experimental Section

### Chemicals

PM2 DNA and exonuclease III from *E. coli* were obtained from Boehringer Mannheim (Lewes, East Sussex), microfilters Microcon-30 were from Amicon (Stonehouse, Gloucestershire). Putrescine, glutathione, Lys-Trp-Lys, Lys-Tyr-Lys and sodium borohydride were purchased from Sigma (Poole, Dorset). Potassium chromate was from BDH (Lutterworth, Leicestershire). Chelex 100 resin and agarose were obtained from Biorad (Hemel Hempstead, Herts). All reagents were of analytical grade.

### The Detection of AP-Sites

The possible generation of AP-sites in the supercoiled DNA of the *Pseudomonas* bacteriophage PM2 was studied by making use of the fact that certain repair enzymes (AP-endonucleases) and polyamines such as putrescine or the tripeptide Lys-Trp-Lys incise the DNA phosphate backbone were the loss of a base has occurred. The induction of a single strand break in supercoiled DNA (form I) causes the molecule to relax to the open circular form (form II). The two forms are easily separated by gel electrophoresis and can be quantified as explained in Appendix I. The reduction of chromate by GSH is known to cause SSB in PM2 DNA (Kortenkamp *et al.*, 1989). Thus, a further increase

in the level of breakage in Cr(VI)/GSH-treated DNA upon post-incubation with either putrescine, tripeptide or exonuclease III is evidence for the generation of AP-sites.

### Acid Heat Depurination

The effectiveness of putrescine, exonuclease III and the tripeptide to reveal AP-sites was tested in a model system, in which AP-sites were induced by exposing DNA to acid pH and high temperatures (70°C). It was important to establish optimal incubation times and concentrations of the agents, so that all the AP-sites generated were detected. This system allowed us to establish the proper reaction conditions for future experiments with chromate and GSH.

Isolated PM2 DNA (16 μg/ml) was incubated in hot sodium citrate (10 mM, pH 5.5) for eleven minutes at 70°C. Cold neutralisation buffer (20 mM HEPES, 1 mM $CaCl_2$, 2 mM mercaptoethanol, pH 7.4) was added and the sample plunged into ice. Aliquots (20 μl), containing 8 μg/ml of PM2 DNA, were placed in different tubes and the AP-sites revealed by treatment with either putrescine (10 mM), exonuclease III (0.5 units/μl) or Lys-Tyr-Lys (0.1 mM) for different time periods (0 - 120 min) at 37°C. Control samples contained the same components but were not heated. The reactions were stopped by the addition of electrophoresis loading buffer and gel electrophoresis was carried out immediately, as described below.

### General DNA Treatment. AP-Sites Detection

Isolated PM2 DNA (8 μg/ml) was incubated with potassium chromate and GSH (the concentrations and duration of treatment are described in the following sections) in phosphate buffer (pH 6.8, 0.1 M) unless otherwise stated, in a final volume of 20 μl. Control samples contained DNA and GSH or DNA and Cr(VI) and were subjected to the same treatments as the rest of the samples. Before post-incubation with either of the agents used to reveal AP-sites, the residual reactants were removed from DNA.

The removal of unreacted chromate and GSH was essential in order to obtain meaningful results. It was observed that when post-incubations with putrescine were carried out without prior removal of the reactants, the level of AP-sites paradoxically decreased the longer DNA was treated with chromate and GSH. It was shown that this

effect was caused by an interaction between the reactants and putrescine, which resulted in a high level of DNA breaks. The more unreacted GSH and chromate remained (short incubation times) the more pronounced the effect became. In order to remove the residual reactants, samples were originally dialysed for 4 hours at 4°C. During the first two hours they were dialysed against HEPES buffer (15 mM, pH 6.8), subsequently, samples to be treated with exonuclease III were dialysed against a buffer containing citrate (50 mM), HEPES (10 mM), calcium chloride dihydrate (0.5 mM) and mercaptoethanol (1 mM), pH 6.3 while the remaining ones were further dialysed against fresh HEPES buffer (15 mM, pH 6.8). The function of calcium and citrate in the exonuclease III incubation buffer was to minimise the exonucleolytic properties of the enzyme while preserving its AP-endonuclease activity (Rogers and Weiss, 1980). Putrescine (10 mM), Lys-Trp-Lys (0.1 mM) or exonuclease III (0.5 units/µl) were added and the samples post-incubated for 1 hour at 37°C.

Modifications of the above procedures yielded more consistent results. The time consuming dialysis steps for the exchange of buffer were replaced by microfiltration (Microcon-30). The resulting filtration concentrates were resuspended in a buffer containing citrate (5 mM), HEPES (10 mM), calcium chloride dihydrate (0.5 mM) and mercaptoethanol (1 mM), pH 7.4 and then incubated with exonuclease III. When putrescine was used to reveal AP-sites, the DNA concentrate was diluted with HEPES (15 mM, pH 6.8). The experimental conditions of the post-incubation step were slightly modified. DNA was post-incubated at room temperature instead of 37°C as before. Use of microfiltration for the exchange of buffers seemed to render the DNA prone to breakage; an excessive level of DNA breaks was observed in all DNA samples when post-incubations were carried out at 37°C. The problem was solved by carrying out the post-incubation with putrescine and exonuclease III at room temperature. In order to maintain the same effectiveness in revealing AP-sites, it was necessary to increase the enzyme concentration to 1 unit/µl. The putrescine concentration could remain unchanged (10 mM).

Post-incubations were stopped, in both methods, by addition of gel electrophoresis loading buffer (5 µl, 0.25 % bromophenol blue and 40 % sucrose). Agarose gel electrophoresis was carried out immediately after the termination of the incubations, as described below.

### The Effect of Chromium(VI) Concentration on the Level of DNA Lesions

Isolated PM2 DNA was incubated with GSH (5 mM) and potassium chromate (0.075 - 0.2 mM) or with each reactant alone in phosphate buffer (0.1 M, pH 6.8) for three hours at room temperature. The residual reactants were removed by dialysis and the AP-sites revealed by post-incubation with either exonuclease III, putrescine or Lys-Trp-Lys in the appropriate buffers.

### Sodium Borohydride Treatment

After treatment with chromate/GSH (0.175/5 mM respectively) for 3 hours the DNA was concentrated using microfilters and resuspended in 0.5 M phosphate buffer (pH 6.7). Aliquots of $NaBH_4$ (0.4 M initial concentration, in water) were added three times, at 30 minutes intervals, until a final concentration of 0.2 M was reached. After incubation for 90 minutes, $NaBH_4$ was removed from the samples by extensive washing with HEPES buffer (15 mM) (microfiltration) and then treated with putrescine or exonuclease III in appropriate buffers. The level of strand breaks observed after $NaBH_4$ treatment was considerably higher than in other samples throughout the present investigation. This can be easily explained by a shearing effect due to the additional steps of centrifugation during microfiltrations, which were necessary to remove unreacted $NaBH_4$ before adding putrescine or exonuclease III.

### Time Course Studies

The time course of the induction of SSB and AP-sites were carried out by treating PM2 DNA with chromate/glutathione (0.175/5 mM respectively) for time periods ranging from 1 to 5 hours. AP-sites were determined using putrescine as described.

In another series of experiments, chromate and glutathione (0.175/5 mM) were mixed in the absence of DNA and kept for different periods of time ('aged') (0 - 4 h) before being added to PM2 DNA and incubated for four hours. AP-sites were revealed by using exonuclease III.

### Gel Electrophoresis

Gel electrophoresis (0.8 % agarose, TBE buffer, 1 hour at 2.3 V/cm) was performed immediately after addition of electrophoresis loading buffer (0.25 % bromophenol blue, 40 % sucrose). The gels were stained with ethidium bromide (1 mg/ml) in the dark for 15 minutes, destained in water overnight and photographed under UV light. The intensity of the DNA bands was measured directly from the photographs using a densitometer equipped with digitalized image analysis using the computer programme PC-Image Plus, version 1.54. Peaks were integrated and the number of breaks per DNA molecule calculated as described in the Appendix I.

### Removal of Catalytic Metal Ions

Where indicated, traces of iron and copper were removed from buffer solutions by stirring overnight with Chelex 100 resin. The resin beads were removed by filtration; it was observed that an increase in buffer pH occurred when the resin was allowed to stand in the buffer. Successful demetallation was verified using the ascorbic acid method of Buettner (1988), in which the stability of ascorbic acid in the chelex-treated buffers was monitored by following its absorbance at 265 nm. In the presence of catalytic metal ions, autoxidation of ascorbic acid occurs with a decay of absorbance at 265 nm. Buettner observed that in metal free solutions ascorbic acid was stable at neutral pH. This phenomenon could be exploited as a quick and easy test to determine if near-neutral buffers were metal-free. Metal contamination was regarded as minimal if AsA was stable (loss of absorbance < 0.5 %) for at least 30 minutes.

Traces of metals were removed from PM2 DNA by overnight incubation with diethylenetriaminepentaacetic acid (DTPA, 2 mM) at 4°C, followed by extensive washing using microfiltration and resuspension in demetallated phosphate buffer (pH 6.8, 0.1 M). The DNA was considered free of metals if ascorbic acid, at 1 mM, did not induce SSB. The main source of metal contamination was found to be phosphate buffer (da Cruz Fresco and Kortenkamp, 1994).

## 2.3 Results

### Validation of the Method Used for the Detection of AP-Sites: Acid/Heat Depurinated DNA

A system, in which AP-sites are known to be induced was utilised for these initial studies in order to establish adequate reaction conditions for the detection of AP-sites. Incubation of DNA in an acid/heat environment causes hydrolytic cleavage of the N-glycosidic bond generating apurinic/apyrimidinic sites. The rate of bond breakage depends on factors such as: temperature, pH, and base and sugar structure (Lindahl and Andersson, 1972; Lindahl and Nyberg, 1972). The AP-sites can then be revealed by using agents known to cause breakage of the DNA strand where the loss of a base has occurred. The level of breakage can be determined as described in the experimental section. Of the many agents known to induce DNA scission at an AP-site three were selected for our studies, the DNA repair enzyme exonuclease III from *E.coli*, which presents AP-endonuclease activity, and the amine containing agents putrescine and Lys-Tyr-Lys.

An acidic solution of PM2 DNA in citrate buffer pH 5.5 was heated for 11 minutes at 70°C in order to induce AP-sites. After neutralising the solutions, the abasic sites were revealed by post-incubation with either putrescine (10 mM), exonuclease III (0.5 units/µl) or Lys-Tyr-Lys (0.1 mM) for different time periods. It was necessary to establish the optimum incubation time for the different agents at the given concentrations so that all the AP-sites could be revealed.

Whereas exonuclease III revealed all AP-sites even at short incubation periods, the reaction with putrescine reached its optimum after 60 minutes (**Figure 2.3-1**). In contrast to a report in which the tripeptide was found to be 100-2000 fold more effective than spermine and spermidine, agents structurally similar to putrescine (Pierre and Laval, 1981), the tripeptide was found to be much less effective than putrescine and exonuclease III.

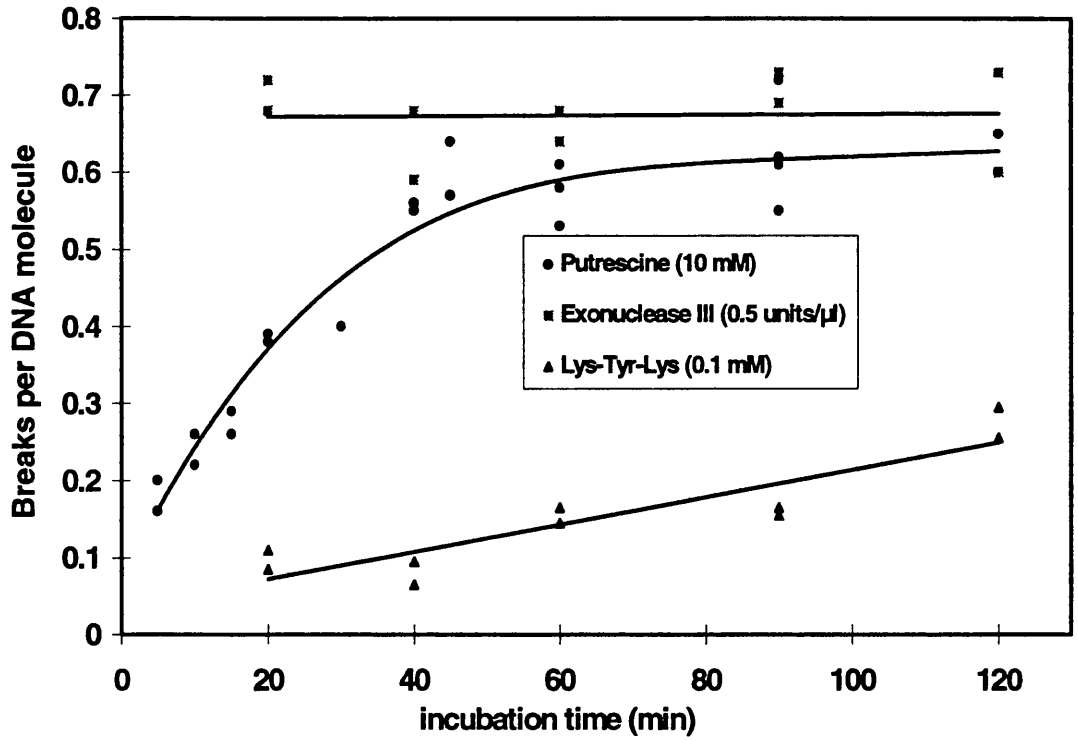



**Figure 2.3-1**: Detection of AP-sites in acid-heat depurinated DNA by exonuclease III, putrescine and Lys-Tyr-Lys. PM2 DNA was acid/heat treated as indicated in the text and then post-incubated with one of the three agents for different time intervals at 37°C.

From these results, post-incubation conditions for studies with chromate and GSH were established: one hour incubation time at 37°C with 10 mM putrescine and 0.5 units /µl exonuclease III. The tripeptide (0.1 mM), despite its low effectiveness in revealing acid/heat induced AP-sites was used in initial experiments.

## The Formation of AP-Sites During the Reduction of Chromate by GSH

Having assessed the ability of exonuclease III, putrescine and the tripeptide to reveal acid/heat induced AP-sites and established suitable post-incubation conditions, the potential of chromate and GSH to induce the release of DNA bases was explored.

Isolated PM2 DNA was incubated with various chromate concentrations (0.075 - 0.2 mM) in the presence of 5 mM GSH for three hours. Upon post-incubation with either exonuclease III, putrescine or Lys-Trp-Lys an increase in DNA cleavage relative to non-post-incubated samples was observed (**Figure 2.3-3**, see next page). The increase in the level of breaks relative to samples which were not post-incubated was evidence that AP-sites were being induced in PM2 DNA by chromate and GSH.

The level of AP-sites steadily increased with rising chromate concentrations, and paralleled the rise in SSB (**Table 2-1**, **Figure 2.3-2**). The number of AP-sites was obtained as the difference between the total number of breaks determined in samples which were post-incubated with any of the agents (total breaks = frank single strand breaks + AP-sites) and the level of breakage in samples where the post-incubation step was omitted (level of "frank single strand breaks", hereafter referred to as SSB).

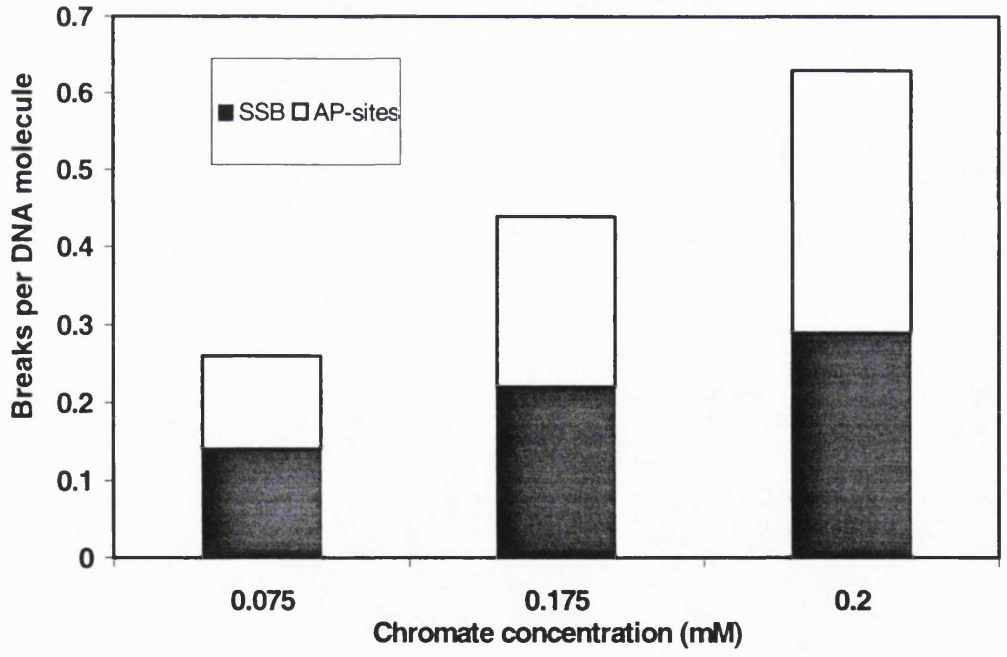



**Figure 2.3-2**: Total level of breaks induced in chromate (0.175 mM)/GSH (5 mM) treated PM2 DNA upon post-incubation with putrescine for 1 hour at 37°C. The fraction of breaks caused by "frank" single strand breaks (SSB) was obtained from samples that had not been post-incubated. All data have been corrected for the level of breaks induced in appropriate control samples (see footnote in Table 2-1).

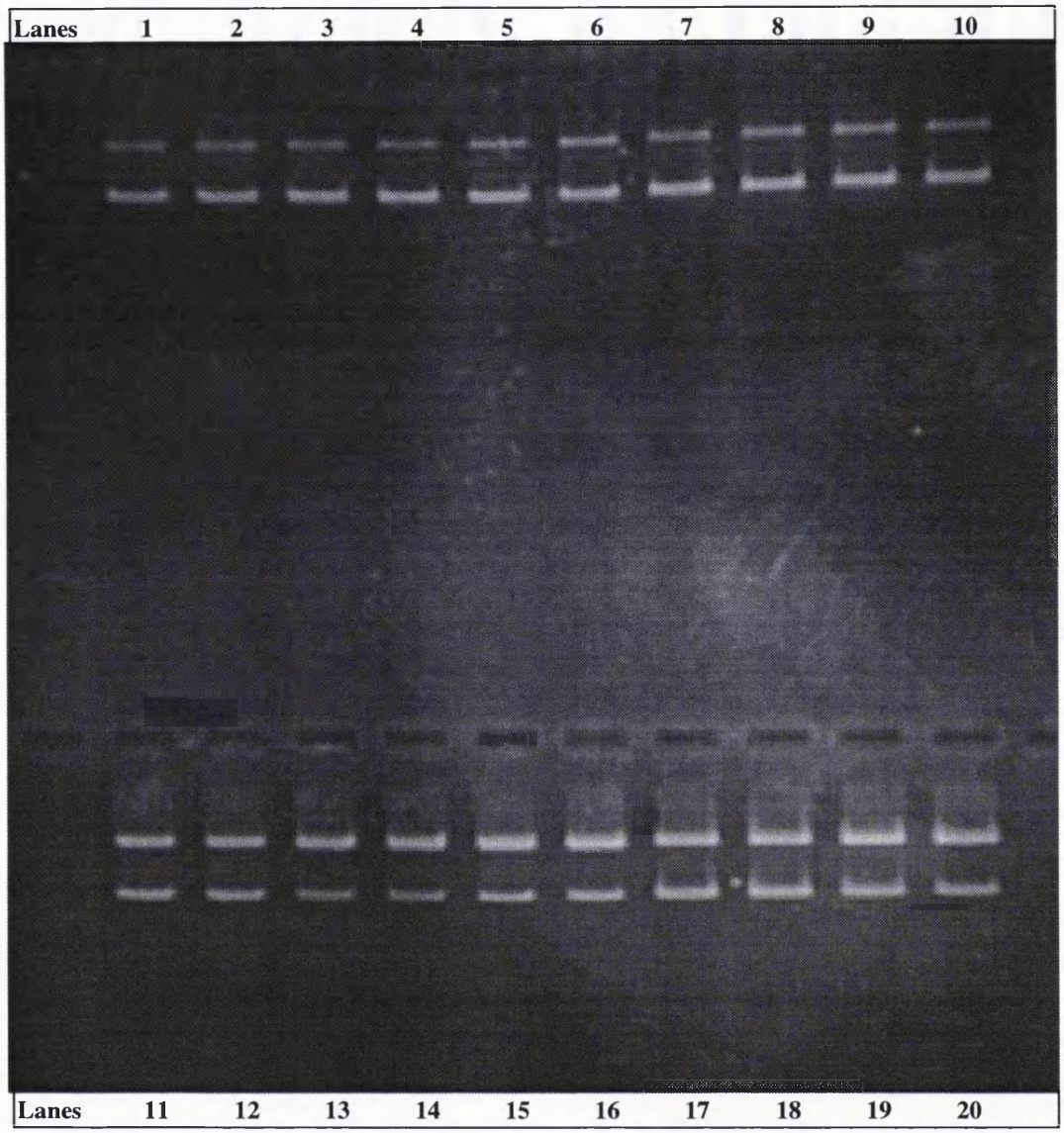



**Figure 2.3-3:** The formation of SSB and AP-sites in PM2 DNA during the reduction of chromate by GSH. DNA was incubated with chromium(VI) (0.175 mM) and GSH (5 mM) or with GSH alone (controls) for three hours. AP-sites were revealed by post-incubation with putrescine, Lys-Trp-Lys or exonuclease III. Before post-incubation reactants were removed by dialysis against the appropriate buffers (see experimental section). Lanes 1-10 were controls and contained DNA with GSH; 1-2 alone dialysed against HEPES buffer; 3-4 post-incubated with putrescine; 5-6 post-incubated with Lys-Trp-Lys; 7-8 alone dialysed against citrate buffer and 9-10 as 7 and 8 post-incubated with exonuclease III. Lanes 11-20, all samples contained DNA, chromium(VI) and GSH. The order of the lanes is the same as for controls. The two bands observed in the agarose gels, upon staining with ethidium bromide and exposure under UV light, correspond to the PM2 DNA in the open circular form (top band) and in the supercoiled form (bottom form). In control samples (lanes 1-10) certain amount of broken PM2 DNA is already present. Upon treatment of DNA with chromate/GSH an increase in the intensity of the open circular form becomes apparent, with a corresponding decrease in the amount of supercoiled DNA (compare lanes 1-2 and 11-12), which is evidence for the induction of DNA strand breaks.

**Table 2-1** : Level of SSB and AP-sites[a] in DNA treated with GSH (5 mM) and various chromate concentrations, residual reactants removed by dialysis

| | Chromate concentration (mM) | | |
|---|---|---|---|
| | 0.075 | 0.175 | 0.2 |
| SSB / HEPES[b] | 0.14 ± 0.04 (4) | 0.22 ± 0.09 (6) | 0.29 ± 0.03 (7) |
| Putrescine (AP-sites) | 0.12 ± 0.07 (3) | 0.22 ± 0.04 (6) | 0.34 ± 0.03 (6) |
| Lys-Trp-Lys (AP-sites) | 0.04 ± 0.01 (3) | 0.20 ± 0.03 (6) | 0.24 ± 0.03 (5) |
| SSB / citrate[b] | 0.07 ± 0.03 (4) | 0.33 ± 0.06 (6) | 0.40 ± 0.07 (6) |
| Exonuclease III (AP-sites) | 0.03 ± 0.003 (4) | 0.11 ± 0.01 (6) | 0.16 ± 0.02 (5) |

[a] Average number of SSB and AP-sites per DNA molecule observed upon incubation with chromate and GSH for 3 hours, corrected for the breaks in appropriate controls. For samples not post-incubated (SSB) controls contained GSH, for the remaining samples controls contained GSH and the respective cleaving agent. Means ± SEM × t (95 %), number of samples indicated in parentheses.

[b] The level of AP-sites was obtained by subtracting the level of "frank" single strand breaks (SSB) seen with chromate and GSH after dialysis against the appropriate buffer from the breakage observed after post-incubation with either agent. Especially at the higher chromate concentrations dialysis against citrate buffer used for post-incubation with exonuclease III resulted in a slightly higher level of SSB when compared to dialysis with HEPES buffer.

The tripeptide presented a low effectiveness in revealing chromate/GSH-induced AP-sites, it was unable to detect levels of AP-sites higher than 0.2 AP-sites per DNA molecule in agreement with the poor results obtained in the acid/heat depurination studies.

When the DNA was treated with either chromate or GSH alone neither SSB nor AP-sites were observed (**Figure 2.3-3**). In order to investigate whether AP-sites were caused by the final product of the reduction of chromate by GSH, the effect of aged solutions (18 h) of chromate (0.175 - 0.2 mM) and GSH (5 mM), containing mainly a Cr(III)-GSH complex, was also studied. As with the reactants administered individually neither SSB nor AP-sites were detected. The results clearly show that neither the initial reactants alone nor the final product of the chromate-GSH reduction process are capable of inducing AP-sites in PM2 DNA. Therefore, an intermediate generated during the reduction process is likely to be responsible for the generation of this DNA lesion.

In these preliminary studies, when dialysis was used to remove residual reactants, putrescine and exonuclease III differed in their ability to reveal the AP-sites generated by the chromate/GSH system. Upon post-incubation with exonuclease III fewer nicks were observed when compared with the results obtained with putrescine. It is also worth noticing that due to some unknown reason, the level of "frank" single strand breaks after dialysis against citrate buffer was slightly higher than when DNA was dialysed against HEPES buffer, especially at the higher chromate concentrations (see **Table 2-1**). When the dialysis process was replaced by microfiltration, the levels of SSB were very similar in all the buffers used. Moreover, it was found that putrescine and exonuclease III were equally effective in revealing AP-sites (**Table 2-2**).

**Table 2-2**: Level of SSB and AP-sites* in DNA treated with chromate (0.175 mM) and GSH (5 mM), when residual reactants were removed by microfiltration

| | Putrescine | | Exonuclease III | |
|---|---|---|---|---|
| **Incubation time (h)** | **SSB** | **AP-sites** | **SSB** | **AP-sites** |
| 3 | 0.37 ± 0.04 | 0.44 ± 0.11 | 0.32 ± 0.03 | 0.29 ± 0.06 |
| 4 | 0.49 ± 0.04 | 0.51 ± 0.09 | 0.45 ± 0.06 | 0.35 ± 0.03 |

* All data have been corrected for the level of breaks induced by the appropriate controls. Results shown are means ± SEM × t (95 %), n = 3 - 7. The null hypothesis that the number of SSB and AP-sites in the two systems did not differ significantly was confirmed using Student's *t*-test with $P < 0.01$.

The formation of AP-sites by chromate and GSH was not restricted to solutions where phosphate was used as a buffering agent. Abasic sites were also detected when isolated PM2 DNA was treated with chromate/GSH in unbuffered solutions (with 0.1 M NaCl, pH adjusted to 7), albeit at lower yields relative to phosphate buffered systems. With chromate at 0.8 mM and 5 mM GSH, the level of AP-sites after three hours incubation was 0.19 ± 0.03 per DNA molecule (means ± SEM × t (95 %), n = 4, two independent experiments).

These studies provide evidence for the formation of AP-sites in PM2 DNA during the reduction of chromate by GSH. Subsequent experiments were undertaken to gain further information about the chemical nature of the chromate/GSH induced AP-sites and the mechanism(s) by which DNA SSB and AP-sites are caused.

## Chromate/GSH Induced AP-Sites Are Aldehydic

The chemical structure of an AP-site can provide important information about the mechanism by which it was induced. AP-sites generated by simple hydrolytic cleavage of the N-glycosidic bond present an aldehyde group at C-1', those formed by oxidation of the deoxyribose sugar at C-4', C-2' or C-1' will lead to a 4'-hydroxylated AP-site, an erythrose with an aldehyde at C-2' or a lactone type AP-site respectively (see **Figure 1.3-1** and **1.3-2**) (Povirk and Steighner, 1989).

The presence of an aldehyde group in chromate/GSH induced AP-sites can be probed by using $NaBH_4$, which has the ability to reduce an aldehyde to alcohol. The cleavage of the phosphodiester bond at an AP-site by putrescine involves the formation of a Schiff base between one of its amino groups and the aldehyde group of the AP-site (**Figure 2.3-4**). The acidity of the α-hydrogens at C-2' in the formed imine are thought to facilitate the β-elimination of the phosphate at the 3' side of the AP-site (Mazumder *et al.*, 1991; Nishikawa *et al.*, 1982). Putrescine would therefore fail to reveal an AP-site which was previously reduced by $NaBH_4$ (Pierre and Laval, 1981). The AP-endonuclease activity of exonuclease III however recognises the absence of a DNA base stereochemically and acts independently of the presence of an aldehyde group (Kow, 1989).

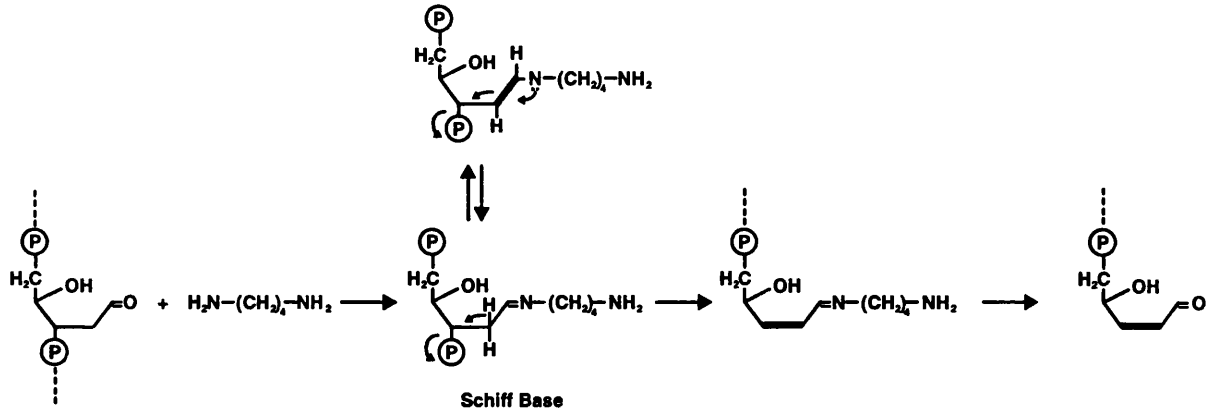



**Figure 2.3-4**: Summarised chemical pathway of the reaction between putrescine and the C-1' aldehyde group of an AP-site.

PM2 DNA treated with chromate (0.175 mM) and GSH (5 mM) was reduced with $NaBH_4$ prior to post-incubation with either putrescine or exonuclease III. After treatment with $NaBH_4$ no additional strand breaks were observed upon post-incubation with putrescine (**Table 2-3**), whereas exonuclease III was still able to reveal AP-sites. These findings clearly demonstrate that the AP-sites generated by chromate and GSH contain an aldehyde group at the carbon atoms of the sugar moiety.

**Table 2-3**: The influence of $NaBH_4$ on the detection of AP-sites induced by chromate/GSH in PM2 DNA

| | Cr/GSH + $NaBH_4$ treated DNA | |
|---|---|---|
| | SSB[a] | SSB + AP-sites[b] |
| Putrescine | 0.68 ± 0.12 | 0.76 ± 0.08 |
| Exonuclease III | 0.67 ± 0.08 | > 2[c] |

[a] The level of SSB in PM2 DNA treated with chromate (0.175 mM) and GSH (5 mM) for 3.5 hours and reduced with $NaBH_4$.

[b] The total level of breaks, after post-incubation with either putrescine or exonuclease III. Results shown are means ± SEM × t (95 %), n = 4. The null hypothesis that the level of SSB and SSB + AP-sites revealed by putrescine after sodium borohydride treatment is not significantly different was confirmed using Student's *t* test with $P < 0.01$.

[c] Levels of breakage exceeding an average number of two breaks per DNA molecule can not be reliably quantified using our experimental method.

It is worth noting that the level of strand breaks observed in these experiments was considerably higher than throughout the present investigation. This inconsistency is easily explained by a shearing effect due to the additional steps of centrifugation during microfiltrations, which were necessary to remove unreacted $NaBH_4$ before adding putrescine or exonuclease III.

## Time Course Studies

In order to investigate whether AP-sites are generated independently from SSB or whether there is an association between the formation of the two lesions, time course studies were carried out. The treatment of PM2 DNA with chromate (0.175 mM) and

GSH (5 mM) for increasing periods of time caused a steady increase in the level of both AP-sites (revealed with putrescine) and SSB. The formation of both lesions followed an identical temporal pattern with a plateau reached after 4 hours and a 1:1 ratio of AP-sites to SSB (**Figure 2.3-5**). Kinetic studies of the reaction of chromate with GSH under identical experimental conditions revealed that four hours were sufficient to completely reduce chromium(VI) (see section 6.3).

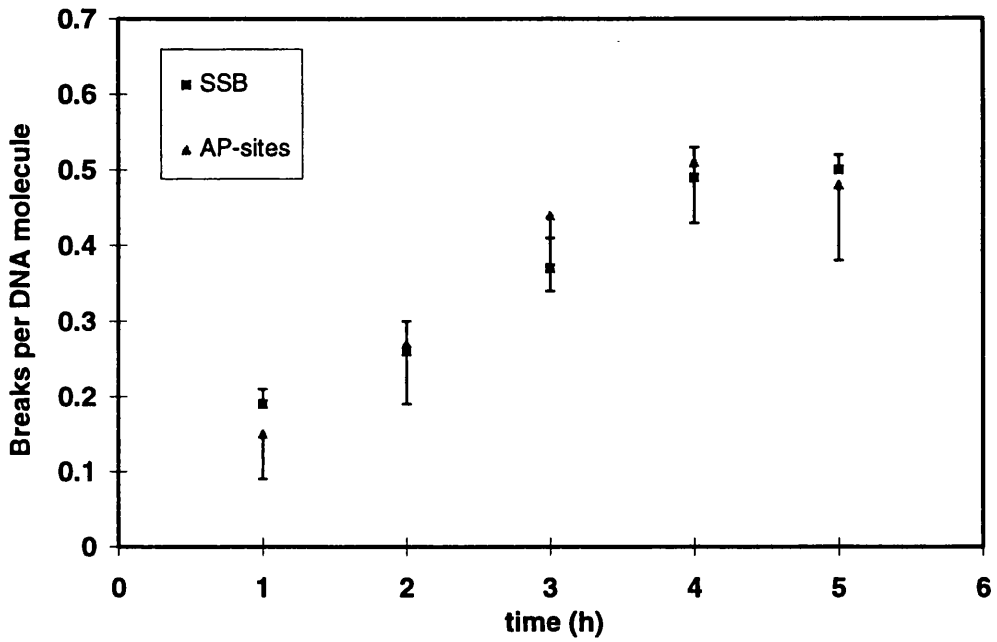



**Figure 2.3-5**: The number of SSB and AP-sites induced in PM2 DNA treated with chromate (0.175 mM) and GSH (5 mM) for different time intervals. AP-sites were revealed by post-incubation with putrescine. All data were corrected for the level of breaks induced in control samples. Results shown are means ± SEM × t (95 %), n = 5 - 10. The null hypothesis that the number of SSB and AP-sites is not significantly different was confirmed using Student's *t*-test with $P < 0.01$.

When solutions of chromate and glutathione were aged for different time intervals prior to addition to DNA, the level of both DNA lesions was found to decrease exponentially, again following a similar temporal pattern (**Figure 2.3-6**). As in the time course studies, the ratio of AP-sites to SSB was close to 1:1. After four hours of ageing the reaction mixtures had lost the ability to induce either AP-sites or SSB, an observation consistent with the results yielded by the kinetic studies described in chapter 6.

These observations provide further evidence for the involvement of an intermediate species generated during the reduction of chromate by GSH in the steps leading to the formation of AP-sites and SSB. The longer the reaction was allowed to proceed in the absence of DNA, the less of the damage-causing species remained to react with DNA. If the final products of the reaction were responsible for the induction of the lesions, the level of damage would be expected to remain largely unchanged or to increase over time.

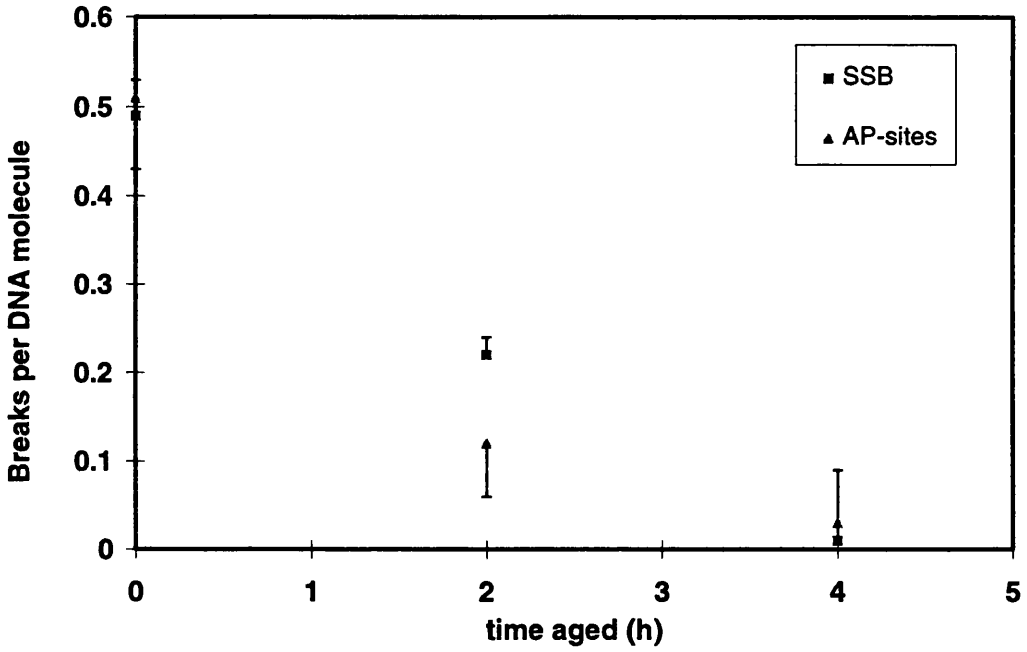



**Figure 2.3-6**: The level of SSB and AP-sites induced in PM2 DNA after treatment with chromate (0.175 mM) and GSH (5 mM) aged in the absence of DNA for the time intervals indicated. All data were corrected for the level of breaks in the appropriate controls. Results shown are means ± SEM × t (95 %), n = 4. The null hypothesis that the number of SSB and AP-sites is not significantly different was confirmed using Student's *t*-test with $P < 0.01$.

The results yielded by the time course studies are striking, not only because the formation of SSB and AP-sites by chromate and GSH followed a similar temporal pattern, but also because the ratio of the lesions was close to 1:1 in all the experiments. These results suggests that there is a link between the formation of SSB and AP-sites.

## 2.4 Discussion

### The Formation of SSB and AP-Sites in Isolated DNA During the Reductive Conversion of Chromium(VI)

The reduction of chromate by intracellular components leads to the formation of a broad spectrum of DNA lesions which are thought to mediate chromate carcinogenicity. At the beginning of this project the DNA lesions identified included: single strand breaks, DNA-protein cross-links, DNA-chromium binding and alkaline labile sites (Standeven & Wetterhahn, 1991a). There had been no attempts to elucidate if AP-sites could be partly responsible for rendering the DNA phosphate backbone sensitive to alkali. Considering the evidence showing that AP-sites are potentially premutagenic (Loeb and Bradley, 1986), we decided to investigate the possible formation of this DNA lesion during the reductive conversion of chromate.

Using a well defined *in vitro* system and the ability of certain agents, putrescine, Lys-Trp-Lys and exonuclease III, to cleave the DNA phosphate backbone where the loss of a base has occurred, we were able to demonstrate that AP-sites are induced in isolated PM2 DNA during the reduction of chromate by GSH. The level of AP-sites increased linearly with rising chromate concentration, a similar trend was observed for the DNA SSB in agreement with previous observations by Kortenkamp *et al.* (1989).

The formation of AP-sites in PM2 DNA not only occurs in phosphate buffer, although higher chromate concentrations were required for the formation of DNA SSB and AP-sites when the incubations were carried out in unbuffered solutions. The rate of reduction of chromate by GSH has been shown to be affected by the type of buffer used (Kortenkamp *et al.*, 1989), the reduction being slowest in phosphate buffer when compared to HEPES, Tris-HCl or unbuffered solutions. It is known that chromate and phosphate anions can condense forming heteropolyanions (Frennesson *et al.*, 1968), which could account for the slower reduction rate of chromate in phosphate buffer, and favour the reaction of any intermediate with DNA.

The "wrong" choice of buffer may completely influence the outcome of some studies. The generation of SSB in chromate/ascorbic acid treated DNA occurs in unbuffered solutions and in phosphate buffer but not in HEPES buffer (da Cruz Fresco and Kortenkamp, 1994). Tris-HCl buffers affect the reduction of chromate and prevent Cr(V) species from being observed, probably by formation of unstable intermediates (O'Brien *et al.*, 1992). Studies on the formation of DNA lesions by NCS had to be carried out in HEPES buffer because AP-sites were found to be unstable in Tris-HCl buffers (Povirk *et al.*, 1988). These examples show that buffers in *in vitro* studies should be carefully chosen and in some cases results confirmed using a second buffer.

The AP-sites induced by chromate and GSH could be protected from putrescine-induced cleavage by treatment with $NaBH_4$, indicating that they contain an aldehyde group. The absence of such a protective effect upon post-incubation with exonuclease III is easily explained since the enzyme recognises AP-sites stereochemically and, unlike putrescine, does not require an aldehyde group to cleave the DNA phosphate backbone. These results seem to suggest that the abasic sites arose during the reduction of chromium(VI) as a consequence of base-adduct formation or oxidation of the C-4' of the deoxyribose sugar, as in both cases an AP-site with an aldehyde group at C-1' is generated (Povirk and Steighner, 1989).

Attack at C-1' leading to a lactone-type AP-site is unlikely; although putrescine has been shown to be able to recognise these type of AP-sites (Dedon *et al.*, 1992), $NaBH_4$ reduces esters (lactone is a cyclic ester) only very slowly (Fessenden and Fessenden, 1982 p. 538) and putrescine therefore would retain its capacity of detecting the abasic sites even after $NaBH_4$ treatment. Erythrose-containing sites formed by oxidation of C-2' contain an aldehyde group at this position, which may be recognised by putrescine and be reduced by $NaBH_4$. However, this type of AP-sites is extremely rare. They have only been identified on DNA treated with γ-radiation, and not much is known about their chemistry (Sugiyama *et al.*, 1993). If recognised by putrescine, the mechanism leading to phosphate backbone cleavage would be different from the one operating for abasic sites containing an aldehyde group at C-1'. However, on the basis of the results

from our $NaBH_4$ studies, the formation of abasic sites in chromate/GSH system via C-2' oxidation cannot be completely ruled out.

It is also worth pointing out, that the AP-endonuclease, exonuclease III, and putrescine were equally effective at revealing chromate/GSH induced AP-sites, in spite of the different mechanisms operating in the detection of this DNA lesion. Povirk and Houlgrave (1988) observed that NCS and bleomycin induced AP-sites were easily recognised by putrescine but were much less sensitive to AP-endonucleases such as exonuclease III and endonuclease III. This resistance to endonucleolytic cleavage was attributed to the presence of SSB closely opposed to an AP-site which was likely to disturb the DNA structure. In view of these findings, our results suggest that chromate/GSH induced AP-sites are "undisturbed" by SSB.

## Mechanism of Induction of SSB and AP-Sites

### The Formation of SSB and AP-Sites Occurs by Attack of a Single Species at a Single DNA Site

The time course studies afforded information about the possible mechanisms underlying the generation of DNA SSB and AP-sites by chromate and GSH. It was observed that both DNA lesions were formed not only with an identical temporal pattern, but also with similar probability (ratio close to 1:1). Moreover, when solutions of chromate and GSH were "aged" for different time periods and then added to the DNA, the ratio of SSB and AP-sites formed was again very similar and they decreased over time at similar rates.

These observations suggested to us that one single species was responsible for the induction of both DNA lesions. In considering the alternative situation, with two species being responsible for the formation of the two different DNA lesions, it is necessary to postulate that these species are formed at the same time (to account for a similar temporal pattern of induction of DNA lesions) and have similar half-lives (to account for the similar decline on DNA lesions observed with "aged" solutions). In addition,

they should have similar reactivities with DNA in order to form the two DNA lesions with equal probability (ratio 1:1). It is highly unlikely that two different species would fulfil all these requirements. Therefore, the idea that a single reactive species causes both SSB and AP-sites seems to be the most likely explanation. However, how can we explain the fact that both lesions are generated with a 1:1 ratio?

In addressing this question one could suggest a mechanism whereby one lesion is the precursor of the other, e.g. SSB evolving from AP-sites or vice versa. It is well documented that AP-sites are susceptible to alkaline hydrolysis. However, the chain cleavage proceeds at extremely slow rates in neutral buffer at 37°C. Data published by Lindahl and Andersson (1972) suggest that under such experimental conditions the lifetime of a phosphodiester bond at an AP-site is *ca.* 190 hours. The number of SSB deriving from AP-sites is therefore negligible under the conditions used throughout these studies.

Furthermore, the suggestion of one lesion evolving from the other is unlikely given the nature of the assay used to estimate the level of DNA cleavage. The system measures conformational changes of DNA molecules which arise from the relaxation of supercoils to the open circular form. At the level of an individual DNA molecule this is an all-or-none response, and additional SSB in an already nicked supercoil will not lead to further conformational changes of the DNA molecule. Thus, an AP-site present in a DNA molecule with a SSB would not be detected and therefore the additional breaks observed in samples post-incubated with either putrescine or exonuclease III must have arisen from DNA molecules which did not already carry SSB (**Figure 2.4-1**).

In view of these features it can be postulated that the interaction of a reactive species deriving from chromate/GSH with DNA damages the macromolecule in a way which subsequently causes the formation of either a SSB or an AP-site. One lesion does not evolve from the other.

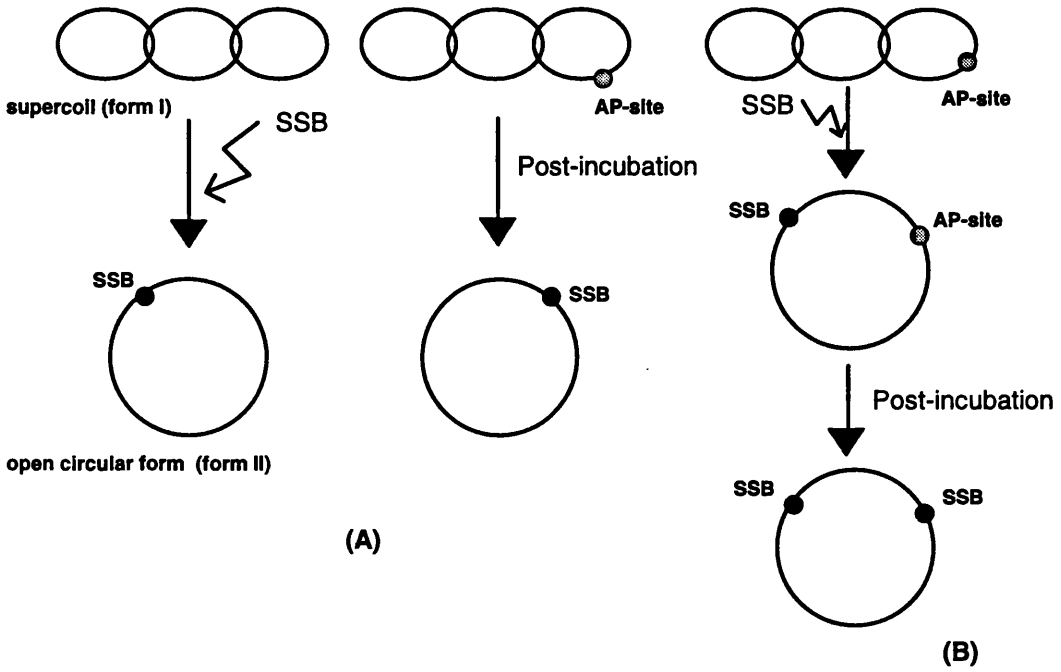



**Figure 2.4-1**: Chromate/GSH-induced SSB and AP-sites are present in different PM2 DNA molecules; one lesion does not evolve from the other. A) The formation of a SSB or the DNA scission at an AP-site upon post-incubation with putrescine or exonuclease III leads to conformational change of the PM2 DNA molecules, from supercoil (form I) to the open circular form (form II). B) However, the presence of an AP-site in the same DNA molecule that has a SSB will remain undetected, as cleavage at an AP-site upon post-incubation will not lead to further conformational changes.

The formation of SSB can occur by attack at the DNA phosphodiester bond or at the deoxyribose moiety, whereas AP-sites arise by attack at the deoxyribose sugar or by labilization of the N-glycosidic bond upon formation of DNA base adducts. If AP-sites were to be generated via the latter route it would be necessary to invoke an attack of a reactive species deriving from chromate/GSH not only at the bases, but also at the deoxyribose or phosphodiester bond in order to explain the generation of SSB. However, such a suggestion is difficult to reconcile with the observation that the induction of SSB and AP-sites followed a similar temporal pattern. Therefore it is very likely that both DNA lesions, AP-sites and SSB, arise from attack of single species at the deoxyribose moiety.

Mechanistic studies on the formation of DNA lesions by agents such as NCS, bleomycin, copper-phenanthroline and ionising radiation have provided extensive information about the consequences hydrogen abstractions from the different carbon atoms of the deoxyribose sugar may have (Dedon and Goldberg, 1992a; Sigman, 1986; von Sonntag, 1987). Whereas hydrogen abstraction from C-3' and C-5' will lead to SSB but not AP-sites, oxidation of C-1', C-2' or C-4' can result in the formation of AP-sites.

Hydrogen abstraction from C-1' by NCS leads to the formation of a lactone type AP-site (see **Figure 1.3-2** for AP-site structures) (Kappen and Goldberg, 1989), which in the case of copper-phenanthroline, has been proposed to further react leading to a SSB (Sigman, 1986). Therefore hydrogen abstraction from C-1' will lead to either a SSB or an AP-site, depending on the agent, but not to both. Not much is known about hydrogen abstraction from C-2', at present the only lesion described formed via this mechanism is an erythrose-containing AP-site (Sugiyama *et al.*, 1993). Of particular relevance, in view of our results, is the effect observed upon hydrogen abstraction from C-4' by agents such as bleomycin or NCS; the initial C-4' radical formed due to hydrogen abstraction is quickly quenched by molecular oxygen leading to the formation of a peroxyl radical, which depending on the conditions, may evolve to form either a 4'-hydroxylated AP-site or a SSB with release of malondialdehyde (MDA) -like products, i.e. both lesions can arise from a common precursor (**Figure 2.4-2**).

A mechanism similar to that presented by bleomycin or NCS (**Figure 2.4-2**) provides an attractive explanation for our observations on the formation of SSB and AP-sites during the reduction of chromate by GSH:

- A single reactive species would lead to the formation of both DNA lesions.

- The formation of both DNA lesions from a common precursor would explain the identical temporal pattern observed for the formation of SSB and AP-sites.

- The partitioning pathway regulated by a random factor would account for the formation of both DNA lesions in similar amounts.

- AP-sites derived from hydrogen abstraction at C-4' contain an aldehyde group.

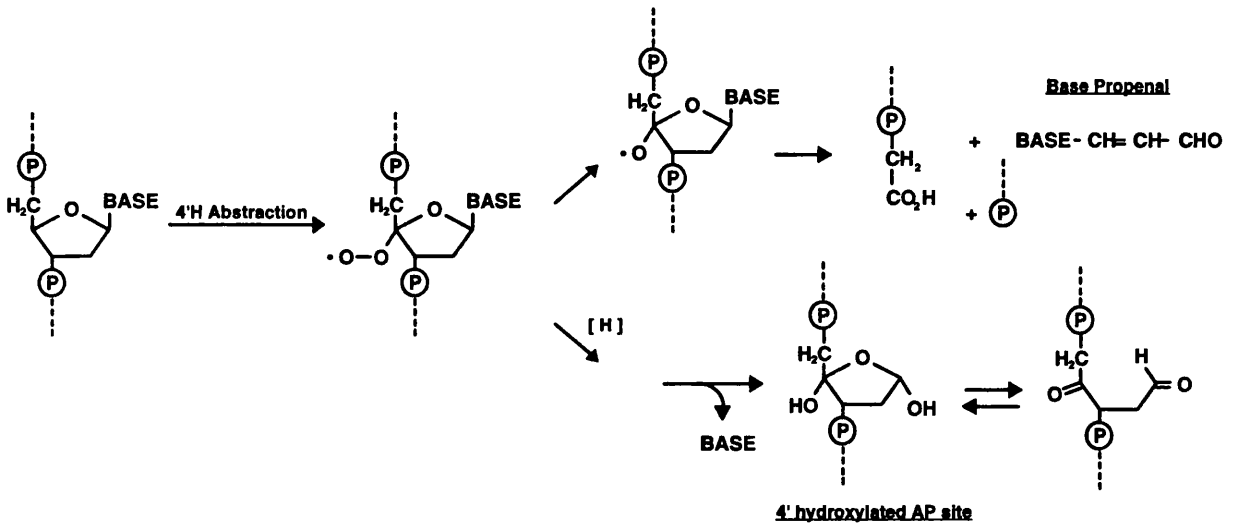



**Figure 2.4-2**: Proposed mechanism of neocarzinostatin-induced DNA damage at C-4', modified from Dedon and Goldberg (1992a).

Although our studies provide evidence that SSB and AP-sites induced by chromate and GSH are generated by a single reactive species attacking at a single DNA site, further studies are required in order to confirm the mechanism proposed.

i) The nature of the reactive intermediate needs to be established, as only highly oxidative species are able to abstract the hydrogens from the deoxyribose moiety. The role of oxygen species on the formation of SSB and AP-sites by chromate and GSH is investigated in Chapter 3.

ii) Further evidence that attack at C-4' occurs is required. The formation of SSB via attack at the C-4' of the deoxyribose by agents such as NCS, bleomycin or ionising radiation results in concomitant release of malondialdehyde-like products (e.g. base propenals) (Dedon and Goldberg, 1992a; Henner *et al.*, 1983). If the generation of SSB by the chromate/GSH system occurs via the proposed mechanisms, then MDA-like products should be formed. Studies addressing the formation of such products by chromate/GSH are presented in Chapter 4.

# *CHAPTER 3*

# INVESTIGATION OF THE NATURE OF THE SPECIES RESPONSIBLE FOR THE FORMATION OF SSB AND AP-SITES DURING THE REDUCTION OF CHROMATE BY GSH

## *3.1 Introduction*

A reactive intermediate generated during the reduction of chromate by GSH has been shown to be responsible for the formation of AP-sites and SSB in PM2 DNA. The reduction process leads to the formation of a variety of intermediates, including Cr(IV), Cr(V), Cr(II) and GS·, and any of them could be involved in the generation of the DNA lesions (Bose *et al.*, 1992; O'Brien *et al.*, 1985; Perez-Benito *et al.*, 1994a). In order to gain further information about the nature of the reactive species, the effect of molecular oxygen and catalase on the formation of SSB and AP-sites in PM2 DNA was studied. Observations from these studies led us to investigate if hydroxyl radicals could be the damaging species; the possible formation of DNA base-adducts characteristic of hydroxyl radicals was monitored by using chromatographic techniques.

## *3.2 Experimental Section*

### Materials

PM2 DNA and exonuclease III from *E. coli* were obtained from Boehringer Mannheim (Lewes, East Sussex), microfilters Microcon-30 were from Amicon (Stonehouse, Gloucestershire). Calf-thymus DNA, GSH, putrescine and catalase were purchased from Sigma (Poole, Dorset). Potassium chromate, hydrogen peroxide were obtained from BDH (Lutterworth, Leicestershire). All reagents were of analytical grade.

### Incubations in the Presence of Catalase and under Anoxic Conditions

Isolated PM2 DNA (8 μg/ml) was incubated with potassium chromate (0.175 mM) and GSH (5 mM) in the presence of catalase (10 μg/ml) for three hours. Some samples contained catalase which was denaturated by heating at 65°C for 10 minutes, in order to exclude the possibility of a non-specific scavenging of the protein. AP-sites were revealed by post-incubation with putrescine.

In order to investigate the role of molecular oxygen in the induction of AP-sites, reagents and buffers were degassed for 45 minutes under vacuum (water pump) in an ultrasonic bath. This treatment did not affect the concentrations of either chromate or GSH as established by its absorbance at 372 nm or by Ellman's assay respectively (see section 6.2 for more details). Sample handling was carried out under a nitrogen atmosphere in a tent. Prior to use, a solution of PM2 DNA in water was washed three times with degassed phosphate buffer in order to minimise the level of oxygen present in the DNA solutions. It is not possible to remove oxygen from DNA using the same procedure as for the reagents and buffers because ·OH radicals are formed in the cavitation bubbles produced by ultrasound (Makino *et al.*, 1983), which would lead to complete cleavage of the DNA molecules. All samples were prepared inside a nitrogen tent and treated with chromate/GSH (0.175/5 mM) for three hours. Aerobic samples were removed from the tent immediately after the addition of chromate/GSH to DNA and exposed to the atmosphere for three hours. AP-sites were revealed by using putrescine or exonuclease III as described in section 2.2.

### Determination of 8-Hydroxydeoxyguanosine (8-OH-dG) by High Performance Liquid Chromatography (HPLC)

Calf-thymus DNA (0.25 mg/ml), in a final volume of 2 ml, was incubated for 16 hours with chromate (0.4 mM) and GSH (5 mM), $FeSO_4$/EDTA (42 μM/ 208 μM) and $H_2O_2$ (50 μM) or with chromate, GSH or $H_2O_2$ alone, in phosphate buffer (0.1 M, pH 6.8) at room temperature. Subsequent to incubations, phosphate buffer was exchanged by dialysis (24 h) against HEPES buffer (15 mM, 0.1 M NaCl, pH 7), in order to prevent phosphate/ethanol interferences, and the DNA precipitated by addition of trichloroacetic acid (TCA, 370 μl, 33 % w/v), ice-cold ethanol (5 ml) and sodium acetate (200 μl, 0.3 M). Samples were kept in ice and transported to the Institute of Occupational Health,

University of Birmingham, where the measurements were carried out with the assistance of Dr. Stephen P. Faux.

Following precipitation, the DNA was pelleted by centrifugation and dried by vacuum desiccation. The pellet was dissolved in ultra high quality water and digested with deoxyribonuclease I, endonuclease (*Neurosporra crassas*), phosphodiesterase I (*Crotalus atrox*) and alkaline phosphatase (*Escherichia coli*) to individual nucleosides as described previously (Faux *et al.*, 1992). The resulting deoxynucleosides mixture were analysed by reverse phase HPLC with electrochemical detection. The 8-OH-dG was identified by co-chromatography with authentic standard synthesised by the Udenfriend hydroxylation system (Faux *et al.*, 1992 and references therein).

### Determination of DNA Base-Adducts by Gas Chromatography/Mass Spectrometry (GC/MS)

Calf-thymus DNA (0.5 mg/ml), in a final volume of 1 ml, was incubated for 7 hours with chromate (0.4 mM) and GSH (5 mM); $FeSO_4$/EDTA (42 μM/208 μM) and $H_2O_2$ (50 μM) or with the previous reactants alone, in phosphate buffer (0.1 M, pH 6.8) at room temperature. Samples were kept in ice and transported to King's College, University of London, where the measurements were carried out with the help of Dr. Andrew Jenner.

Samples were dialysed against distilled water to remove all reactants and the amount of DNA recovered was determined spectrophotometrically at 260 nm. Internal standards (6-azathymine, 1 nmole and 8-azaadenine, 2 nmole) were added to 0.1 mg of DNA and the aliquots lyophilised. The DNA mixtures were hydrolysed with formic acid (0.5 ml, 60 %), in evacuated and sealed tubes, for 45 minutes at 150°C. Samples were lyophilised and then silylated by treatment with a tris(trimethylsilyl)trifluoroacetamide / acetonitrile (0.1 ml, 4:1 v/v) mixture by heating at 90°C for 60 minutes. Derivatised samples were analysed by a mass selective detector interfaced with a gas chromatograph (Hewlett-Packard 5971A/5890II). A high sensitivity of detection was achieved by operating the MS in the selected ion monitoring (SIM) mode. In this mode, the MS is set to monitor several ions derived by fragmentation of a particular product during the time at which this product is expected to elute from the GC column (Halliwell and Dizdaroglu, 1992).

## 3.3 Results

### The Possible Involvement of Reactive Oxygen Species in the Formation of AP-Sites

Earlier observations by Kortenkamp *et. al.* (1990) of a protective influence of catalase on the induction of SSB by chromate/GSH, led us to probe the involvement of oxygen reactive species in the generation of AP-sites. As shown in **Figure 3.3-1**, the addition of catalase to incubation mixtures containing chromate (0.175 mM) and GSH (5 mM) almost completely prevented both DNA lesions from occurring, suggesting that hydrogen peroxide or a peroxidic species are involved in the process leading to DNA damage. Denaturated catalase failed to elicit a protective influence, confirming that the effect observed was due to the property of catalase to catalyse the dismutation of peroxo species and not to a non-specific scavenging of the protein.

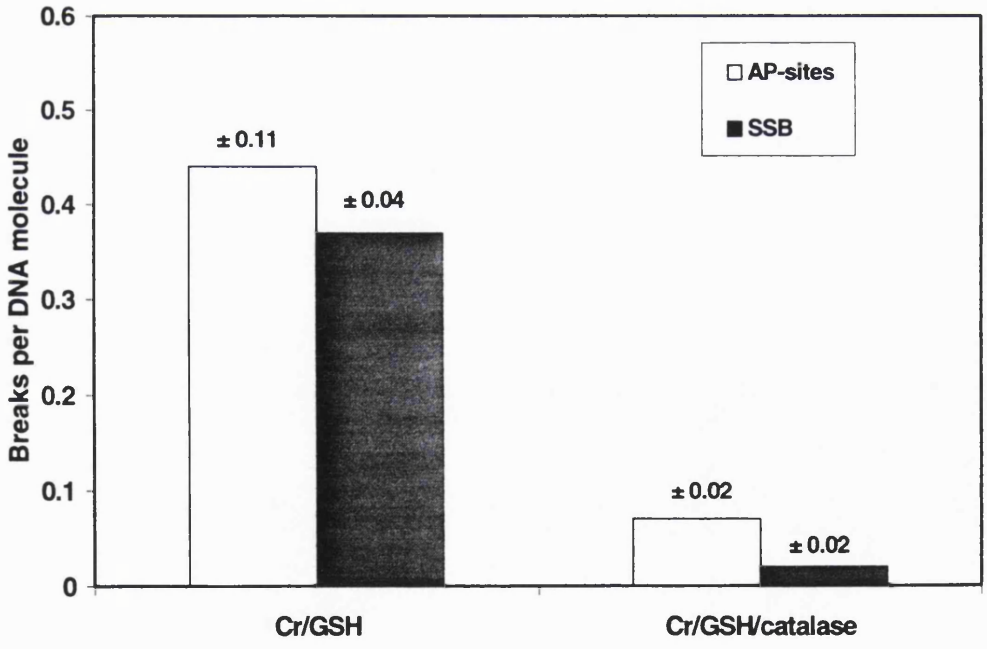



**Figure 3.3-1**: Effect of catalase on the level of SSB and AP-sites induced in PM2 DNA by chromate (0.175 mM) and GSH (5 mM) after 3 hours incubation. Results are means ± SEM × t (95 %), n = 4 - 10. The number of SSB and AP-sites did not differ significantly in the presence of catalase (Student's *t*-test with $P < 0.01$). The same was true in the absence of catalase.

In order to obtain more information about the origin of the peroxidic species involved in the induction of AP-sites and SSB, attempts were made to exclude oxygen from the reaction mixtures. The results shown in **Table 3-1** demonstrate that the level of both AP-sites and SSB was significantly lower under anaerobic conditions when compared to aerobic conditions. In one instance (experiment n° 1) an almost complete inhibition of the formation of DNA damage was observed. It is interesting to note that in the experiments which yielded an incomplete inhibition of DNA damage, the earlier observed 1:1 ratio of AP-sites to SSB was preserved.

**Table 3-1**: The influence of oxygen on the induction of SSB and AP-sites

| Experiment no | aerobic[a] | | anoxic[b] | |
|---|---|---|---|---|
| | SSB | AP-sites | SSB | AP-sites |
| 1[c] | 0.39 | 0.32 | 0.01 | 0.05 |
| | 0.40 | 0.29 | 0.02 | 0.04 |
| 2[c] | 0.36 | 0.50 | 0.22 | 0.19 |
| | 0.36 | 0.55 | 0.22 | 0.15 |
| 3[c] | 0.37 | 0.38 | 0.15 | 0.19 |
| | 0.37 | 0.43 | 0.16 | 0.19 |
| 3[d] | 0.37 | 0.36 | 0.18 | 0.21 |
| | 0.35 | 0.34 | 0.18 | 0.23 |

[a] The number of SSB and AP-sites per DNA molecule induced by chromate (0.175 mM) and GSH (5 mM) in PM2 DNA in aerobic conditions. In experiment 3 all solutions were free from iron and copper ions. Data shown are corrected for the level of breaks in the controls containing DNA and GSH. The level of AP-sites, was obtained by subtracting the level of SSB from the total level of breaks observed after post-incubation with either putrescine or exonuclease III.

[b] The level of SSB and AP-sites in PM2 DNA treated with chromate and GSH under a nitrogen atmosphere.

[c] AP-sites were revealed by post-incubation with putrescine.

[d] AP-sites were revealed by post-incubation with exonuclease III.

These results indicate that molecular oxygen is essential for the formation of both SSB and AP-sites and is the source of the peroxidic species which is scavenged by catalase.

In view of these results, the possibility that the oxidative species formed during the reduction of chromate by GSH could be hydroxyl radicals was investigated. The removal of traces of catalytic metal ions such as iron(II) or copper(II) left the level of both AP-sites and SSB unchanged (**Table 3-1**, experiment 3). This is indirect evidence that simple Fenton chemistry, in which the residual catalytic metals would be involved

on the formation of hydroxyl radicals, does not seem to be responsible for the damage observed in the chromate/GSH system. However, it is necessary to examine the ability of chromium species to mediate the formation of hydroxyl radicals via a Fenton type mechanism.

## Detection of Modified DNA Bases by HPLC and GC/MS Chromatography

Hydroxyl radicals are extremely powerful oxidants that react at nearly diffusion-limited rates with most organic substrates. If formed in the presence of DNA they would attack indiscriminately any of its components generating, among other DNA lesions, single-strand breaks and adducts with any of the four DNA bases (Imlay and Linn, 1988; Halliwell and Aruoma, 1991).

A possible source of ·OH, apart from ionising radiation, is the oxidation of transition metals, such as iron and copper, by hydrogen peroxide via Fenton chemistry (although ·OH has never been conclusively identified in these systems), equation 1:

$$M^{(n-1)+} + H_2O_2 \rightarrow M^{n+} + \cdot OH + H_2O \quad \text{(equation 1)}$$

It has been suggested, that chromium (V) or (IV) could act as Fenton-like metals, thereby producing hydroxyl radicals (Kawanishi *et al.*, 1986; Aiyar *et al.*, 1989; Kortenkamp *et al.*, 1989; Shi and Dalal, 1992; Shi *et al.*, 1994).

The possible generation of ·OH radicals during the reduction of chromate by GSH was investigated by monitoring the formation of 8-hydroxydeoxyguanosine, one of the major base adducts formed after attack on DNA by ·OH. This DNA base-adduct is not specific for ·OH and can also be formed by other reactive oxygen species (Halliwell and Aruoma, 1991). Therefore, formation of 8-OH-dG would be an indication that such species are formed in the system, but would not provide conclusive evidence that ·OH is indeed responsible for the damage. However, hydroxyl radicals can produce a unique

number of other base modifications which can be used as a fingerprint for its detection (Halliwell and Dizdaroglu, 1992). In order to conclusively establish the possible formation of hydroxyl radicals in the chromate/GSH system, the range of base modifications can be measured using gas chromatography and mass spectrometry in the selected ion monitoring mode (SIM).

## Formation of 8-Hydroxydeoxyguanosine

Calf-thymus DNA was incubated with chromate (0.4 mM) and glutathione (5 mM), or with either reactant alone in phosphate buffer. Mixtures of iron/hydrogen peroxide, a system known to produce high levels of 8-OH-dG, were used as a positive control. The possible formation of 8-OH-dG was monitored using HPLC coupled to an electrochemical detector.

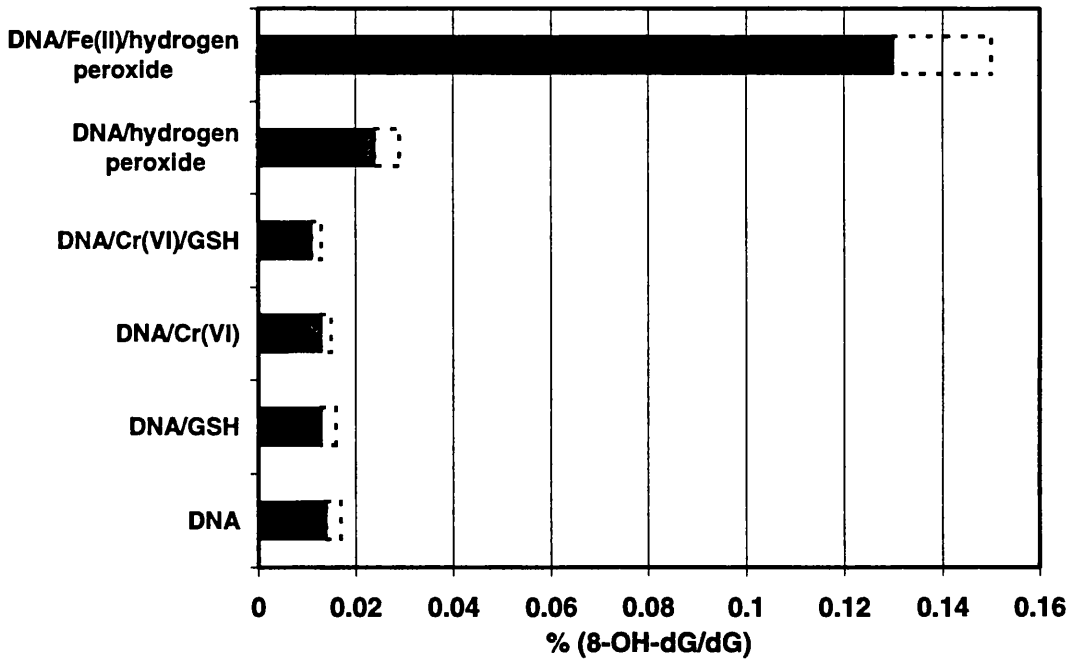



**Figure 3.3-2:** The levels of 8-OH-dG in calf-thymus DNA following treatment with chromate (0.4 mM), GSH (5 mM), chromate and GSH, $H_2O_2$ (50 μM) or iron sulphate (42 μM) and $H_2O_2$ for 16 hours. The DNA base-adducts were measured using HPLC with electrochemical detection. Results are means ± s.d. (broken lines) (n = 3).

No significant increase in the level of 8-OH-dG was observed in the chromate/GSH samples compared to controls (**Figure 3.3-2**). In contrast, treatment of calf-thymus DNA with Fe(II)/$H_2O_2$ induced, as expected, high levels of 8-OH-dG.

In order to firmly establish that hydroxyl radicals were not formed during the reduction of chromate by GSH, the possible generation of other base modifications by hydroxyl radicals was monitored using GC/MS.

### Detection of DNA Base-Adducts by GC/MS

Calf-thymus DNA was treated with Cr(VI)/GSH under the same experimental conditions used for the detection of 8-OH-dG. The presence of the different base-adducts, 5-hydroxyuracil (5-OH-Ura), 5-(hydroxymethyl)uracil (5-OH-Me-Ura), 4,6-diamino-5-formamidopyrimidine (FAPy-Ade), 8-hydroxyadenine (8-OH-Ade), 2,6-diamino-4-hydroxy-5-formamidopyrimidine (FAPy-Gua) and 8-hydroxyguanine (8-OH-Gua) (**Figure 3.3-3**), was monitored with GC/MS in SIM mode.

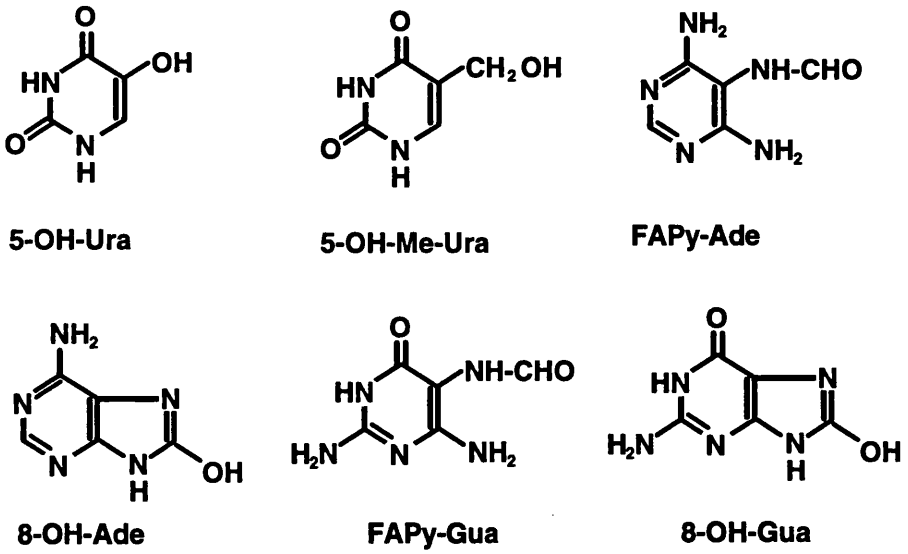



**Figure 3.3-3**: The chemical structures of some DNA base-adducts formed as consequence of hydroxyl radical attack.

The level of modified DNA bases in chromate/GSH treated DNA was similar to that of non-treated DNA or DNA treated with either reactant alone (**Table 3-2**). On the other hand, the formation of oxidative base damage is evident for DNA treated with $Fe^{2+}/H_2O_2$. In Appendix II the quantification of the different base modifications in nmoles/mg DNA from the area of the SIM spectra peaks is explained.

None of the typical DNA base-adducts induced by hydroxyl radicals were observed in chromate/GSH treated calf-thymus DNA by either of the techniques used. One can conclude that there is no evidence that hydroxyl radicals are being formed during the reduction of chromate by GSH, therefore these species are highly unlikely to be responsible for the induction of the DNA lesions observed.

**Table 3-2**: Yield of base-products (nmoles/mg DNA) upon treatment of calf-thymus DNA with different reaction systems

| | DNA | DNA/Cr (VI) | DNA/GSH | DNA/Cr(VI) /GSH | DNA/ $Fe^{2+}$ / $H_2O_2$ |
|---|---|---|---|---|---|
| 5-OH-Ura | 0.033 ± 0.019 | 0.047 ± 0.017 | 0.039 ± 0.011 | 0.041 ± 0.008 | 0.18 ± 0.015 |
| 5-OH-Me-Ura | 0.015 ± 0.014 | 0.006 ± 0.004 | 0.015 ± 0.010 | 0.009 ± 0.007 | 0.05 ± 0.011 |
| FAPy-Ade | 0.160 ± 0.080 | 0.150 ± 0.080 | 0.132 ± 0.081 | 0.108 ± 0.055 | 0.75 ± 0.13 |
| 8-OH-Ade | 0.141 ± 0.041 | 0.128 ± 0.072 | 0.094 ± 0.048 | 0.126 ± 0.098 | 0.63 ± 0.18 |
| FAPy-Gua | 0.164 ± 0.015 | 0.168 ± 0.040 | 0.164 ± 0.077 | 0.153 ± 0.060 | 1.10 ± 0.20 |
| 8-OH-Gua | 0.067 ± 0.027 | 0.042 ± 0.029 | 0.062 ± 0.029 | 0.057 ± 0.039 | 1.04 ± 0.25 |

Results are means ± s.d. from 3 independent experiments.

## *3.4 Discussion*

### Oxygen is Essential for the Formation of the DNA Damaging Species

It was established that an intermediate(s) generated during the reduction of chromate by GSH were responsible for the formation of SSB and AP-sites, as neither Cr(III), the final product of the reduction, nor the initial reactants were able to induce these DNA lesions. Various studies were carried out in order to gain further insight in the nature of the damaging species.

Upon addition of catalase to the reaction mixture the formation of SSB and AP-sites was almost completely inhibited, suggesting that hydrogen peroxide or a peroxo species was involved in the process leading to DNA damage. Catalase is known to catalyse the dismutation of hydrogen peroxide to water and oxygen, however, studies have shown that it can also act as a peroxidase in the presence of a hydrogen donor when the concentration of substrate is below $10^{-4}$ M or when the substrate is an alkyl peroxide (Deisseroth and Dounce, 1970).

Studies conducted under anoxic conditions revealed that the presence of molecular oxygen is essential for the formation of SSB and AP-sites. The oxygen requirement could be at two different levels:

1) Oxygen may be necessary for the formation of the damaging species that attacks DNA.

   and/or

2) Oxygen may be required for the fixation of a nascent DNA lesion after attack of the damaging species. Carbon centred radicals at any of the 5 positions of the deoxyribose sugar, formed upon hydrogen abstraction by an oxidative species, react extremely fast with oxygen to form peroxy radicals, $R\cdot + O_2 \rightarrow ROO\cdot$, $k \approx 10^9\ M^{-1}s^{-1}$ (Simic *et al.*, 1989). This has been shown to be an essential step for the formation of certain DNA lesions by NCS, bleomycin and ionising radiation (Dedon and Goldberg, 1992a; Stubbe and Kozarich, 1987).

At present, our data suggest that the requirement for oxygen is during the formation of the intermediate species. The ability of catalase to prevent the DNA damage points to a peroxo species free in solution. It is highly unlikely that catalase could exert its effect after the initial attack on DNA, as the steric hindrance would be too great for catalase to be able to access a DNA peroxyl species. Further support for the idea may come from the kinetic studies on the reduction of chromate by GSH (see chapter 6), in which a variation of the rate of reduction under anoxic conditions could be an indication that

molecular oxygen is involved in a minor or side pathway of the reduction process, leading to the formation of the DNA damaging species.

Molecular oxygen may also be essential for the fixation of the nascent DNA lesion, however, it will be difficult to prove, as without oxygen no damaging species seems to be formed and therefore no DNA damage is initiated.

The involvement of active oxygen species in the steps leading to DNA damage has been stressed in other studies. da Cruz Fresco and Kortenkamp (1994) showed that oxygen was required for the formation of SSB in isolated DNA by chromate and ascorbic acid, these DNA lesions may have been induced by an oxygen-activated species of strong oxidising behaviour described by Lefebvre and Pézerat, (1992). Snyder (1988) showed that in the presence of catalase the induction of DNA strand breakage on human diploid fibroblasts treated with chromate was completely inhibited, and Witmer *et al.* (1994) provided evidence that reactive oxygen species could be formed upon exposure of A549 lung cells to chromate. The more important piece of evidence came from mutagenicity studies (Sugden *et al.*, 1990), the mutations induced by potassium dichromate on the *Salmonella* strains TA2638 and TA102 were found to be dependent on the presence of molecular oxygen. In anaerobic conditions the induction of mutations in strain TA102 was completely eliminated.

## Hydroxyl Radicals Are Not Generated During the Reduction of Chromate by GSH

Chromatographic studies using HPLC and GC/MS provided evidence that no DNA base oxidative damage occurred upon treatment of calf-thymus DNA with chromate and GSH. The level of DNA base-adducts used as markers for ·OH formation (8-OH-dG, 5-OH-Ura, 5-OH-Me-Ura, FAPy-Ade, 8-OH-Ade, FAPy-Gua and 8-OH-Gua) remained unchanged, even after incubation with chromate and GSH at concentrations (0.4 and 5 mM respectively) in which high levels of SSB and AP-sites were observed. The suggestion that chromium may act as a Fenton-like metal ($H_2O_2 + M^{(n-1)+} \rightarrow M^{n+} + \cdot OH$

+ $H_2O$) (Shi and Dalal, 1992), thereby producing ·OH, is not supported by these findings. Our results indicate that these highly reactive species are not formed during the reduction of chromate by GSH and therefore do not play a role in the induction of either SSB or AP-sites in isolated PM2 DNA.

Although SSB and AP-sites were measured using supercoiled PM2 DNA and modified bases with calf-thymus DNA, the conclusions drawn from these studies should be valid for both systems. SSB have been shown to be induced in DNA from different sources (human fibroblasts, Chinese hamster ovary cells, V-79 hamster cells) at concentrations lower or as high as those used in our studies (Snyder, 1988; Standeven and Wetterhahn, 1991a). One can therefore expect, that SSB and AP-sites are also induced in calf-thymus DNA. An inference further supported by the studies presented in Chapter 4, which provided indirect evidence that SSB were being induced in calf-thymus DNA upon treatment with chromate and GSH.

Our results are in good agreement with other studies. Aiyar *et al.* (1989) also failed to observe the generation of 8-OH-dG upon treatment of DNA with chromate (1.8 mM) and GSH (18 mM) for 30 minutes in Tris-HCl buffer. Kortenkamp and O'Brien (1994) using competition kinetics studies between DNA and ·OH scavengers presented evidence that no ·OH were formed in the Cr(VI)/GSH system, as the rate of reaction of the intermediate species with DNA did not match that of ·OH. Hydroxyl radicals have been shown to be generated in *in vitro* systems in which $H_2O_2$ or microsomes/NADPH were used as chromium (VI) reductants (see section 1.2.3; Kawanishi *et al.*, 1986; Aiyar *et al.*, 1989; Shi *et al.*, 1991). However, there is no evidence to suggest that such species are formed in the presence of the two major chromium (VI) intracellular reductants, GSH and ascorbic acid. In similar studies to those reported here, no evidence of ·OH formation was obtained during the reduction of chromate by ascorbic acid (da Cruz Fresco personal communication).

*In vivo* studies seem to support these suggestions. Upon treatment of male Sprague-Dawley rats with sodium chromate for several hours, Standeven and Wetterhahn (1991a) did not observe a significant increase in 8-OH dG levels in either liver or kidney

DNA. Recent studies on the effect of chromate in chick embryos, showed an increase in the level of 8-oxo-dG in DNA from red blood cells but not in liver DNA. Red blood cells have high levels of haemoglobin which, as the authors pointed out, is likely to be responsible for the oxidative damage observed (Misra *et al.*, 1994).

Further support for the idea that ·OH is not involved in the mediation of chromate mutagenicity is provided by work on the mutational spectrum of chromate in human cells. In their study of chromate treated cells, Chen and Thilly (1994) observed that the distribution of chromate mutations was different from that induced by agents such as $H_2O_2$, $O_2$ and X-rays, which are thought to exert their mutagenicity via oxygen free radical intermediates.

The reduction of chromate by GSH leads to the formation of chromium species in intermediate oxidation states such as Cr(V) and Cr(IV) (Kitagawa *et al.*, 1988; Bose *et al.*, 1992). There is evidence that a Cr(V)-GSH complex isolated from the reduction reaction is capable of inducing SSB in isolated DNA (O'Brien *et al.*, 1990; Kortenkamp *et al.*, 1989). Considering that ·OH radicals do not seem to be responsible for the DNA damage observed, but oxygen is essential for the formation of the intermediate species, one could speculate about the possibility of a Cr(IV) or Cr(V) - activated oxygen species being formed. A species of this kind would be highly oxidising and could account for our observations (see section 6.4 for further development of this idea).

At present, the data available are insufficient for more definitive conclusions about the nature of the intermediate(s) responsible for the induction of DNA lesions. However, a few facts are now well established: hydroxyl radicals are not generated during the reduction of chromate by GSH but molecular oxygen is essential for the formation of the reactive species or one of its precursors.

The role of oxygen on the formation of the reactive intermediate species was further investigated by kinetic studies of the reduction of chromate and GSH under aerobic and anoxic conditions (see Chapter 6 ).

# CHAPTER 4

# THE RELEASE OF MDA-LIKE PRODUCTS FROM CHROMATE/GSH TREATED DNA

## 4.1 Introduction

If the generation of SSB by chromate and GSH occurs as a consequence of attack at C-4' by a reactive species, as proposed in chapter 2, one would expect the release of malondialdehyde-like products in the process leading to DNA scission (see **Figure 2.4-2**). The degradation of the deoxyribose sugar can be studied using the thiobarbituric acid assay (TBA assay).

It has been known for a long time that oxidative degradation of the deoxyribose by periodate yields malondialdehyde (MDA) which reacts at temperatures close to 100°C with thiobarbituric acid to form a pink chromogen (Waravdekar and Saslaw, 1959). Formation of DNA SSB by agents such as ionising radiation (e.g. X-rays), NCS and bleomycin, has been shown to involve attack at the deoxyribose sugar with concomitant release of products, which in a way similar to MDA yield chromogens with TBA, therefore termed MDA-like products (Kapp and Smith, 1970; Hatayama and Goldberg, 1980; Giloni *et al.*, 1981). In most instances, these products are thought to be MDA precursors rather than free MDA; under the harsh conditions of the TBA assay (pH 2, 100°C) these precursors would hydrolyse to form MDA which would subsequently form an adduct with TBA as shown in **Figure 4.1-1**.

The possible release of MDA-like products during the formation of DNA lesions by chromate and GSH was investigated using this assay.

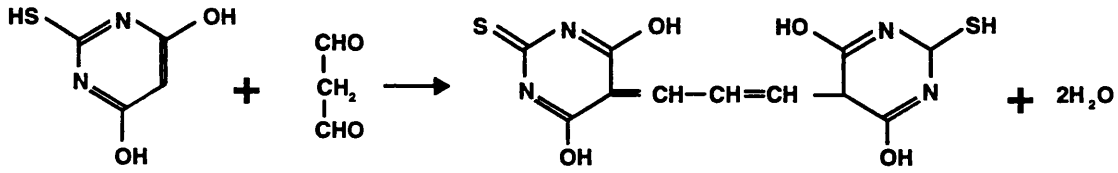



**Figure 4.1-1**: Formation of TBA-MDA adduct, upon reaction of free malondialdehyde or MDA-like products with thiobarbituric acid at 100°C and acidic pH. The pink chromogen has an absorption maximum at 532 nm.

## *4.2 Experimental Section*

### Chemicals

Glutathione, thiobarbituric acid and highly polymeric calf-thymus DNA were purchased from Sigma (Poole, Dorset). Potassium chromate was from BDH (Lutterworth, Leicestershire). Chelex 100 resin and agarose were obtained from Biorad (Hemel Hempstead, Herts). All reagents were of analytical grade. Absorbances were recorded in a Shimadzu MPS-2000 spectrophotometer.

### TBA Assay. The Generation of Malondialdehyde-Like Products

The possible release of MDA-like products from 2-deoxyribose and calf-thymus DNA upon treatment with chromate and GSH was investigated using the thiobarbituric acid. TBA reacts quantitatively with MDA in acid pH at temperatures close to 100°C, forming a pink chromogen which can be monitored at 532 nm.

The TBA assay was tested by constructing a standard curve using 1,1,3,3,-tetraethoxypropane (TEP), which in acid conditions hydrolyses to yield MDA (Sinnhuber and Yu, 1958). An aliquot of TEP stock solution was diluted with a 1 % solution of $H_2SO_4$ to a final concentration of 10 mM and allowed to stand for two hours at room temperature (Cheeseman *et al.*, 1988). After hydrolysis, aliquots of increasing MDA concentration were prepared (5 - 10 μM, in water) and assayed with TBA (0.3 ml sample + 0.6 ml TBA (0.67 %) + 50 μl HCl (1 M)). The mixtures were boiled for 20 minutes in a water bath, and the absorbance of the samples measured at 532 nm. A

linear increase in absorbance with rising MDA concentrations was observed, the slope of the curve gave an extinction coefficient of $0.48 \times 10^5$ $M^{-1}$ $cm^{-1}$. In a previous work, Sinnhuber and Yu (1958) established that $\varepsilon_{MDA} = 1.5 \times 10^5$ $M^{-1}$ $cm^{-1}$; a possible explanation for our much lower value is that the hydrolysis of TEP to MDA may not have been quantitative, and therefore the concentrations of MDA measured were lower that expected. Nevertheless, with this study it was confirmed that using the TBA assay increasing amount of MDA could be detected.

### Treatment of 2-Deoxyribose:

In initial experiments, it was observed that incubation of deoxyribose with chromate alone led to pronounced increases in absorbance at 532 nm in the TBA assay. The level of absorbance did not increase with prolonged incubation times, and therefore it was concluded that the effect was due to chromate interfering with the TBA assay, rather than chromate being able to cause oxidative damage on the sugar. The absorbance at 532 nm decreased when the chromate concentrations were lowered, and at around 100 µM chromate the absorbance fell to control levels (same absorbance as deoxyribose alone). In view of these results, it was important to carefully choose the reaction conditions for the chromate/GSH system, so as to minimise any interference of chromate.

2-deoxyribose (2 mM) was incubated with potassium chromate (0.4 - 0.8 mM) and GSH (5 mM) in demetallated phosphate buffer (0.1 M, pH 6.9) for 6 hours in a final volume of 1 ml ("samples"). Under these conditions, the residual levels of chromate after 6 hours were lower than 100 µM. However, in order to ensure that any increase in absorbance observed was due to MDA formation and not caused by traces of residual chromate, control samples were carefully prepared. Chromate and GSH were incubated under the same experimental conditions than the "samples" but without deoxyribose, which was added immediately before carrying out the TBA assay from a concentrated stock (5 µl, 0.4 M) to minimally affect the final concentration of the reactants. Any increase in absorbance in the control samples would be caused by residual chromate. Aged solutions of chromate and GSH consisting of a Cr(III)-GSH complex did not interfere with the TBA assay. Therefore, the difference between the absorbance at 532

nm of the chromate/GSH samples and the absorbance of the control samples, can be attributed to MDA formed by interaction with deoxyribose.

After incubation, all samples were transferred to glass tubes, and upon addition of TBA (1.5 ml, 0.67 % w/v in 50 mM NaOH) and trichloroacetic acid (TCA) (0.5 ml, 20 %) giving a pH ≈ 2 they were boiled in a water bath for 20 minutes. After cooling, the absorbance at 532 nm was monitored using a Shimadzu spectrophotometer. Blank samples containing phosphate buffer were treated in the same way.

When stated, catalase (10 μg/ml) was added at the beginning of the incubation period; denatured catalase was obtained by heating for 10 minutes at 70°C.

### Calf-Thymus DNA

For practical and economic reasons PM2 DNA could not be used in these studies. The detection limit of the TBA assay is $10^{-7}$ moles of MDA/l (Sinnhuber and Yu, 1958). Assuming that 1 mole of MDA is equivalent to 1 mole of PM2 DNA breaks and considering that under our experimental conditions approximately 1 break per DNA molecule is induced (section 2.3), then the minimum amount of DNA required per sample would be around 200 μg. Solutions of DNA at high concentrations had to be prepared and calf-thymus DNA was the best option.

In these experiments, control samples had to be carefully prepared not only to mimic the amount of residual chromate in chromate/GSH samples ("samples"), but also the amount of Cr(III)-GSH formed after the reduction process. It was observed that the reduction of chromate by GSH in the presence of calf-thymus DNA led, after the TBA assay, to an increase in the baseline absorbance relative to samples that contained DNA and one of the reactants (chromate or GSH). This effect was due to the formation of a fine precipitate of Cr(III)-GSH in the "samples". Unlike deoxyribose, it is not possible to prepare solutions of calf-thymus DNA of high concentration (max. 2 mg/ml), and a different approach had to be taken to prepare the control samples. In order to mimic the final amount of Cr(III)-GSH encountered in "samples", chromate and GSH were

incubated for the same period of time at concentrations twice that of the "samples". Prior to the TBA assay, an identical volume of calf-thymus DNA solution was added, thereby halving the concentrations of Cr(III)-GSH to the desired ones. In this way, control samples showed the same increase in baseline absorbance as "samples". Under these conditions, all Cr(VI) had been reduced, therefore an extra amount of Cr(VI) was added just before TBA assay to account for residual Cr(VI) remaining in the "samples". For controls and "samples", similar spectra were observed after incubation, in the region 360 - 700 nm.

In order to investigate the possible release of MDA-like products from DNA upon treatment with chromate/GSH, highly polymerised calf-thymus DNA (1 mg/ml) was incubated with potassium chromate (0.3 - 0.7 mM) and GSH (5 mM) in demetallated phosphate buffer for 7 hours in a final volume of 500 µl. Control samples were prepared as described above. After incubation, samples were transferred to glass capped tubes, together with HCl (70 µl, 1 M) and TBA (570 µl, 0.67 % w/v in 50 mM NaOH) giving a final pH ≈ 2, and boiled for 20 minutes in a water bath. The samples were cooled and the absorbance at 532 nm monitored. Blank samples containing phosphate buffer were treated in the same way.

Some experiments were carried out in demetallated systems: A stock solution of calf-thymus DNA (2 mg/ml) was demetallated by incubation with DTPA (6.2 mM) overnight at 4°C. After dialysis (24 h, 4°C) against demetallated phosphate buffer the DNA concentration was established by measuring the absorbance at 260 nm and demetallation was ascertained by using the Buettner assay as described in section 2.2.

## *4.3 Results*

### Detection of Oxidative Damage of 2-Deoxyribose

Preliminary studies were carried out using 2-deoxyribose as a substrate. The sugar was incubated with chromate (0.4 - 0.8 mM) and GSH (5 mM) in demetallated phosphate

buffer for six hours. The TBA assay was performed as described by Cheeseman *et al.* (1988) and the absorbance was monitored at 532 nm. In order to account for any interference of residual chromate with the measurement of the absorbance at 532 nm, controls were carefully prepared, as described in the experimental section.

The generation of MDA-like products upon incubation of deoxyribose with chromate and GSH was revealed by the presence of a peak at 532 nm after incubation with TBA, characteristic of the MDA-TBA adduct (**Figure 4.3-1**).

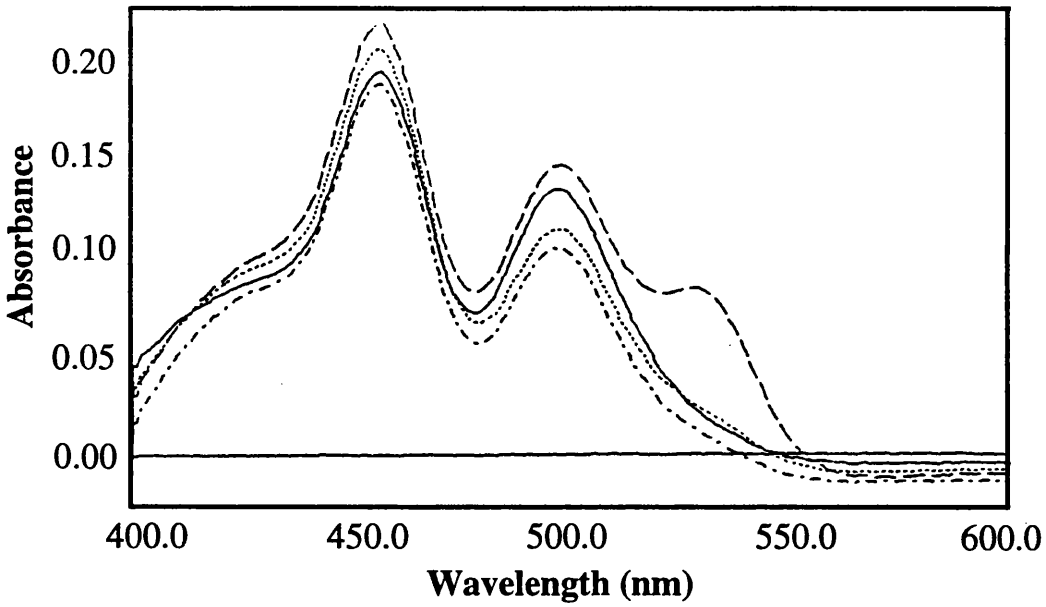



**Figure 4.3-1**: Release of MDA-like products from chromate (0.6 mM)/GSH (5 mM) treated deoxyribose as revealed by the increase in absorbance at 532 nm relative to controls after the TBA assay. Deoxyribose alone (—); controls (- · - · -); Cr(VI)/GSH treated deoxyribose (-----); Cr(VI)/GSH treated deoxyribose in the presence of catalase (·····).

The difference spectrum obtained between the control sample and Cr(VI)/GSH samples was identical to that of standard MDA (obtained from TEP hydrolysis) (**Figure 4.3-2**). The peaks at 496 and 455 nm have been previously observed upon reaction of DNA with the reactant TBA (Kapp and Smith, 1970; Hatayama and Goldberg, 1980).

A linear increase in the level of the MDA-TBA adduct was observed with rising chromate concentrations (**Figure 4.3-3**). Neither GSH alone nor the final product of the reduction (Cr(III)-GSH complex) led to the formation of the pink chromogen, pointing to an intermediate formed during the reduction process as being responsible for the degradation of the sugar.

In the presence of catalase the formation of MDA-like products was completely inhibited, however no decrease in absorbance was observed with denatured catalase.

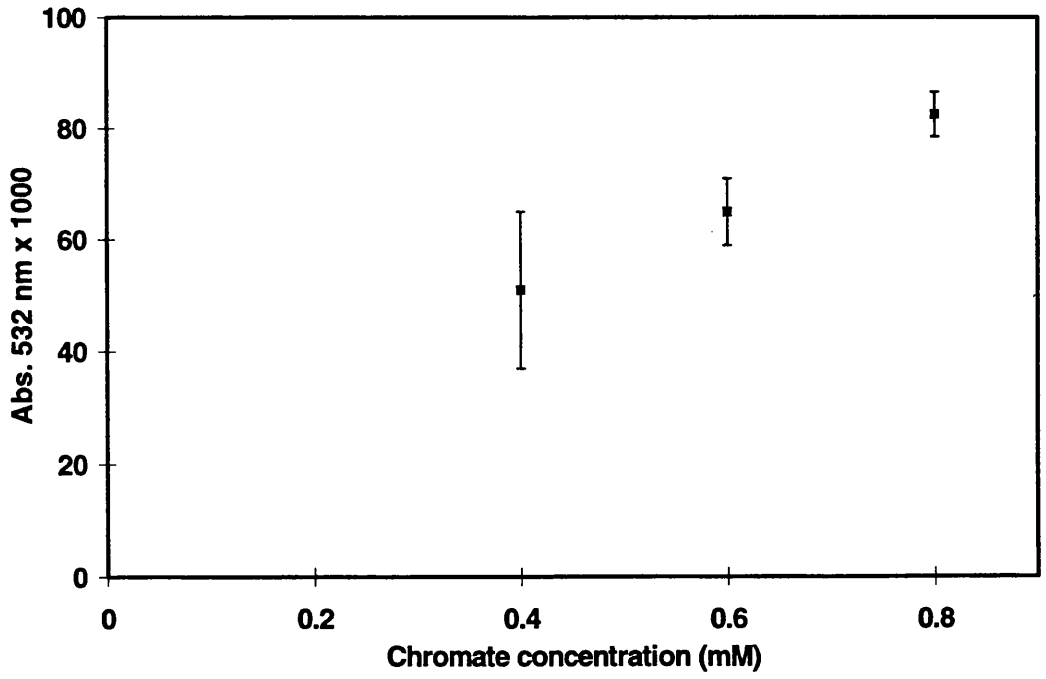
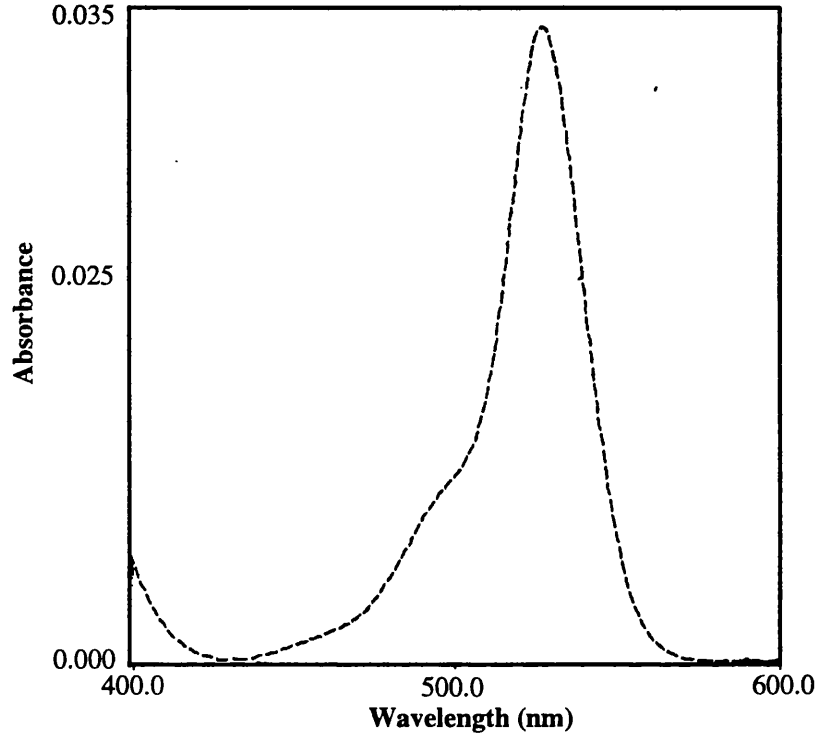



**Figure 4.3-3**: The release of MDA-like products from 2-deoxyribose treated with GSH (5 mM) and various Cr(VI) concentrations for 6 hours, as measured by using the TBA assay. All data were corrected for the absorbance in control samples. Results are means ± SEM × t (95 %), n = 5.



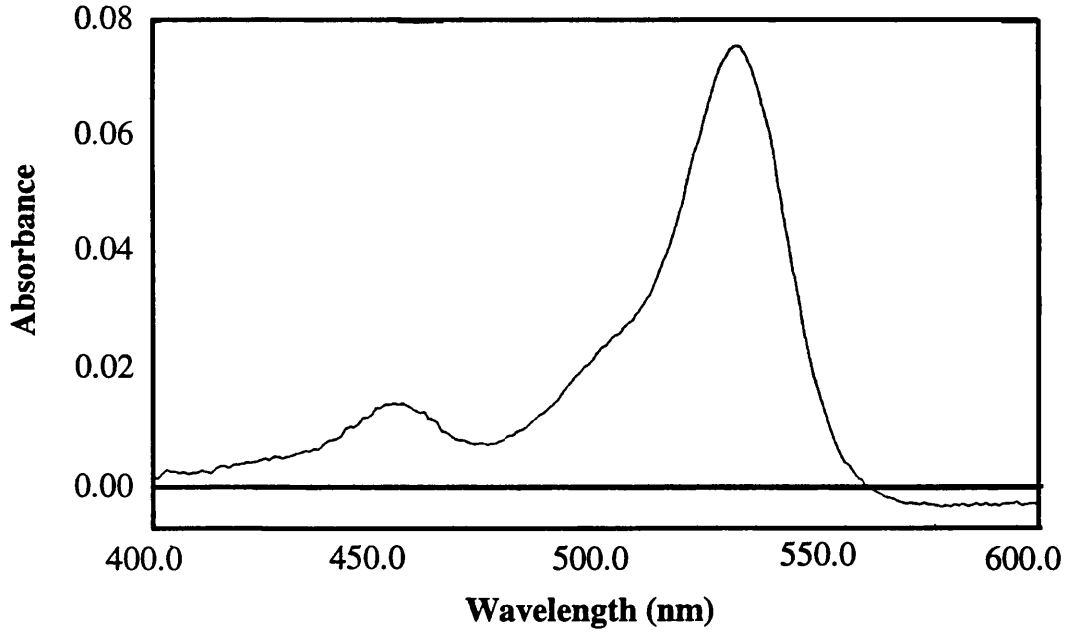




**Figure 4.3-2**: Absorption spectra of MDA at 532 nm after reaction with TBA. A) Standard MDA obtained from TEP hydrolysis; B) Difference spectrum of chromate/GSH (0.8 mM/5 mM) treated deoxyribose and control samples, prepared as described in the experimental section.

## The Release of MDA-Like Products from Calf-Thymus DNA

The demonstration that MDA-like products are release from 2-deoxyribose during the reduction of chromate by GSH prompted us to probe whether MDA-like products were also formed when using calf-thymus DNA as a substrate.

Treatment of calf thymus DNA with chromate and GSH for seven hours led to an increase in absorption at 532 nm relative to controls, upon incubation with TBA, which was taken as evidence of sugar degradation. The pink/yellow chromogen behaved like a TBA-MDA adduct upon addition of alkali, as described in a study by Waravdekar *et al.* (1959), in which the formation of adducts between TBA and several conjugated aldehydes was compared. The presence of alkali induced a spectral shift to higher wavelengths (from 532 to 545 nm) resulting in a colour change from pink to purple; the colour persisted for several hours.

However, MDA or MDA-like products were not released free in solution after treatment of DNA with Cr(VI) and GSH, but remained attached to the DNA. When the DNA was acid precipitated prior to the TBA assay, all the TBA reactive products were recovered with the resuspended DNA pellet. Hence, a combination of acid/heat conditions was required for the release of MDA and the formation of the TBA adduct.

The level of MDA-like products increased with rising chromate concentrations as observed by an increase in the absorbance at 532 nm (**Figure 4.3-4**). An intermediate generated during the reduction was responsible for the damage, as neither GSH alone nor the final product of the reduction led to any increase in absorbance at 532 nm.

The removal of traces of catalytic metals such as iron(II) and copper(II) from DNA (demetallated DNA) left the level of TBA-MDA adduct unchanged (**Table 4-1**), suggesting that hydroxyl radicals, formed via Fenton chemistry involving residual catalytic metal ions were very likely not responsible for the damage observed in the system.

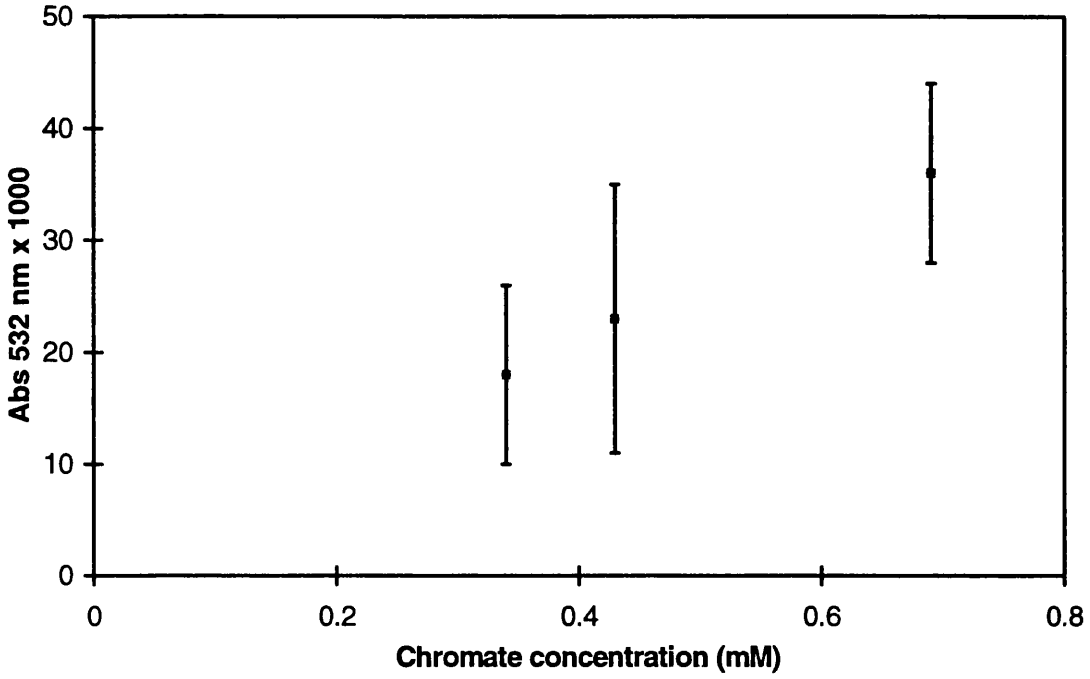



**Figure 4.3-4**: The release of MDA-like products from calf-thymus DNA treated with GSH (5 mM) and various Cr(VI) concentrations for 7 hours, as measured by using the TBA assay. All data were corrected for the absorbance in control samples. Results are means ± SEM × t (95 %), n = 5.

| **Table 4-1**: Influence of residual metal ions on the formation of MDA-like products | | |
|---|---|---|
| **0.69 mM Cr(VI)/5 mM GSH** | **conventional system** | **demetallated system** |
| **Absorbance 532 nm*** | 0.036 ± 0.008 | 0.034 ± 0.007 |

* Results are means ± SEM × t (95 %) n = 5 - 6.

As shown in **Table 4-2**, the addition of catalase to samples containing Cr(VI)/GSH prevented the formation of MDA-like products, suggesting that hydrogen peroxide or a peroxo species are involved in the process leading to sugar damage. Denatured catalase did not elicit any effect.

| Table 4-2: The effect of catalase on the release of MDA-like products from chromate/GSH treated DNA | | | |
|---|---|---|---|
| | **controls** | **Cr(VI)/GSH*** | **Cr(VI)/GSH/catalase** |
| **Absorbance 532 nm** | 0.056 ± 0.006 | 0.1 ± 0.01 | 0.054 ± 0.02 |

* Calf-thymus DNA was treated with chromate (0.52 mM) and GSH (5 mM) for 7 hours. TBA assay was then performed as described in the experimental section. Results are means ± SEM × t (95 %), n = 3 - 4.

These results provide evidence that an intermediate generated during the reduction of chromate by GSH is able to cause oxidative degradation of the DNA deoxyribose sugar, releasing MDA-like products, which can be detected by the TBA assay. This process would provide a route for the formation of DNA SSB, as the release of MDA results in DNA phosphate backbone scission. Although these studies were carried out using calf-thymus DNA instead of PM2 DNA, it is reasonable to assume that the induction of the SSB by chromate and GSH in isolated PM2 DNA also occurs via attack at the deoxyribose sugar.

## *4.4 Discussion*

### The Formation of SSB Occurs Via Attack at the DNA Deoxyribose Sugar

The generation of MDA-like products, as determined using the TBA assay, during the treatment of calf-thymus DNA with chromate and GSH, provides evidence that oxidative degradation of the deoxyribose had occurred.

The formation of MDA precursors has also been observed for DNA damaging agents such as bleomycin, NCS, X-rays and γ-rays (Giloni *et al.*, 1981; Hatayama and Goldberg, 1980; Kapp and Smith, 1970; Ward and Kuo, 1976). MDA has been proposed to derive from C-1', C-2' and C-3' of the deoxyribose sugar, and is thought to arise from hydrogen abstraction from C-4' and bond cleavage between C-3' and C-4' (Kapp and Smith, 1970; Giloni *et al.*, 1981; Schulte-Frohlinde and von Sonntag, 1985).

The release of MDA-like products results in a DNA SSB with a 3'-phosphoglycolate end. It is very likely that chromate and GSH generate MDA-like products via a similar mechanism. The fact that all the TBA reactive products were recovered in the acid precipitated pellet seems to indicate that either the chromophore-yielding material remained bound to the DNA or that upon release the MDA-precursor suffered condensation with the DNA bases.

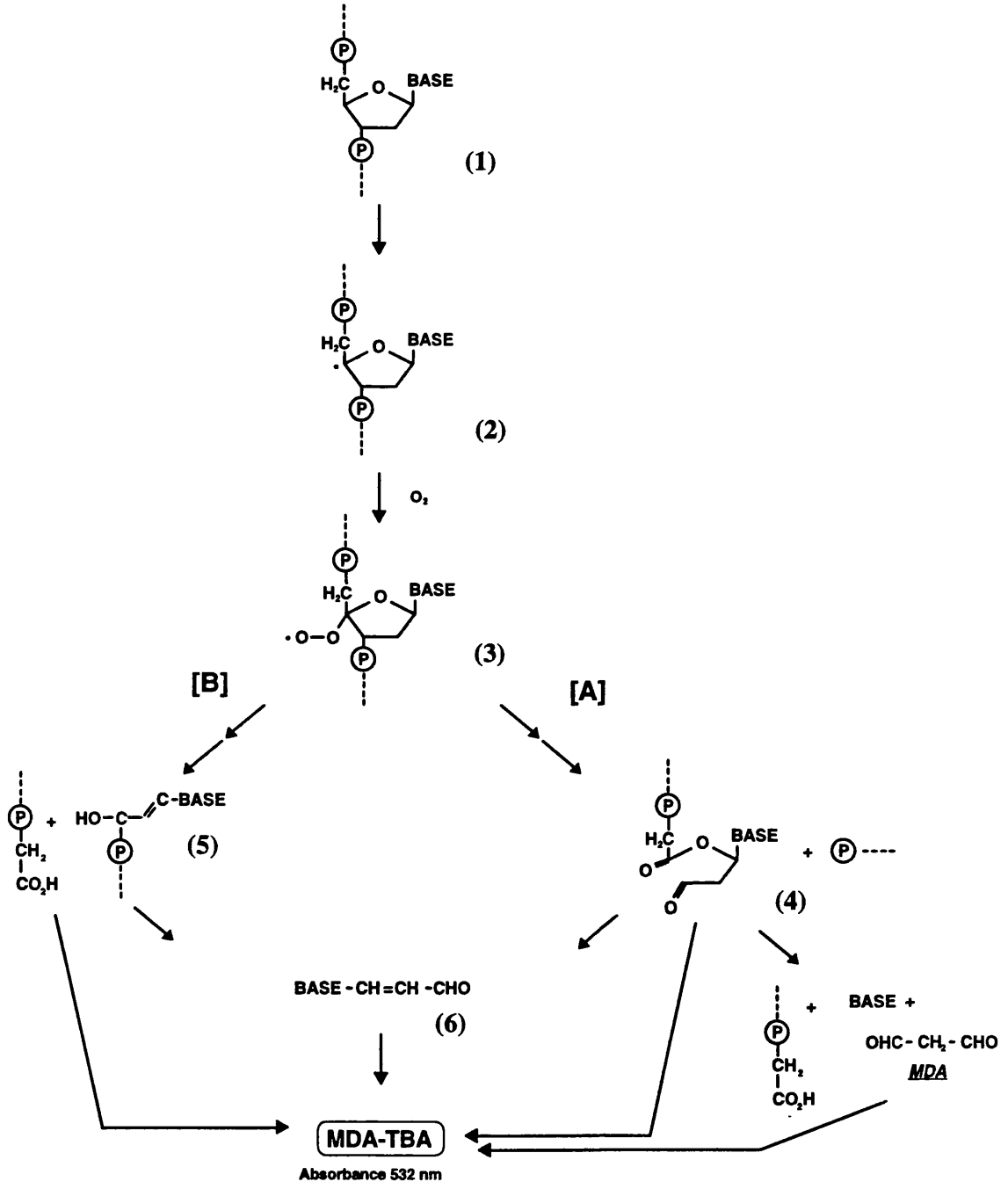



**Figure 4.4-1**: Possible pathways for the formation of free MDA or MDA like products upon attack at C-4' of the deoxyribose moiety.

**Figure 4.4-1** summarises what might occur in the system. Upon abstraction of the hydrogen from C-4' by an oxidative species, the radical formed (2) can be quenched by oxygen in an extremely fast reaction (Simic *et al.*, 1989), leading to a peroxyl radical (3), which, depending on the system, may evolve to form either (4) or (5) via two different pathways. Pathway *A* has been suggested to occur upon treatment of DNA with ionising radiation (Schulte-Frohlinde and von Sonntag, 1985; von Sonntag, 1987, pp 247-248), species (4) could either react to form base-propenals (6), free MDA or remain unchanged, releasing MDA during the TBA assay. On the other hand, Burger *et al.* (1986) proposed pathway *B* to account for the formation of base propenals upon attack of DNA by bleomycin. They observed that the release of the TBA reactive materials from species (5) occurred during a period of 40 minutes at 4°C.

Although the TBA assay studies were carried out using calf-thymus DNA instead of PM2 DNA, it is very likely that the SSB formed by chromate and GSH in PM2 DNA were at least partly derived from deoxyribose degradation. This assumption is supported by the following observations:

a) The level of SSB and MDA-like products increased linearly with rising chromate concentration. In contrast, when ascorbic acid was used as a reductant (data not shown) the level of MDA-like products increased exponentially, in a similar fashion to the SSB induced in that system.
b) An intermediate generated during the reduction of chromate by GSH is responsible for the induction of SSB and MDA-like products, and both are inhibited when catalase is added to the system.
c) Considering the differences between the systems, the level of MDA-like products released correlates reasonably well with the level of DNA SSB:

0.34 mM Cr (VI)/5 mM GSH, calf-thymus DNA 7 hours incubation:
Abs 532 nm = 0.018 ≡ 0.27 nmoles MDA/mg DNA ($\varepsilon_{MDA} = 1.5 \times 10^5\ M^{-1}\ cm^{-1}$)

0.175 mM Cr (VI)/5 mM GSH, PM2 DNA 4 hours incubation:
0.5 breaks per DNA molecule ≡ 0.07 nmoles of breaks/mg DNA

Further evidence for the generation of MDA-like products via abstraction of the hydrogen at C-4' can be obtained by proving the presence of a 3'-phosphoglycolate residue at the 3' terminal end of the SSB, by using radioactive labelled DNA and denaturing polyacrylamide gels in conjunction with the Maxam-Gilbert degradation process (see Chapter 5).

Halliwell and co-workers (Halliwell *et al.*, 1988) have defined the TBA assay as a simple "test tube" assay for detection of ·OH radicals. The results obtained in these studies together with those obtained previously with the bleomycin system (Gajewski *et al.*, 1991) question the validity of this statement. Both systems, bleomycin and chromate/GSH, induce MDA-like products but do not lead to DNA base-adducts characteristic of ·OH. Therefore, species other than hydroxyl radicals are able to generate TBA reactive materials.

## 4.5 Conclusions

The results from chapter 2 indicated that a single reactive species acting on a single site of DNA was responsible for the formation of SSB and AP-sites in PM2 DNA by chromate and GSH. These observations led us to propose that both lesions could arise from attack at C-4' of the deoxyribose sugar by a reactive intermediate. Further support for this suggestion has been obtained from investigations on the nature of the reactive species (chapter 3) and from TBA assay studies (this chapter).

Molecular oxygen is essential for the formation of the reactive species or one of its precursors, probably peroxidic in nature. Therefore, it is possible that this species is oxidising enough to be able to abstract the hydrogen from C-4' of the deoxyribose sugar. Moreover, the release of MDA-like products from calf-thymus DNA during the reduction of chromate by GSH is proof that the SSB are formed by the degradation of the deoxyribose sugar, very likely via abstraction of the hydrogen from C-4'.

All the data support the hypothesis that the formation of SSB and AP-sites by chromate and GSH occurs via a mechanism similar to that of NCS or bleomycin (see **Figure 2.4-2**): Initial formation of a C-4' radical due to hydrogen abstraction by an oxidising species, which may evolve to form either a 4'-hydroxylated AP-site or a SSB with release of MDA-like products.

In an attempt to provide more definitive evidence for this model the following questions should be addressed:

**A) The Generation of a 4'-Hydroxylated AP-Site**

Further support for the suggestion that the aldehydic AP-sites generated by chromate and GSH arise by attack at C-4' could be obtained by using hydrazine for the detection of AP-sites. Whereas putrescine can react with virtually all abasic sites to produce SSB, hydrazine reacts selectively with the C-4'-hydroxylated AP-sites leading to DNA scission with 3'phosphopyridizine and 5'phosphate ends (Dedon and Goldberg, 1992b).

**B) Specific Attack of a Reactive Intermediate at C-4'**

It is necessary to envisage a mechanism by which the reactive intermediate arising from chromate/GSH would have easy and preferential access to C-4'. Considering that intercalation of a chromate/GSH intermediate into the minor groove of the DNA is unlikely to take place (C-4' is easily reached through the minor groove), initial binding of the intermediate species to the phosphodiester bond may provide such a situation.

There is evidence of chromium binding to DNA subsequent to the reduction of chromate by GSH, and although the binding site is far from clear (Borges and Wetterhahn, 1989; Hneihen *et al.*, 1993; Salnikow *et al.*, 1992), some data support the idea that interaction of chromium with the DNA phosphate backbone can occur. In their examination of DNA isolated from [$^{51}$Cr]chromate-treated cells, Salnikow *et al.* (1992) observed that after enzymatic DNA degradation the $^{51}$Cr was released from the DNA but did not elute with any of the nucleotides, suggesting that it was bound to the DNA phosphate backbone. Kortenkamp *et al.* (1991) using $^{31}$P NMR showed that

chromium(III) complexes can bind to the phosphate groups of ATP via formation of outer-sphere complexes. Further studies are required to definitively establish the nature and the site of chromium binding. The main problem one faces when carrying out binding studies using $^{51}Cr$ and DNA is that during the isolation or digestion procedure the original chromium-DNA binding may be disturbed (Salnikow *et al.*, 1992; Hneihen *et al.*, 1993). A way forward for the study of such interactions could be the use of non-invasive techniques such as $^{31}P$ or $^{1}H$ NMR in combination with short oligonucleotides.

**C) The nature of the "random factor" causing the formation of either SSB or AP-sites**

The factor regulating the formation of either SSB or AP-sites may be difficult to elucidate. In the case of bleomycin, it has been shown that molecular oxygen is the key factor; the partitioning does not take place after the formation of the peroxyradical (see **Figure 2.4-2**), as is the case with NCS, but in the previous step (formation of C-4' radical). Oxygen has been shown to be essential for the formation of SSB but not 4'-hydroxylated AP-sites (Wu *et al.*, 1985a, b; Dedon and Goldberg, 1992a). As previously discussed, in the chromate/GSH system oxygen is essential for the formation of the DNA damaging species, therefore any further requirement for molecular oxygen cannot, for the time being, be established.

The formation of both DNA lesions, SSB and AP-sites, by NCS is dependent on the presence of molecular oxygen (see **Figure 2.4-2**). The partitioning takes place after the formation of the peroxyl radical and it is probably mediated by the presence of thiols (Dedon and Goldberg, 1992a, b). It is well established that thiols are able to donate a hydrogen atom, from their SH groups, to oxygen and carbon centred DNA radicals which may prevent, in some cases, the formation of DNA lesions (Lafleur and Retèl, 1993). In a short experiment, the effect of GSH concentration (1 - 7 mM) in the chromate/GSH mediated DNA lesions was investigated. The level of both DNA lesions, SSB and AP-sites, decreased with increasing GSH concentration, but the ratio of the DNA lesions was close to 1:1 at all times (**Table 4-3**).

**Table 4-3:** The level of SSB and AP-sites* induced in PM2 DNA upon incubation with chromate (0.175 mM) and various GSH concentrations

| [GSH] (mM) | SSB | AP-sites |
|---|---|---|
| 1 | > 2 | > 2 |
| 3 | 0.92 ± 0.05 | 0.92 ± 0.06 |
| 5 | 0.66 ± 0.04 | 0.69 ± 0.16 |
| 7 | 0.31 ± 0.04 | 0.23 ± 0.01 |

* Samples were incubated for three hours. AP-sites were detected by putrescine. Results presented are means ± s.d., n = 2.

Two factors are likely to contribute to the decrease in DNA damage with rising GSH concentrations, a protective effect of GSH and an increase in the rate of chromate reduction with increasing GSH concentration.

The fact that the ratio of both DNA lesions remained the same could be an indication that the thiol is not the partitioning factor, an increase in the level of AP-sites with increasing GSH concentrations would be expected according to the mechanism proposed in **Figure 2.4-2**. However, there is not enough data to firmly support this suggestion. At present, neither molecular oxygen nor GSH can be ruled out as the partitioning factors mediating the formation of SSB and AP-sites by the chromate/GSH system.

# *CHAPTER 5*

# THE DISTRIBUTION OF CHROMATE/GSH INDUCED DNA LESIONS ALONG THE DNA STRANDS

## *5.1 Introduction*

Our studies of the generation of SSB and AP-sites in PM2 DNA during the reduction of chromate by glutathione have provided new mechanistic insights into the mechanisms by which these DNA lesions may arise. The release of MDA-like products from chromate/GSH treated DNA was an indication that the formation of SSB occurred via deoxyribose sugar degradation, and the time course studies suggested that AP-sites and SSB originated from a common precursor lesion.

Studies using 5'-end radiolabelled DNA and denaturing polyacrylamide gels were carried out in order to further substantiate these ideas. Used in conjunction with the Maxam-Gilbert sequencing process, these techniques can provide the means to study the distribution of SSB and AP-sites along the DNA strand and the possibility to gain information about the chemical nature of SSB 3'-terminal ends.

**Distribution of Lesions along the DNA Strands:**

The induction of single strand breaks in a double stranded DNA fragment leads to a set of fragments of different length (oligonucleotides) upon denaturation. DNA molecules that differ in length by just a single nucleotide can be separated into distinct bands by electrophoresis through polyacrylamide gels (PAGE). The exact position of DNA damage along the phosphate backbone can be elucidated by comparison with the bands obtained by the Maxam-Gilbert degradation process, in which each family of

nucleotides (A, C, G, T) is specifically cleaved by a unique set of reactions (Maxam and Gilbert, 1980; Brown, 1994).

The distribution of chromate/GSH induced DNA damage, SSB and AP-sites (after being revealed by putrescine or exonuclease III) can therefore be studied using this technique. Two different outcomes are possible: If the damage was base or sequence specific, bands would be observed at certain positions along the DNA fragment. On the other hand, if breaks were randomly induced the set of bands generated would not present any particular pattern.

The time course studies using PM2 DNA suggest a common precursor lesion and the same probability for the induction of SSB and AP-sites. If this hypothesis is correct, it should be reflected in the distribution of both DNA lesions along the DNA fragment. One would expect the same type of distribution for SSB and AP-sites along the DNA strand, i.e. the band patterns reflecting the location of the two DNA lesions, should be either both base specific or both randomly distributed. If a different band distribution was observed for each DNA lesion, the conclusion drawn from our time course studies would need to be reassessed.

### Chemical Structure of 3'Cleaved Ends:

DNA SSB are very rarely caused by a simple cleavage of the phosphate backbone. Usually parts or the whole of the nucleotide are released in the process. Characterisation of the two ends of the DNA strand formed as a consequence of a SSB can provide information on the mechanism underlying SSB induction. Using the same technique as described above, 3'ends can be characterised by radioactively labelling the 5'ends and vice versa, by comparing the migration of the bands of the nicked DNA with those of the DNA degraded by Maxam-Gilbert reactions. The breaks induced by the latter carry 3'phosphorus terminal ends, variations in the electrophoretic mobility relative to these markers would be an indication that a different type of end was formed. Various possible structures of the 3'ends are depicted in **Figure 5.1-1**.

Studies of this kind have been successfully conducted for other DNA damaging agents such as NCS and ionising radiation (Kappen *et al.*, 1991; Henner *et al.*, 1982; Henner *et al.*, 1983). The formation of a glycolic acid residue (**Figure 5.1-1 C**), which migrated quicker than the markers, was observed and was attributed to the formation of MDA-like products upon fragmentation of the C4'- C3' sugar bond. Detection of such a residue in chromate/GSH treated DNA would be unambiguous confirmation that SSB were induced by attack at the deoxyribose sugar and would be further proof that MDA-like materials were being formed as concluded from the TBA assays.

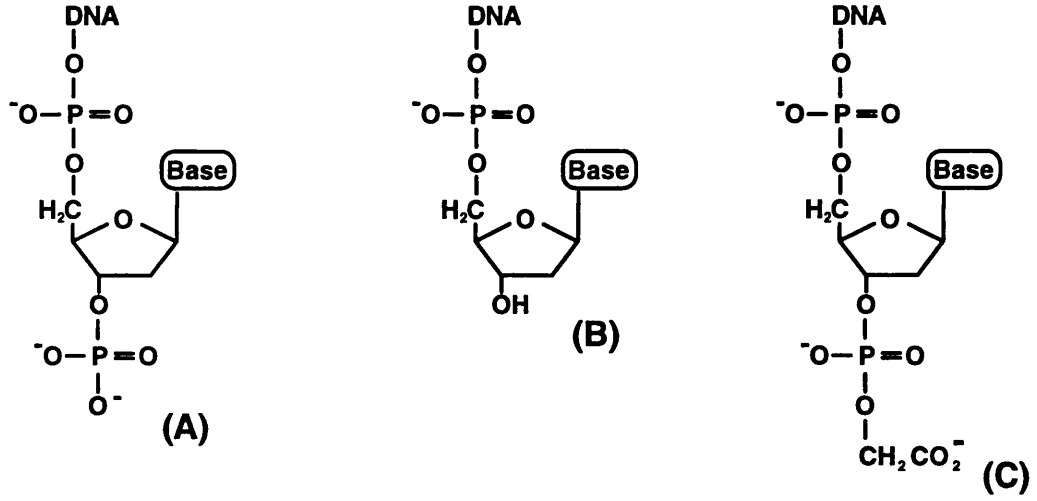



**Figure 5.1-1**: Possible chemical structures at the 3'terminal end of a DNA SSB: A) 3'phosphorous terminal; B) 3'hydroxy terminal; C) 3'glycolate ester.

## *5.2 Experimental Section*

### Materials

The enzymes T4 polynucleotide kinase and ClaI were purchased from Gibco (Life Technologies, Renfrew Road, Paisley). Taq polymerase and dNTP mix were obtained from Stratagene (Milton Road, Cambs). A programmable thermal controller, model PTC-100, MJ. Research Inc., (USA), was used for the amplification of DNA fragments using the polymerase chain reaction (PCR). SpinBind recovery systems were supplied

by Flowgen (Sittingbourne, Kent) and DE-81 filters by Whatmann (Maidstone, Kent). Agarose, polyacrylamide and bromophenol blue were purchased from Biorad (Hemel Hempstead, Herts). [$\gamma^{32}$P] ATP (5000 Ci/ µl) and Hyperfilm-MP were obtained from Amersham (Little Chalfont, Bucks); urea and xylene cyanol from Sigma (Poole, Dorset).

### Polymerase Chain Reaction (PCR)

The required DNA fragment (1100-bp) was obtained by PCR amplification of the yeast LEU 2 gene coding for β-isopropylmalate (IPM)- dehydrogenase (Andreadis *et al.*, 1984). The following components were added sequentially to an Eppendorf tube: Taq buffer (10 mM Tris-HCl pH 8.8, 50 mM KCl, 1.5 mM $MgCl_2$, 0.01 % w/v gelatine and other stabilisers, 10 µl), deoxynucleotide mix (100 µM each dNTP, 1 µl), primers (25 ng of each, 1 µl each), autoclaved water (for a total final volume of 100 µl) and DNA template (around 10 ng, 1 µl). Control samples contained all components except the DNA template. The samples were placed in a thermocycler; after a "hot start" (94°C for 5 minutes) the enzyme Taq DNA polymerase (5 units, 1 µl) was added to the mixtures. The temperature programme was as follows: 1 minute at 94°C (denaturing), 1 minute at 45°C (annealing), 3 minutes at 72°C (extension). The cycle was repeated 30 times with a final step of 10 minutes at 72°C.

The PCR product was purified from primers by microfiltration using the SpinBind system for PCR purifications. In order to confirm successful purification and to quantify the amount of DNA fragment recovered, a small aliquot was run in an agarose gel (0.8 %, 2.3 V/cm, 1 hour); the DNA was visualised under UV light after staining the gel with ethidium bromide. The intensity of the bands was analysed by densitometry and the amount of DNA calculated from a standard curve constructed with known amounts of lambda DNA (25 - 100 ng).

### $^{32}$P 5'End Labelling

The amplified 1100-bp DNA fragment was 5'-end labelled using the T4 polynucleotide kinase (PNK) forward reaction, which is the most convenient method to label DNA containing a terminal 5'-hydroxyl group. Chemically-synthesised oligonucleotides, e.g. by PCR, contain such a group. The reaction mixture consisted of: DNA fragment (500

ng, 1.375 pmoles 5'ends), reaction buffer (70 mM Tris-HCl, 10 mM $MgCl_2$, 100 mM KCl and 1 mM 2-mercaptoethanol), T4 PNK (20 units), [$\gamma^{32}$P] ATP (68.7 μCi, 13.75 pmoles) and autoclaved water to a final volume of 50 μl. The mixture was incubated for 30 minutes at 37°C and the reaction stopped by treatment at 65°C for 10 minutes. The amount of incorporated radiolabel was determined by differential adsorption of the products on positively charged surfaces (DE-81 paper) followed by liquid scintillation counting; 70 % of the DNA fragments were found to be labelled (Mundy *et al.*, 1991). The unincorporated radionucleotides were removed from DNA by ethanol precipitation (Sambrook *et al.*, 1989, p.11.34).

### Enzyme Digestion

In order to obtain asymmetrically labelled DNA (only one of the two 5'ends of the double stranded DNA labelled) the 1100-bp DNA fragment was cleaved into two fragments (150 bp/950 bp) by using the restriction enzyme ClaI, which nicks the DNA at the sequence AT^CGAT.

The DNA pellet was redissolved in ClaI reaction buffer (40 μl, 50 mM Tris-HCl pH 8, 10 mM $MgCl_2$). Upon addition of ClaI enzyme (4 units, 2 μl) the sample was incubated for one hour at 37°C. The two DNA fragments were separated by agarose gel electrophoresis; a non-radioactive DNA fragment which was digested under the same experimental conditions was run in parallel. The gel was cut in half and the part containing the non-radioactive fragments was stained with ethidium bromide, the position of the two fragments was then established by examining the gel under UV light. This was used as a template to locate the radioactive fragments on the other half of the gel. The radioactive fragments were recovered from the excised gel using "SpinBind DNA recovery system for agarose gel samples" following the recommended procedure of the supplier (Flowgen). The counts per minute (cpm)/μl of the purified DNA solutions were determined by liquid scintillation counting as described above.

### DNA Treatment

The asymmetrically labelled 150-bp DNA fragment (4 μl, 2805 cpm/μl) was incubated with different reaction mixtures; GSH (5 mM)/potassium chromate (0.8 mM), hydrogen peroxide (14 mM)/potassium chromate (1.4 mM) and ferrous sulphate-disodium EDTA

(160 μM/500 μM)/hydrogen peroxide (0.5 mM) in phosphate buffer (0.1 M pH 6.8) in a final volume of 6 μl for 4 hours at room temperature. Controls samples contained DNA without any further addition. Incubations were terminated by the addition of gel electrophoresis loading buffer (4 μl, 80 % formamide, 10 mM NaOH, 1 mM EDTA, 0.1 % (w/v) xylene cyanol, 0.1 % (w/v) bromophenol blue) to the mixtures, and all the samples, except one control, denatured for 2 minutes at 90°C and quick chilled in ice. Aliquots (4 μl) were immediately loaded onto a pre-warmed (45°C) denaturing polyacrylamide gel (15 %, 21 × 40 cm) and the gel run for 2 hours at 50 W, with a temperature close to 50°C. The gel was removed from the glass plates using 3MM paper and wrapped in cling film before exposure to an X-ray film. Autoradiography was carried out in a light-tight cassette for 48 hours at room temperature. The X-ray film was developed manually.

## *5.3 Results*

### Characterisation and Distribution of DNA Damage Induced by Chromate/GSH

A DNA fragment was obtained by amplifying the yeast LEU 2 gene coding for β-IPM dehydrogenase (1100-bp) using PCR, which yielded an average of 900 - 700 ng of DNA per amplification. The DNA fragment was then radioactively labelled at the 5'ends using [γ $^{32}$P]ATP in a reaction with T4 polymerase kinase; under the experimental conditions used 70 % of the DNA fragments were labelled. After removal of unincorporated nucleotides by ethanol precipitation, asymmetrical labelling was achieved by digestion of the DNA fragment with ClaI. The two new fragments (150, 950-bp) were recovered by purification from agarose gel.

The 150-bp DNA fragment was incubated with different systems known to induce DNA SSB, Cr(VI)/GSH, Cr(VI)/$H_2O_2$ and Fe(II)/$H_2O_2$, for four hours at room temperature. Upon addition of loading buffer all the samples, except one of the controls, were

denatured for two minutes at 90°C. The samples were immediately loaded onto a polyacrylamide gel. The obtained autoradiograph is presented in **Figure 5.3-1**.

Inspection of the autoradiograph revealed no detectable differences between the band pattern obtained for control samples (lane 3-4) and that of samples treated with agents known to cleave DNA (lanes 4-10). Therefore, there was no indication that the Cr(VI)/GSH or Cr(VI)/$H_2O_2$ systems induced DNA SSB under these experimental conditions.

The appearance of two main bands in all the lanes, even in those containing DNA alone, was indicative of the presence of DNA fragments of different sizes in the radiolabelled DNA stock. This fact remained undetected during the DNA purification using agarose gel electrophoresis. Because the two bands observed differed only by 10-20 nucleotides it was not possible to distinguish these small differences using 0.8 % agarose gels. It is difficult to establish at which stage the generation of DNA fragments of different size occurred; the two most likely steps are the PCR amplification (a shorter DNA fragment may have been amplified at the same time that the 1100 bp) or the enzyme digestion process (the enzyme may not have been as selective as expected and may have cleaved at unexpected sites). In future experiments it would be advisable to purify the DNA fragments using polyacrylamide gels instead of agarose gels, as bands differing in only a few nucleotides can be resolved and the DNA fragment of interest easily purified.

The faint bands observed in between the two main sharp bands, present in all the lanes except in 1-2 (non denatured DNA), were very likely caused by "silent breaks", breaks that were already present in the original DNA, but were only revealed upon separation of the two DNA strands. The origin of these breaks in the original DNA is unclear.

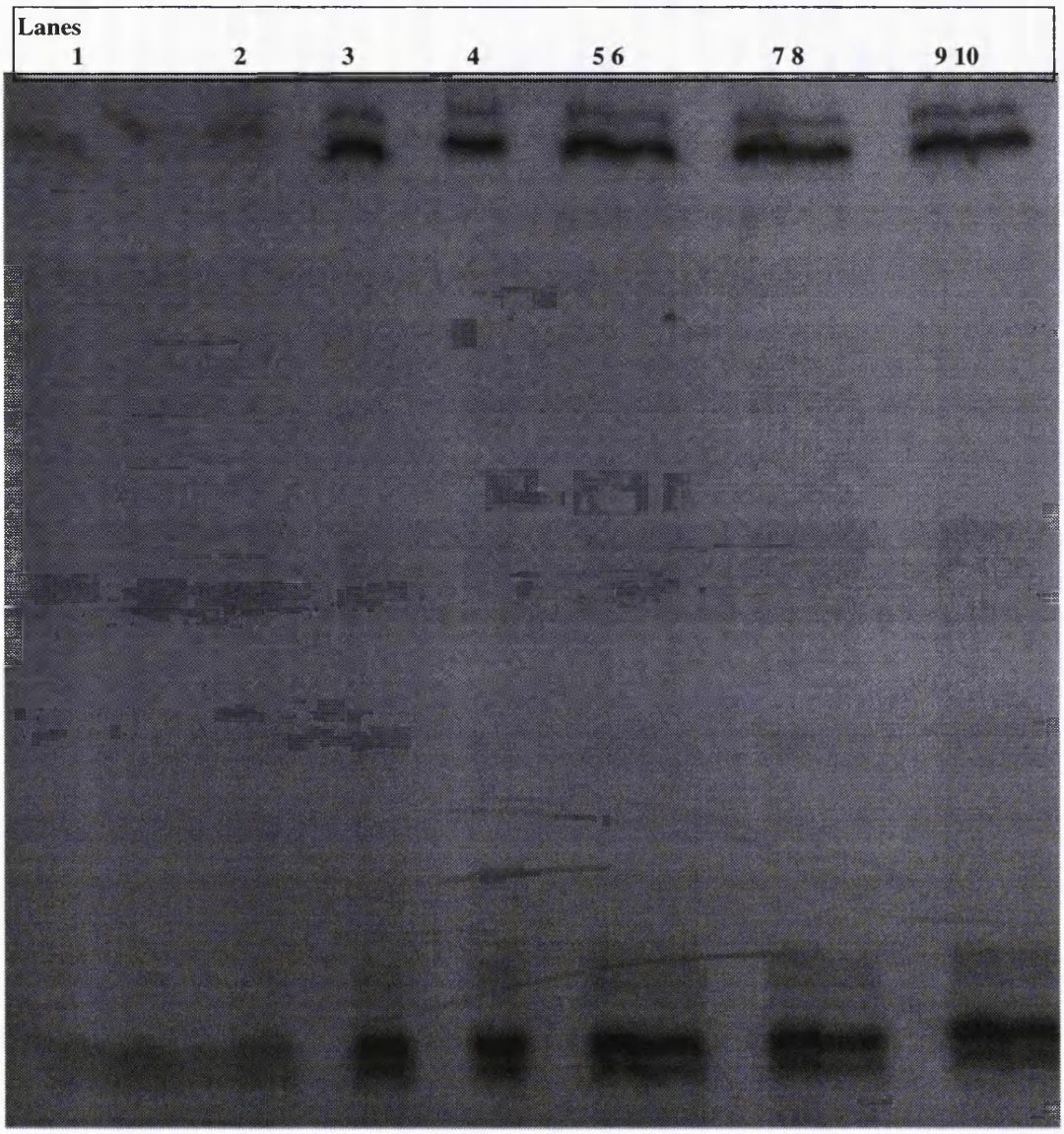



**Figure 5.3-1**: Autoradiograph of a PAGE after treatment of a $^{32}P$ 5'end labelled DNA fragment (150 bp) for four hours with: Lanes 1-2: DNA non treated and non-denatured; lanes 3-4: DNA non treated but denatured; lanes 5-6: DNA treated with Cr(VI) (0.8 mM) and GSH (5 mM); lanes 7-8: DNA treated with Cr(VI) (1.4 mM) and $H_2O_2$ (14 mM); lane 9-10: DNA treated with Fe(II) (160 μM) and $H_2O_2$ (0.5 mM).

## 5.4 Discussion

The investigations of the distribution of the SSB induced by chromate and GSH along the DNA strand using $\gamma^{32}$P-end labelled DNA and PAGE techniques were unsuccessful. Upon incubation of a 150-bp oligonucleotide with different reaction mixtures, known to induce DNA breakage in other systems, it was not possible to detect any sign of DNA scission.

Kawanishi *et al.* (1986), in similar studies, also failed to observe the formation of additional bands indicative of SSB upon treatment of a 110-bp DNA fragment with Cr(VI)/GSH. In contrast to our findings, they were able to detect the generation of oligonucleotides of different sizes upon incubation of the DNA fragment with Cr(VI)/$H_2O_2$. They concluded that intermediates generated during the reduction of chromate by GSH were not able to induce DNA SSB, unlike those formed in the presence of $H_2O_2$ as the reductant.

The fact that no additional bands were observed in the present studies for either reductant, should not be taken as evidence that no SSB are induced by these systems. It is well documented that Cr(VI)/GSH, Cr(VI)/$H_2O_2$ and Fe(II)/$H_2O_2$ mixtures, under very similar experimental conditions are able to induce DNA SSB in supercoiled DNA (Kortenkamp *et al.*, 1989; Aiyar *et al.*, 1989), and are easily detected by agarose gel electrophoresis techniques, which are highly sensitive (1 SSB in $10^6$ DNA base pairs can be detected). A plausible explanation for the apparent lack of SSB induction in the 150-bp fragment is that the PAGE/$^{32}$P system under the conditions used in these experiments may not be sensitive enough to detect the level of SSB being induced. For a SSB to appear as a band on the polyacrylamide gel, it is necessary to have a certain number of oligonucleotides of equal length. At the molecular level that would imply that a population of DNA molecules must be cleaved at a particular nucleotide several times in order for the resulting random fragments to be detectable. Considering that the DNA fragment used in these experiments consisted of 300 nucleotides (150-bp) the probability of the same nucleotide being hit more than once in independent DNA

fragments was likely to be quite low. A similar line of argumentation would explain why Kawanishi *et al.* (1986) did not observe breaks with the Cr(VI) /GSH system using a 110-bp DNA fragment.

In future studies, the sensitivity of the assay could be improved by either using a shorter DNA fragment (10-30 bp) or by increasing the amount of DNA per lane.

# *CHAPTER 6*

# KINETIC STUDIES OF THE REDUCTION OF CHROMATE BY GSH UNDER ANOXIC AND AEROBIC CONDITIONS

## *6.1 Introduction*

The mechanism of reduction of chromate by GSH has been the focus of extensive research (McAuley and Olatunji, 1977; Connett and Wetterhahn, 1985; O'Brien *et al.*, 1992; Bose *et al.*, 1992; Perez-Benito *et al.*, 1994c) due to the important role of GSH in the mediation of chromate carcinogenicity.

The reduction of chromate Cr(VI) to Cr(III) requires the transfer of 3 electrons, which are provided by GSH, essentially a one electron reductant; the reduction occurs via several steps involving 1 or 2 electron transfers. The scheme generally proposed in previous studies for the reduction is summarised below (McAuley and Olatunji, 1977; Connett and Wetterhahn, 1985; O'Brien *et al.*, 1992; Bose *et al.*, 1992; Perez-Benito *et al.*, 1994b). The initial step of the reduction process involves the formation of a chromium(VI)-thioester (**1**), followed by a 2 electron reduction step involving another molecule of GSH (**2**). Cr(IV) could then dismutate to form Cr(V) and Cr(III) (**4**). One electron reduction of the thioester also occurs at neutral pH (O'Brien and Wang, 1992) and although it seems to be a minor pathway (at least with an excess of GSH) it could have important physiological consequences due to the formation of a thiyl radicals in the process (**3**).

Spectroscopic evidence has been obtained for the various reactive intermediates, Cr(IV), Cr(V)-complexes and GS·, which, as already stated in section 1.2.4, are likely to play an

important role in the induction of DNA lesions and the mediation of chromate carcinogenicity.

$$Cr(VI) + GSH \rightleftharpoons Cr(VI)^{-}SG \qquad K = k_1/k_{-1} \qquad (1)$$

$$Cr(VI)^{-}SG + GSH \rightarrow Cr(IV) + GSSG \qquad k_2 \qquad (2)$$

$$Cr(VI)^{-}SG \rightarrow Cr(V) + GS\cdot \qquad (3)$$

$$2\ Cr(IV) \rightarrow Cr(V) + Cr(III) \qquad (4)$$

The observation that the formation of SSB and AP-sites in PM2 DNA was prevented by removal of oxygen (see section 3.3) led us to investigate the effect of oxygen on the reduction of chromate by GSH. In order to address this question, the reduction of chromate by GSH was studied at different concentrations under aerobic and anoxic conditions, by monitoring the decay of $CrO_4^{2-}$ and the formation and disappearance of the Cr(VI)-thioester.

## *6.2 Experimental Section*

### Materials

Glutathione and 5-5'-dithio-bis(2-nitrobenzoic acid) (DTNB) were obtained from Sigma (Poole, Dorset) and potassium chromate from BDH (Lutterworth, Leicestershire). All reagents were of analytical grade. GSH solutions were prepared freshly at the beginning of each experiment. Absorbances were recorded in a Shimadzu MPS-2000 spectrophotometer. Non-linear least squares fits were carried out using a computer programme adapted from Bevington "Data reduction and error analysis for the physical science" McGraw-Hill, 1969: Program 11-5,6,7.

### Monitoring the Reduction of Chromate by GSH

Kinetic studies of the reduction of chromate by GSH were conducted by monitoring the decay of Cr(VI) and the appearance and disappearance of the Cr(VI) thioester intermediate, by following the variation in absorbance at 372 and 433 nm, respectively,

with time. In a typical experiment, stock solutions of GSH and potassium chromate in demetallated phosphate buffer (pH 6.9, 0.1 M) were mixed, immediately transferred to a spectrophotometer cell and the measurements recorded with a Shimadzu spectrophotometer at room temperature (≈ 20°C). The range of concentrations studied was as follows: 0.175 mM chromate with 3, 5, 10 or 20 mM GSH and 0.058 mM chromate with 3 mM GSH; the studies were carried out under aerobic and anoxic conditions. No changes of pH were observed upon completion of the reduction, $pH_{final} = pH_{initial} \pm 0.05$. The excess of GSH (15 - 115 fold) over chromate ensured that the experiments were carried out under pseudo-first-order conditions. The concentrations chosen for the experiments were similar to those used in the studies of the induction of DNA lesions by chromate/GSH.

For the anoxic studies, all the solutions were degassed for 45 minutes under vacuum (water pump) in an ultrasonic bath. All sample handling was carried out under a nitrogen atmosphere in a tent, using the same experimental conditions as described above. The spectrophotometer cells were tightly sealed and the measurements recorded as described. The degassing process did not affect the concentrations of either GSH, as determined by Ellman's assay (see below), or $CrO_4^{2-}$, established by measuring the absorbance at 372 nm.

Demetallated phosphate buffer (traces of iron and copper removed form buffer using resins, as described on section 2.2) was used in all the studies in order to minimise autoxidation of GSH, which is catalysed by redox metals such as iron and copper (Misra, 1974). The rate of chromate reduction by GSH was the same in "conventional" and in demetallated phosphate buffer.

### The Stoichiometry of Chromate Reduction by GSH in an Anoxic System

The stoichiometry of the reduction of chromium(VI) by GSH under anoxic conditions was determined by allowing chromate (0.175 mM) to react with GSH (5 mM) for 2-2.5 hours (demetallated phosphate buffer pH 6.9, 0.1 M) and determining the level of residual reactants. The stoichiometry was obtained as the ratio of $\Delta$ [GSH]/$\Delta$[Cr(VI)] ($\Delta$ = initial concentration - final concentration). The amount of residual chromate was

determined spectrophotometrically (372 nm). The level of residual GSH was determined using the Ellman's assay: an aliquot of the reaction mixture (200 µl) was diluted in phosphate buffer (pH 8, 0.1 M) and DTNB (10 µl, 10 mM in 0.05 M phosphate buffer pH 7) was added. After thoroughly mixing, the absorbance at 412 nm of the resulting yellow solution was measured and the GSH concentration estimated from a standard curve. The assay was not affected by the presence of residual chromate. It was essential to determine the amount of the two residual reactants simultaneously.

In initial studies the stoichiometry of the reduction process was investigated by spectrophotometric redox titration, using the method described by Kwong and Pennington (1984) for the reduction of Cr(VI) by L-cysteine. Different molar ratios of GSH:Cr(VI) (from 1:1 to 5:1) were allowed to react for 15 hours, and the absorbance at 372 nm of the remaining chromate was measured in the different mixtures. The readings were then plotted against the GSH:Cr(VI) ratio of the initial reaction mixture. A sharp break point in the resulting graph was observed, the intersection with the x-axis indicating the stoichiometry of the reaction.

## *6.3 Results*

### The Reduction Kinetics of Chromate by GSH

The kinetics of the reduction of chromium(VI) by GSH were studied by monitoring the decay of $CrO_4^{2-}$ at 372 nm, and by following the appearance and disappearance of an orange intermediate species at 433 nm, which is assigned to the relatively stable chromium(VI)-thioester (Connett and Wetterhahn, 1985; Bose *et al.*, 1992; Perez-Benito *et al.*, 1994b) (**Figure 6.3-1**).

The following concentrations were studied: 0.175 mM chromate with 3, 5, 10 or 20 mM GSH and 0.058 mM chromate with 3 mM GSH, all reactions were carried out in demetallated phosphate buffer pH 6.8, 0.1 M, at room temperature ≈ 20°C. No changes of pH were observed upon completion of the reduction, $pH_f = pH_i \pm 0.05$. The excess of

GSH (15 - 115 fold) over chromate did ensure that the reaction proceeded under pseudo-first-order conditions. The role of oxygen was assessed by conducting the studies under anoxic and aerobic conditions.

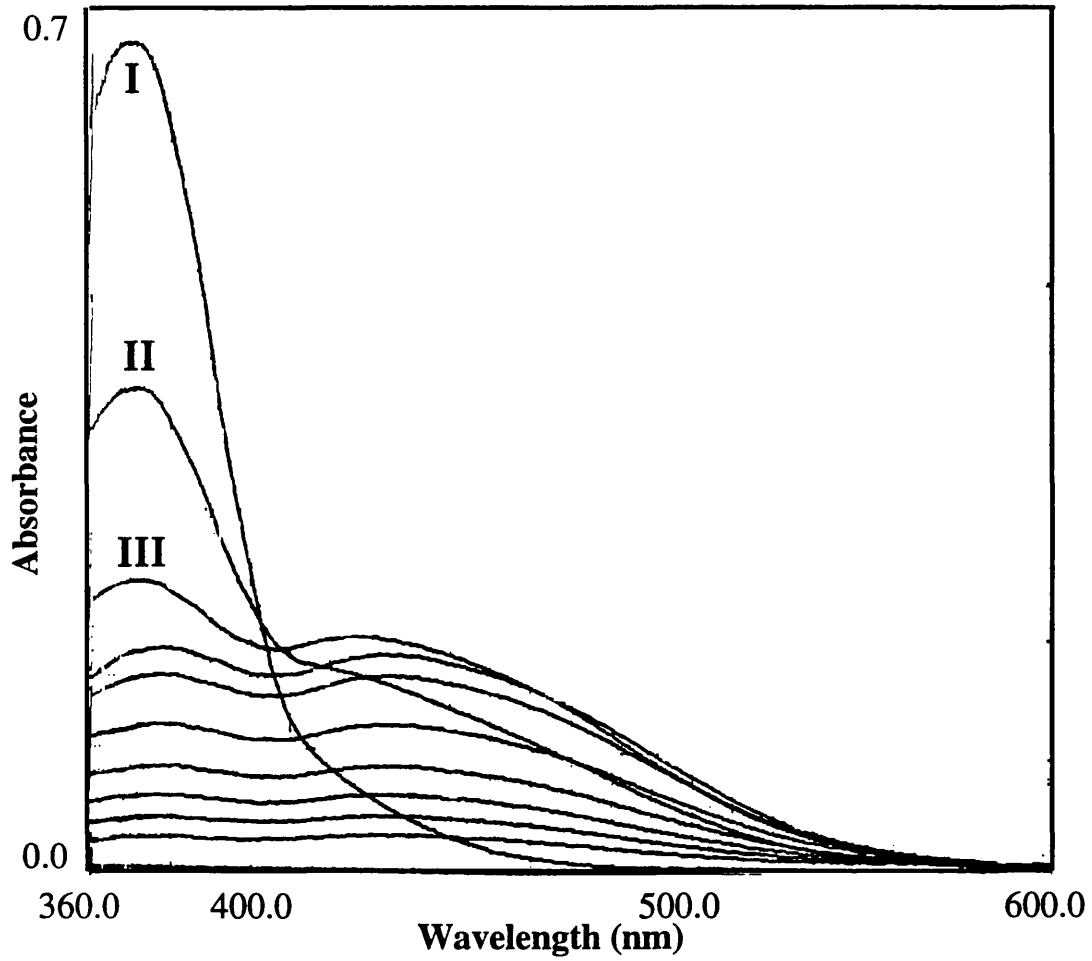



**Figure 6.3-1**: Typical UV-visible spectra of solutions of chromium(VI) (0.175 mM) and GSH (20 mM) recorded at different times after initiation of the reaction: I) Chromium(VI) alone; II) Chromium(VI) with GSH after 45 seconds; III) Spectrum after 4.5 minutes. The reduction was completed after 33 minutes.

### The Disappearance of Chromate with Time, Monitored by Following the Absorbance at 372 nm

**Figure 6.3-2** depicts the variation of absorbance at 372 nm with time during the reduction of chromate by GSH for the four different GSH concentrations studied (3, 5, 10 and 20 mM). The rate of reduction increases with rising GSH concentrations, 0.175 mM of chromate were reduced in 50 minutes by 20 mM GSH, whereas it required more than 6 hours with 3 mM GSH. The shape of the curves suggest a process of biphasic character, in agreement with previous studies (Connett and Wetterhahn, 1985; O'Brien *et al.*, 1992). There is an initial rapid decrease in the absorbance, followed by a much slower decay.

The removal of oxygen from the systems resulted in an increase in the rate of reduction, the fast initial step seemed to be unaffected but the variation was obvious for the second phase of the kinetic curve. The effect was more pronounced at low GSH concentrations (3, 5 mM), when the reduction time was nearly halved relative to the aerobic systems. At higher GSH concentrations the reduction proceeded faster and the effect became less pronounced.

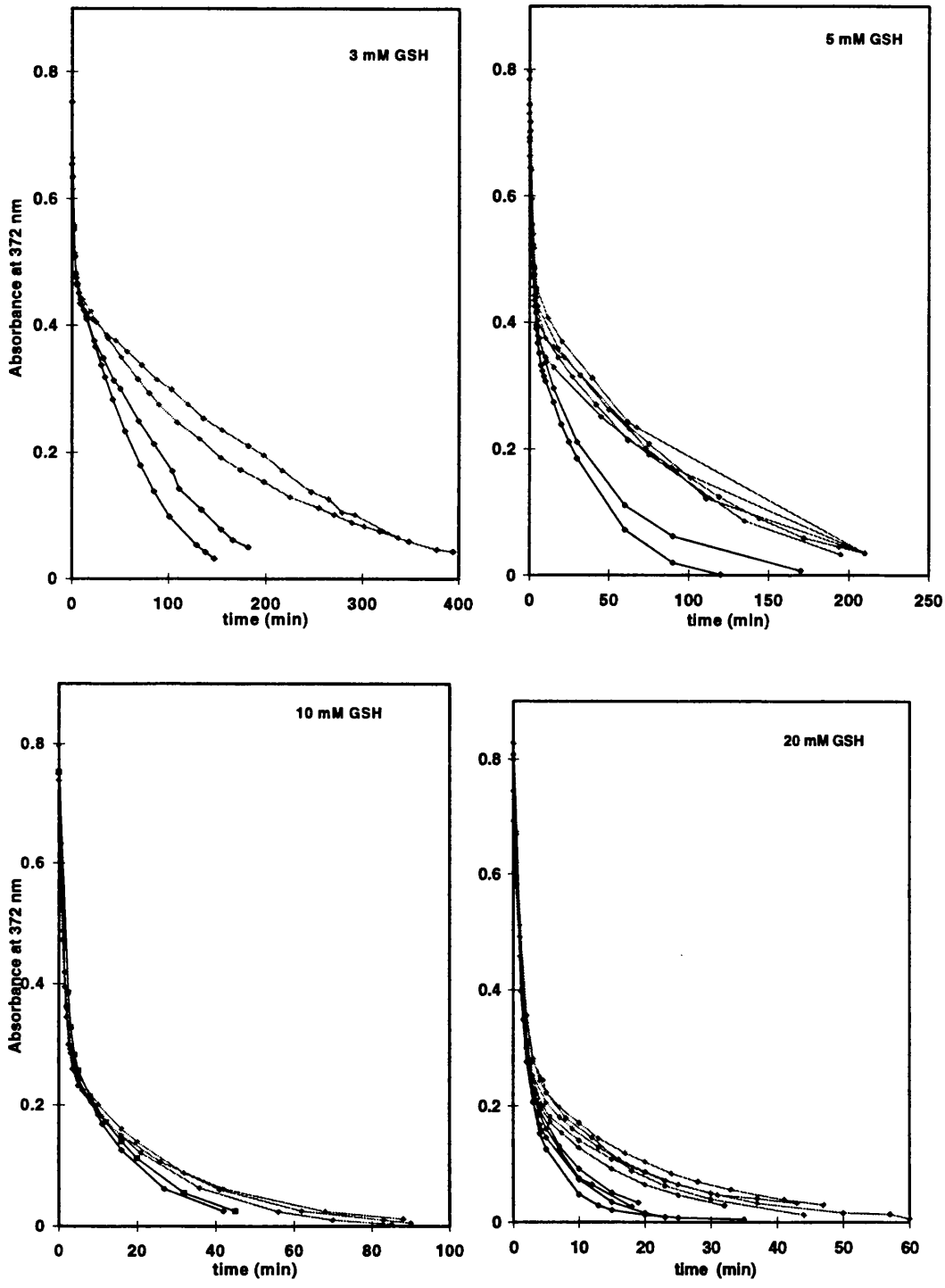



**Figure 6.3-2**: Variation of absorbance at 372 nm with time in solutions containing chromate (0.175 mM) and GSH at different concentrations under aerobic (grey lines) and anoxic conditions (black lines). The various lines represent independent experiments.

Degassing the solutions did not affect the concentration of either GSH or $CrO_4^{2-}$. The increase of the reduction rate observed under anoxic conditions could be reversed when the degassed samples were exposed to air during the reduction process (**Figure 6.3-3**), providing additional evidence that the effect observed was consequence of the removal of oxygen from the system.

The increase in the reduction rate upon removal of oxygen was not restricted to phosphate buffer. A similar effect was observed when the reductions were conducted in HEPES buffer (pH 7, 15 mM HEPES, 0.15 M NaCl).

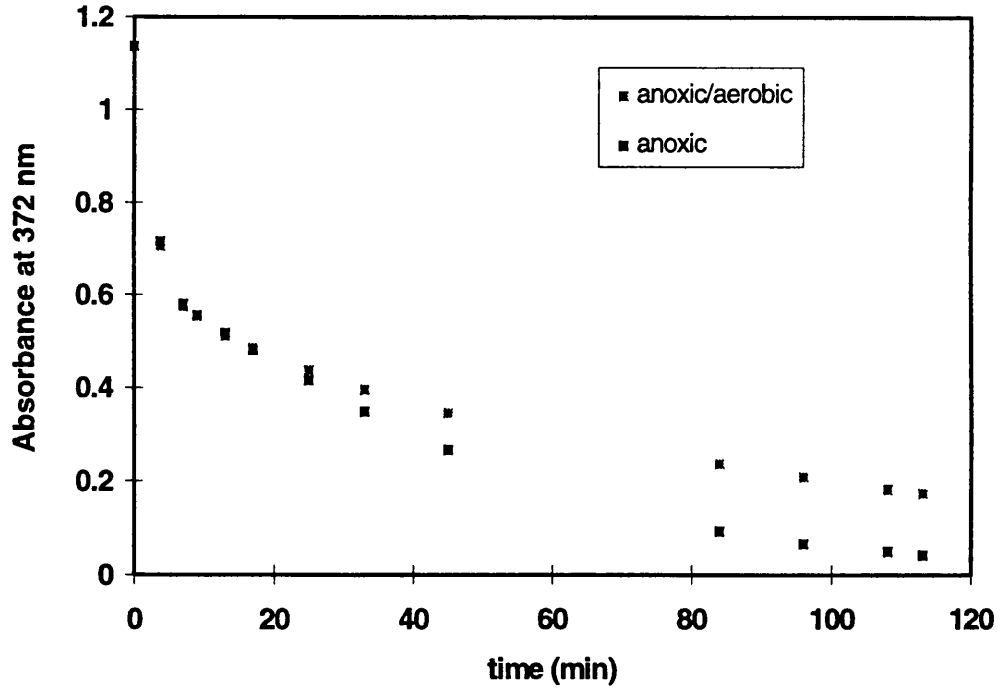



**Figure 6.3-3**: Exposure to air of anoxic mixtures of chromium (0.2 mM) and GSH (5 mM) slows the reduction process. Two anoxic mixtures of Cr(VI) and GSH were prepared and the change in absorbance at 372 nm with time was monitored. After 10 minutes reacting one of the samples was exposed to air.

Previous studies failed to detect an effect of oxygen in the reduction of chromate by GSH; McAuley and Olatunji (1977) and O'Brien and Ozolins (1989) reported that neither degassing the solutions nor saturating them with nitrogen had an effect on the reduction rates. However, the range of concentrations studied was 20 - 140 mM GSH / 0.2 mM $HCrO_4^-$ and 100 - 1000 mM GSH/1 mM $CrO_4^{2-}$, respectively. At these high GSH concentrations the rate of reduction is fast and any effect oxygen may have, is no longer observable.

## Monitoring the Formation and Decay of the Chromate(VI)-Thioester

The appearance and disappearance of the orange chromate(VI)-thioester intermediate was followed at 433 nm. The variation of absorbance with time during the reduction of $CrO_4^{2-}$ (0.175 mM) in the presence of various GSH concentrations (3, 5, 10, 20 mM) is shown in **Figure 6.3-4**. The process presents a rapid increase in absorbance, followed by a slow decay. In a way similar to the absorbance at 372 nm, the removal of oxygen did not affect the fast initial phase, but clearly accelerated the decrease in absorbance during the second phase of the reduction process.

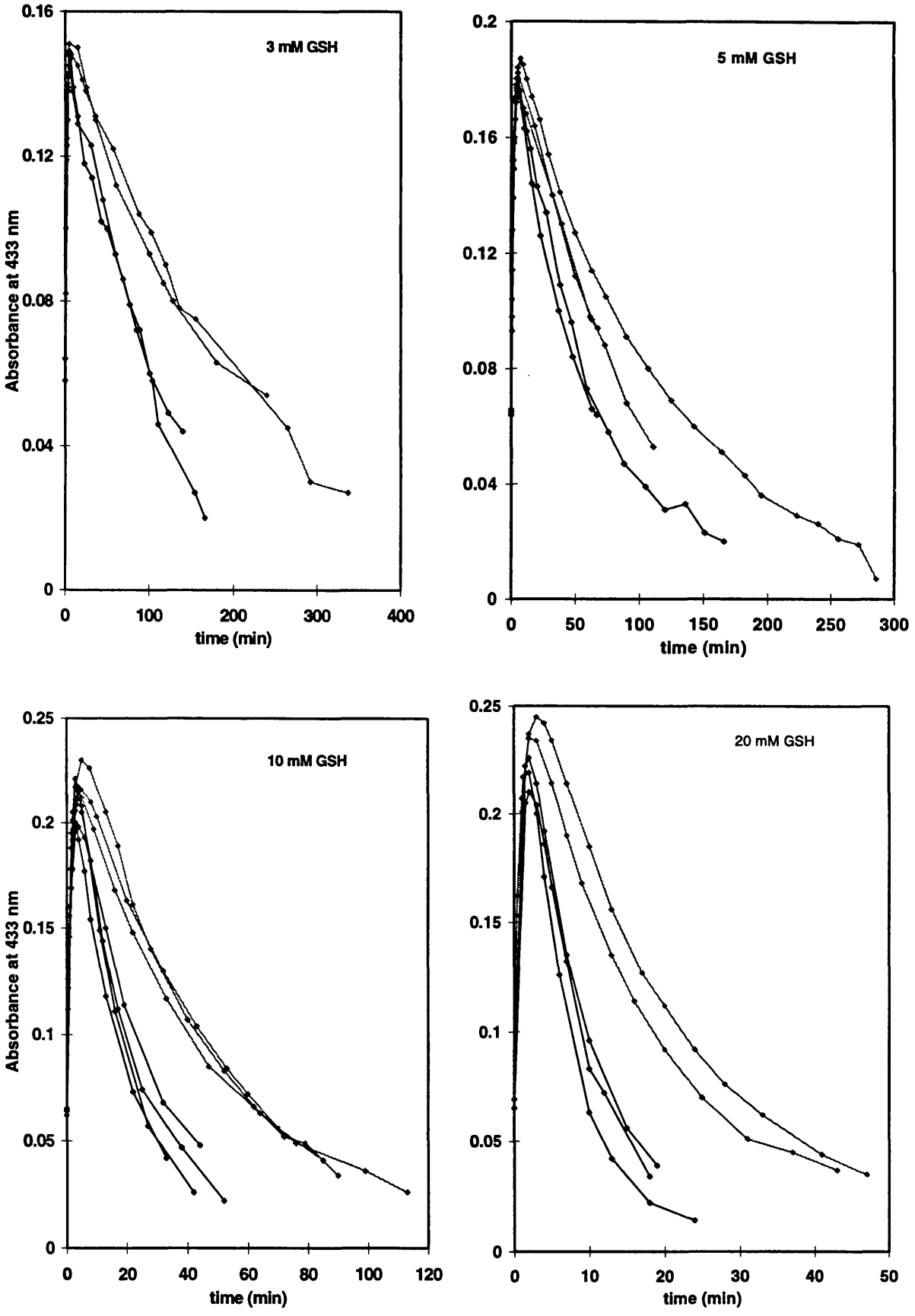



**Figure 6.3-4**: Variation of absorbance at 433 nm with time in solutions containing chromate (0.175 mM) and GSH at different concentrations under aerobic (grey lines) and anoxic conditions (black lines). The various lines represent independent experiments.

## Determination of the Rate Constants

At neutral pH the reduction of chromate by GSH is considered to involve the formation of a chromium(VI)-thioester via a rapid pre-equilibrium step (reaction **(1)** in introduction), followed by a slower redox step involving a second molecule of GSH (reaction **(2)** in introduction). The overall rate law consistent with the kinetic data from previous studies (Connett and Wetterhahn, 1985; O'Brien *et al.*, 1992) is:

$$-d[Cr(VI)] / dt = [k_2 K [GSH]^2 /(1 + K [GSH])] [Cr(VI)]$$

A detailed deduction of the rate law is described in Appendix III.

Under the conditions selected for the experiments, the concentrations of GSH were high enough relative to chromate to be considered effectively constant during the course of the reaction (pseudo-first-order). The pseudo-first-order rate law can be written as:

$$-d[Cr(VI)] / dt = k_{obs} [Cr(VI)] \qquad \text{where } k_{obs} = k_2 K [GSH]^2 /(1 + K [GSH])$$

The apparent first order rate constants were obtained from the plots of absorbance vs. time, by treating the kinetic profiles as two consecutive first order reactions (Alcock *et al.*, 1970; Espenson, 1981):

$$A \rightarrow B \rightarrow C$$

The values of the pseudo-first order rate constants, $k_{1obs}$ ($A \rightarrow B$) and $k_{2obs}$ ($B \rightarrow C$), were evaluated from a non-linear least-squares iterative computer fit of the equation:

$$\text{Absorbance} = (\varepsilon_A - \varepsilon_B) [A_0] \exp(-k_1 t) + k_1 (\varepsilon_B - \varepsilon_C) [A_0] [ \exp(-k_2 t) - \exp(-k_1 t)] / (k_1 - k_2) + \varepsilon_C [A_0]$$

(the deduction of the equation can be found in Alcock *et al.*, 1970 and Espenson, 1981).

Considering that $[A_0]$ = initial concentration of chromate and $\varepsilon$ the extinction coefficients then,

$\varepsilon_A [A_0]$ = Initial absorbance ($Abs_i$)

$\varepsilon_C [A_0]$ = Final absorbance ($Abs_\infty$)

the equation becomes,

$$Abs = (Abs_i - \varepsilon_B[A_0]) \exp(-k_1 t) + k_1 (\varepsilon_B[A_0] - Abs_\infty) [\exp(-k_2 t) - \exp(-k_1 t)] / (k_1 - k_2) + Abs_\infty \quad (5)$$

with three unknown parameters $\varepsilon_B [A_0]$, $k_{1obs}$ and $k_{2obs}$, which were obtained by a non-linear least squares fitting computer programme. This type of kinetic situation admits two possible solutions, with the same numerical values for the rate constants, but with the order of assignment reverse. Alcock *et al.* (1970) summarised it: " An optical density which rapidly increases and slowly declines does not necessarily imply a fast first and slow second reaction". The correct set of solutions of the fittings was easily distinguished, as one set often showed a negative absorption coefficient which is physically meaningless.

Estimates of the apparent rate constant for the first order reaction B $\rightarrow$ C were also obtained from plots of ln $(A_i - A_\infty)$ vs. time for the final stages of the reaction. These first order plots were linear and the value of the slope provided an estimate of the apparent rate constant, named $k^*_{2obs}$, to differentiate from $k_{2obs}$ obtained from non-linear square fitting of equation **(5)**.

The values of $k_{1obs}$, $k_{2obs}$ and $k^*_{2obs}$ obtained from the variation of absorbance at 372 and 433 nm with time, for the different GSH concentrations under aerobic and anoxic conditions, are presented in **Table 6-1** and **Table 6-2**, respectively.

Although for convenience the same notation is used for the apparent rate constants from the measurements at 372 nm and 433 nm, this does not imply that the values should be the same, as different species are being measured in each case and therefore the processes may not be equivalent.

**Table 6-1**: Pseudo-first order rate constants for the disappearance of Cr(VI) at 372 nm

| [GSH] $\times 10^{-3}$ M | 372 nm | $k_{1obs} \times 10^3$ $s^{-1}$ | $k_{2obs} \times 10^4$ $s^{-1}$ | $k^*_{2obs} \times 10^4$ $s^{-1}$ |
|---|---|---|---|---|
| 3 | $O_2$ *(n=5)* | 6.00 ± 0.5 | 1.3 ± 0.3 | 1.3 ± 0.3 |
| 3 | $N_2$ *(n=5)* | 6.30 ± 0.7 | 2.7 ± 0.3 | 3.8 ± 0.7 |
| 5 | $O_2$ *(n=6)* | 10.9 ± 1.0 | 2.0 ± 0.1 | 2.0 ± 0.2 |
| 5 | $N_2$ *(n=4)* | 10.6 ± 0.3 | 4.2 ± 0.3 | 4.5 ± 0.3 |
| 10 | $O_2$ *(n=3)* | 17.8 ± 1.5 | 8.2 ± 0.7 | 8.3 ± 0.1 |
| 10 | $N_2$ *(n=3)* | 16.3 ± 1.8 | 13 ± 1.7 | 13.3 ± 1.6 |
| 20 | $O_2$ *(n=5)* | 19.5 ± 1.6 | 15 ± 2.5 | 15 ± 3.3 |
| 20 | $N_2$ *(n=5)* | 20.3 ± 3.3 | 30 ± 0.5 | 33 ± 1.0 |

Results are means ± s.d. n = number of samples. $[CrO_4^{2-}]$ = 0.175 mM, pH 6.9.

**Table 6-2**: Pseudo-first order rate constants for the appearance and disappearance of the intermediate at 433 nm

| [GSH] $\times 10^{-3}$ M | 433 nm | $k_{1obs} \times 10^3$ $s^{-1}$ | $k_{2obs} \times 10^4$ $s^{-1}$ | $k^*_{2obs} \times 10^4$ $s^{-1}$ |
|---|---|---|---|---|
| 3 | $O_2$ *(n=4)* | 7.8 ± 0.5 | 1.7 ± 0.5 | 1.7 ± 0.1 |
| 3 | $N_2$ *(n=4)* | 8.0 ± 0.5 | 3.2 ± 0.3 | 3.3 ± 1.2 |
| 5 | $O_2$ *(n=2)* | 8.3 ± 0.1 | 2.8 ± 1.0 | 2.5 ± 1.2 |
| 5 | $N_2$ *(n=3)* | 8.5 ± 0.8 | 4.0 ± 0.8 | 5.5 ± 1.7 |
| 10 | $O_2$ *(n=3)* | 11.2 ± 0.9 | 4.8 ± 0.1 | 5.5 ± 1.0 |
| 10 | $N_2$ *(n=4)* | 10.5 ± 1.8 | 12.0 ± 2.7 | 13.0 ± 2.0 |
| 20 | $O_2$ *(n=2)* | 16.6 ± 1.6 | 12.0 ± 1.6 | 13.3 ± 3.3 |
| 20 | $N_2$ *(n=3)* | 13.0 ± 0.9 | 35.0 ± 1.6 | 33.3 ± 2.5 |

Results are means ± s.d. n = number of samples. $[CrO_4^{2-}]$ = 0.175 mM, pH 6.9.

There is an excellent agreement between the values of $k^*_{2obs}$ and $k_{2obs}$, which were obtained by different methods. Under anoxic conditions the values of $k_{2obs}$ and $k^*_{2obs}$ are approximately twice as high as the constants obtained from the aerobic data. However, the values for $k_{1obs}$, obtained from the rapid phase of the biphasic process, are the same under anoxic and aerobic conditions.

Typical fits of absorbance vs. time using equation (5) are shown in **Figure 6.3-5.**

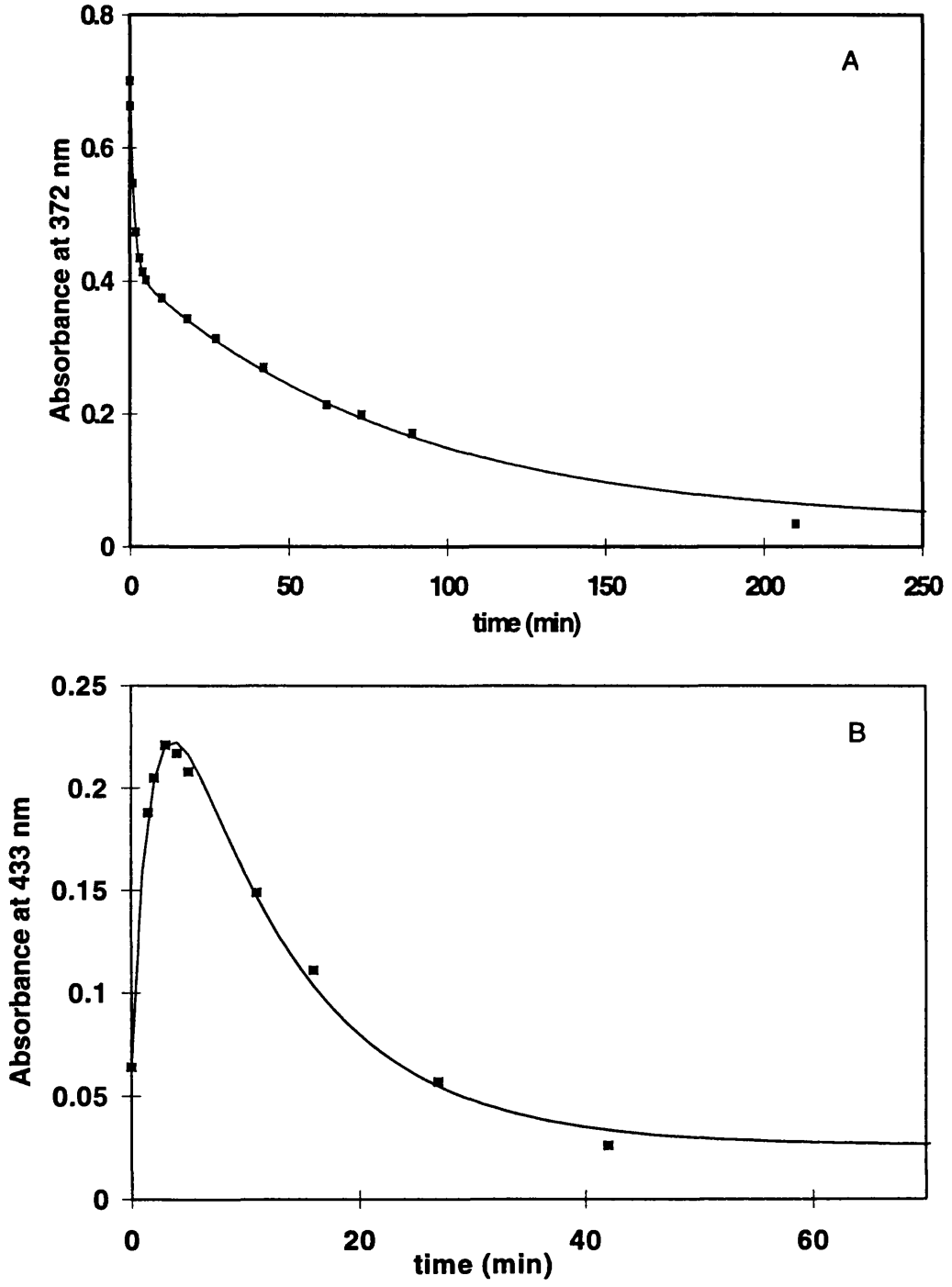



**Figure 6.3-5:** Typical non-linear fits of Abs vs. time (solid lines). Fitted using equation (5) with: A) 372 nm [GSH] = 5 mM $k_{1obs}$ = 0.63 $min^{-1}$, $k_{2obs}$ = 0.020 $min^{-1}$; B) 433 nm [GSH] = 10 mM, $k_{1obs}$ = 0.55 $min^{-1}$, $k_{2obs}$ = 0.09 $min^{-1}$; (■ measured values).

### The Variation of the Pseudo-First Order Rate Constants with GSH Concentration

The dependence of the apparent rate constants on [GSH] was obtained from plots of $k_{obs}$ vs. [GSH], which allowed the estimation of the rate constants $k_1$, $k_{-1}$ and $k_2$.

The variation of $k_{2obs}$ with GSH (**Figure 6.3-6**) was nearly linear, with a slight curvature at low GSH concentrations, in excellent agreement with previous studies (Connett and Wetterhahn, 1985; O'Brien *et al.*, 1992). The data fitted the following expression: $k_{obs} = a\,b\,[GSH]^2/(1 + b\,[GSH])$. At high GSH concentrations $b\,[GSH] > 1$, and the equation can be approximated to $k_{obs} = a\,[GSH]$. Therefore, the slope of $k_{obs}$ vs. [GSH] provides a useful initial estimate of $a$ for further refinement by non-linear least-squares fit. Comparing $k_{obs}$ with the rate law for the reduction of chromate by GSH, it becomes apparent that $a = k_2$ and $b = K$. The values for these parameters for the various studies are presented in **Table 6-3**. Identical results were obtained for $k^*_{2obs}$ (data not shown).

The variation of $k_{1obs}$ with [GSH] is illustrated in **Figure 6.3-7**. A linear dependence with [GSH] can be observed, $k_{obs} = a\,[GSH] + b$. The data at 372 nm deviate from linearity due to a single data point (3 mM, in brackets). This is likely caused by an inaccuracy of the determination of $k_{1obs}$ by the non linear square fit. Fittings of absorbance vs. time using equation **(5)** provide more accurate results when the absorbance first increases and then decreases, as is the case at 433 nm. At 372 nm the turning point between $k_{1obs}$ and $k_{2obs}$ is not so well defined and can lead to errors of the fittings.

$k_{1obs}$ reflects the initial part of the kinetic process, when the rapid equilibrium to form the Cr(VI)-thioester is being established ($CrO_4^{2-}$ + GSH $\rightleftharpoons$ Cr(VI)-SG). At concentrations of GSH in excess over Cr(VI) (pseudo-first order conditions) it can be shown that the rate law for the equilibrium process is (Espenson, 1981):

$$d[Cr(VI)]/dt = k_{obs}\,[Cr(VI)] \quad \text{with} \quad k_{obs} = k_1\,[GSH] + k_{-1}$$

Therefore, linear plots of $k_{1obs}$ vs. [GSH] have a slope = $k_1$ and an intercept = $k_{-1}$. The equilibrium constant is $K = k_1 / k_{-1}$. A summary of the results is presented in **Table 6-3**.

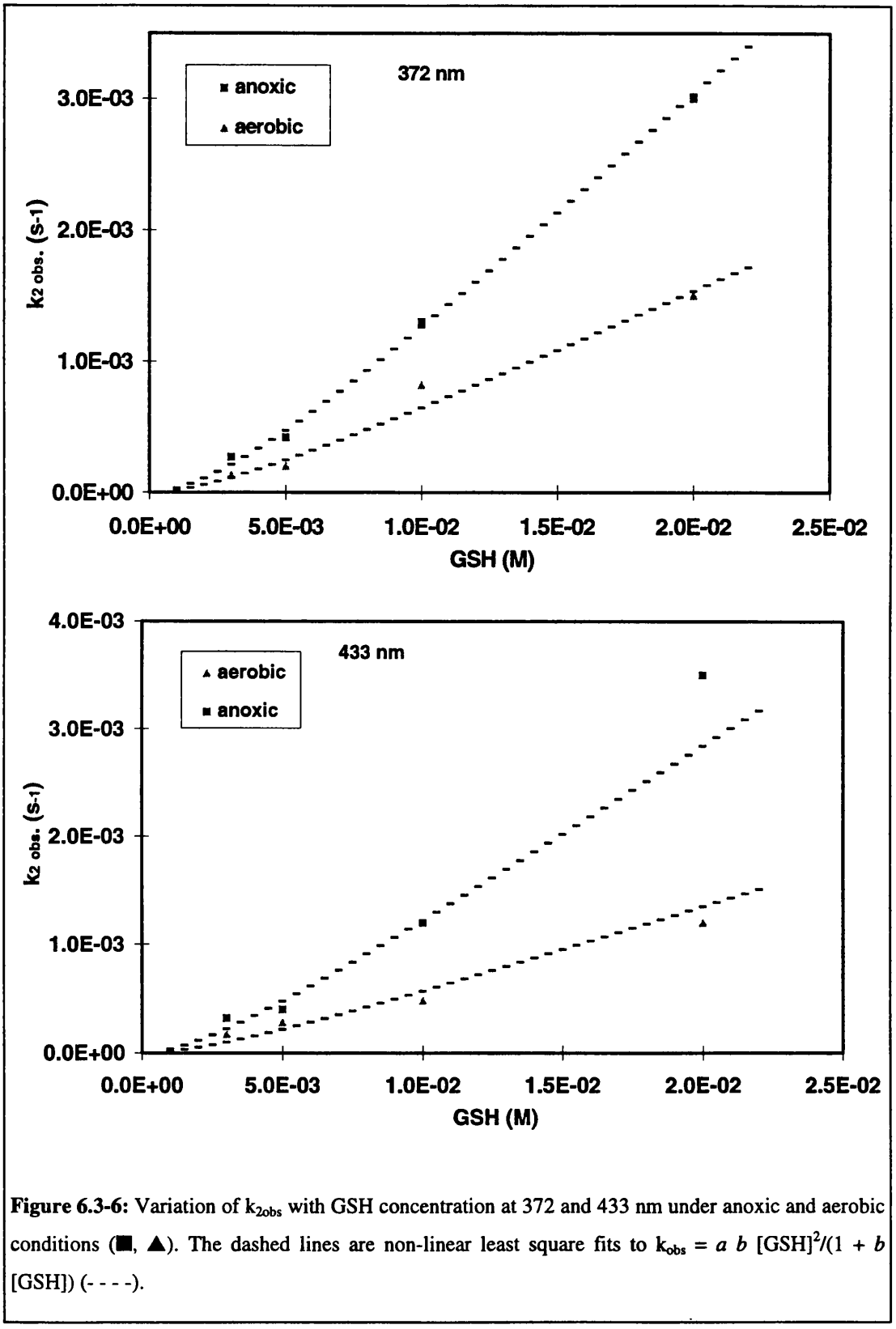
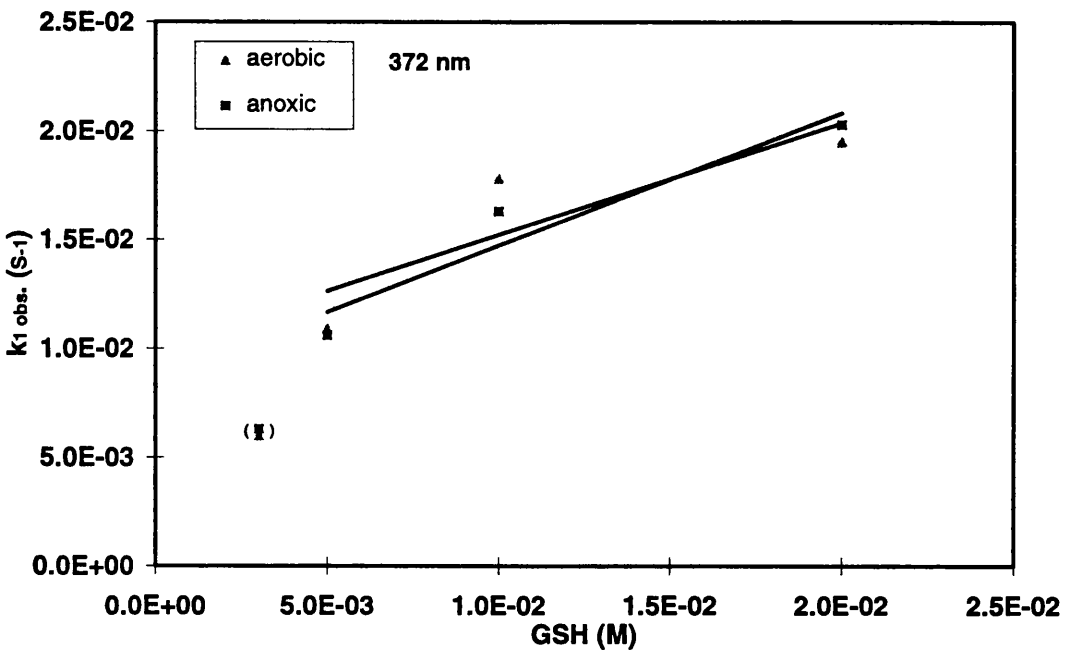



**Figure 6.3-6:** Variation of $k_{2obs}$ with GSH concentration at 372 and 433 nm under anoxic and aerobic conditions (■, ▲). The dashed lines are non-linear least square fits to $k_{obs} = a\ b\ [GSH]^2/(1 + b\ [GSH])$ (- - - -).



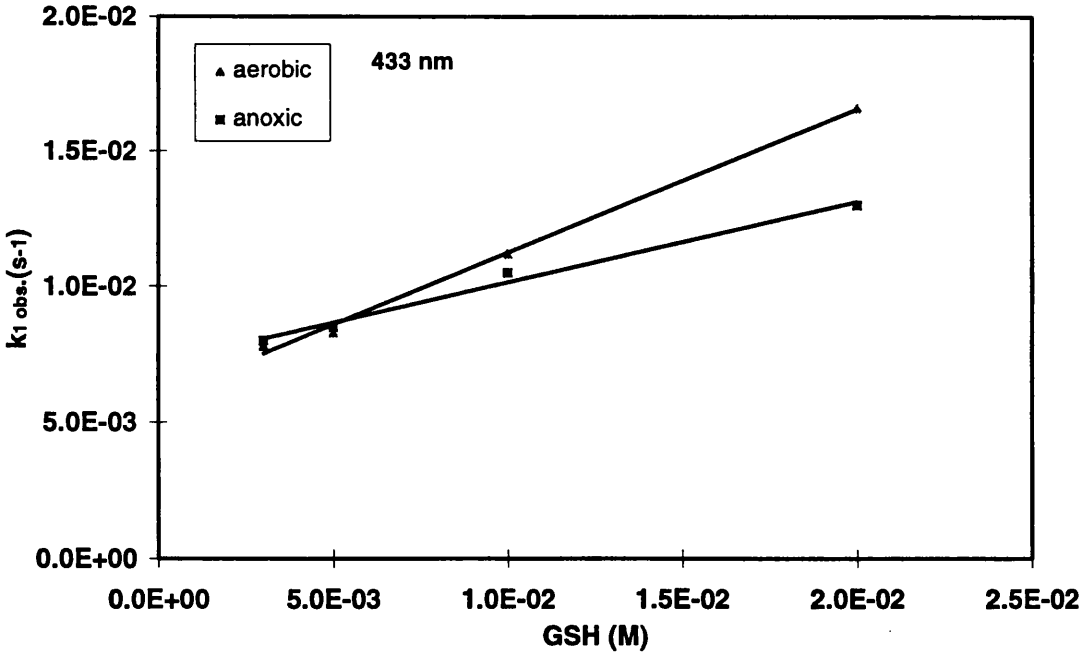




**Figure 6.3-7:** Variation of $k_{1obs}$ with GSH concentration at 372 and 433 nm under anoxic and aerobic conditions. The solid lines are least square fits to $k_{obs} = k_1 [GSH] + k_{-1}$. Points in brackets are outliers.

**Table 6-3**: Rate constants derived from fits of $k_{1obs}$ and $k_{2obs}$

| | | $k_{1obs}$ [a] | | | $k_{2obs}$ [b] | | |
|---|---|---|---|---|---|---|---|
| | | $k_1$ $M^{-1}$ $s^{-1}$ | $k_{-1}$ $M^{-1}$ $s^{-1}$ | K | $k_2$ [c] $M^{-1}$ $s^{-1}$ | $a$ ($k_2$) $M^{-1}$ $s^{-1}$ | $b$ (K) |
| 372 nm | $O_2$ | 0.52 | $10 \times 10^{-3}$ | 52 | 0.084 | 0.092 | 220 |
| | $N_2$ | 0.61 | $8.6 \times 10^{-3}$ | 71 | 0.17 | 0.19 | 197 |
| 433 nm | $O_2$ | 0.53 | $6.0 \times 10^{-3}$ | 88 | 0.063 | 0.083 | 217 |
| | $N_2$ | 0.30 | $7.2 \times 10^{-3}$ | 42 | 0.21 | 0.17 | 253 |

[a] $k_{1obs} = k_1 [GSH] + k_{-1}$
[b] $k_{2obs} = k_2 K [GSH]^2/(1 + K [GSH])$
[c] Obtained from linear approximation

From these results the following observations need to be emphasised:

- Two different values for the equilibrium constant K were obtained, depending on whether the calculations were based on $k_{1obs}$ or $k_{2obs}$ plots. From $k_{1obs}$ vs. [GSH] plots, K was estimated as 60. This value increased to 200 when derived from $k_{2obs}$ vs. [GSH] plots. Two sources of error are likely to contribute to the variation:

1. The value of K from $k_{2obs}$ vs. [GSH] plots was obtained by fitting $k_{obs} = a\ b\ [GSH]^2/(1 + b\ [GSH])$, which takes into account the slight curvature at low GSH concentrations. However, there is only one data point (3 mM) contributing to the curvature, which implies that computer fittings had to rely in that single value to estimate K. The error introduced in this way was probably quite high.

2. The equilibrium constant from $k_{1obs}$ vs. [GSH] plots was obtained considering that an equilibrium was being established between the initial reactants and the thioester. However, the $k_{1obs}$ values had been obtained from computer fittings assuming two consecutive first order reactions, A $\rightarrow$ B $\rightarrow$ C, ignoring the reverse reaction A $\leftarrow$ B of the equilibrium. Therefore, the estimation for $k_{-1}$ is likely to be not very accurate and the resulting error will be reflected in the estimation for the equilibrium constant

K. Nevertheless, the value for K determined here is in good agreement with previous studies (see **Table 6-5** in section 6.4 ).

- The rate constants ($k_1$, $k_{-1}$ and $k_2$) obtained from the analysis of data at the two wavelengths, 433 and 372 nm, are similar, therefore the biphasic kinetics observed at the two wavelengths reflect the same physical processes: During the fast initial phase a pre-equilibrium between Cr(VI) and the thioester is being established, and is followed by a slow decay in which the redox steps become important.

- The rate constants ($k_1$, $k_{-1}$ and $k_2$) estimated in these studies are in good agreement with results obtained in previous studies (see **Table 6-5** in discussion).

- The removal of oxygen does not seem to affect the initial formation of the thiolate ester. However, the values of $k_2$ under anoxic conditions are twice as high as those obtained under anoxic conditions, indicating that oxygen has an effect during the second phase of the reduction, when the redox decay of the thioester becomes important.

## The Stoichiometry of the Reduction of Chromate by GSH at Neutral pH and under Anoxic Conditions

The increase in the rate of reduction of chromate by GSH upon removal of oxygen led us to investigate a possible effect on the stoichiometry of the reduction reaction.

It is well established that under aerobic conditions and acidic pH, 3 moles of GSH are required per mole of Cr(VI), in order to reduce chromate to Cr(III) (McAuley and Olatunji, 1977; Bose *et al.*, 1992). The autoxidation of GSH at neutral pH has prevented the determination of the stoichiometry under these conditions. However it is assumed to be similar to the stoichiometry under acidic conditions. This assumption is supported by the fact that with other thiols such as cysteine or penicillamine 3 moles are required to completely reduce 1 mole of chromate at neutral pH (Kwong and Pennington, 1984; Hojo *et al.*, 1977; Connett and Wetterhahn, 1985).

The autoxidation of thiols has been proposed to be initiated by redox metals, such as iron and copper, followed by a univalent reduction of oxygen by the thiyl radicals (Misra, 1974). Therefore, removal of oxygen from the system and chelation of residual metal ions would ensure that GSH autoxidation is kept to a minimum.

Initial attempts to determine the stoichiometry of the reduction of chromate by GSH at neutral pH using spectrophotometric titration led to results that were not in agreement with those reported in the literature. At neutral pH the ratio GSH:Cr(VI) was 3 - 4 and 5 - 6, under anoxic and aerobic conditions, respectively. These readings are very likely confounded by the autoxidation of GSH. The reduction of chromate by GSH at neutral pH is much slower than under acidic conditions. The long period of time required to complete the reduction (15 hours) allowed GSH to autoxidise even in demetallated buffer, thereby diminishing the amount of thiol available to reduce Cr(VI). The higher levels of residual chromate remaining led to the discrepant stoichiometries. Under anoxic conditions the autoxidation of GSH is minimised compared to the aerobic situation, the reading therefore was closer to the expected value.

Nevertheless, it was possible to determine the stoichiometry under anoxic conditions and neutral pH using a different approach. Chromate (0.175 mM) and GSH (5 mM) were allowed to react for 2 - 2.5 hours. The amount of residual reactants was then determined as described in the experimental section and the stoichiometry was obtained as the ratio of $\Delta$ [GSH] / $\Delta$ [Cr(VI)]. Reaction of the mixtures under anoxic conditions and only for a short period of time ensured that the autoxidation of GSH was minimal and did not affect the determination of the stoichiometry. It was established that under our experimental conditions (anoxic, 5 mM GSH, demetallated phosphate buffer pH 6.9) the autoxidation of thiol was negligible; using Ellman's assay a loss of $0.02 \pm 0.01$ mM of GSH (mean $\pm$ s.d., 3 independent experiments) was observed after allowing a 5 mM GSH solution to stand for 3 hours under anoxic conditions. The results from four independent experiments are presented in **Table 6-4**.

**Table 6-4**: Stoichiometry of the reaction of chromate by GSH under anoxic conditions

| Residual GSH (mM) $[GSH]_i$ = 5 mM | Residual $CrO_4^{2-}$ (mM) $[CrO_4^{2-}]_i$ = 0.175 mM | $\Delta [CrO_4^{2-}] / \Delta [GSH]$ |
|---|---|---|
| 4.49 | 0.0050 | 0.51 / 0.170 = **3.00** |
| 4.49 | 0.0072 | 0.51 / 0.168 = **3.03** |
| 4.53 | 0.0085 | 0.47 / 0.166 = **2.83** |
| 4.54 | 0.0090 | 0.46 / 0.166 = **2.77** |
| ***Stoichiometry of the reduction of chromate by GSH mean ± s.d.*** | | **2.91 ± 0.13** |

The results show that at neutral pH and under anoxic conditions approximately 3 moles of GSH are consumed per mole of Cr(VI). Therefore, the removal of molecular oxygen from the system, although affecting the rate of reduction, does not alter the stoichiometry of the reduction.

## 6.4 Discussion

Kinetic studies of the reduction of chromate by GSH at neutral pH were carried out by monitoring the disappearance of chromate at 372 nm and the formation and decay of a chromium(VI)-thioester at 433 nm with time. Both processes present a rapid initial change of the absorbance, followed by a slow decline.

The values for the rate constants, obtained by kinetic analysis of the data, confirmed the proposed mechanism: A fast initial process ($k_1 \approx 0.52\ M^{-1}\ s^{-1}$, $K \approx 100$) in which an equilibrium is established **(1)**, followed by a second slow process, in which the rate determining redox step becomes important ($k_2 \approx 0.2$ and $8 \times 10^{-3}\ M^{-1}\ s^{-1}$ anoxic and aerobic respectively) **(2)**.

$$Cr(VI) + GSH \rightleftharpoons Cr(VI)^{-}SG \qquad \textbf{(1)}$$

$$Cr(VI)^{-}SG + GSH \rightarrow Cr(IV) + GSSG \qquad \textbf{(2)}$$

In order to investigate a possible role of molecular oxygen in the mechanism of the reduction process, oxygen was excluded from reaction mixtures by degassing the reagents and preparing the samples under a nitrogen atmosphere.

It was observed that under anoxic conditions the decline in absorbance at 372 and 433 nm proceeded at a faster rate during the second phase of the reduction process, when compared to measurements carried out under aerobic conditions. This effect was most pronounced at low GSH concentrations. Consequently, the rate constants $k_1$ and $k_{-1}$, which reflect the initial phase of the reduction process, were unaffected under anoxic conditions, whereas the values for $k_2$ under anoxic conditions were twice as high as those obtained under aerobic conditions. These results suggest that molecular oxygen is involved in the reduction of chromate by GSH at stages subsequent to the formation of the chromate-thioester.

Our observations allow us to clarify a number of inconsistencies in the rate constants for the second phase of the reduction ($k_2$) which are reported in the literature (**Table 6-5**). Whereas Connett and Wetterhahn (1985) and O'Brien *et al.* (1992) reported a $k_2$ in the order of 0.2-0.3 $M^{-1}$ $s^{-1}$, Perez-Benito *et al.* (1994b) estimated $k_2$ at 0.078 $M^{-1}$ $s^{-1}$. The values of the rate constant, $k_2$, reported by Connett and Wetterhahn (1985) and O'Brien *et al.* (1992) (0.2 and 0.3 $M^{-1}$ $s^{-1}$, respectively). are in good agreement with the value obtained in our studies under anoxic conditions ($k_2$ = 0.18 $M^{-1}$ $s^{-1}$). In these two studies, the concentrations of GSH used were between 10 - 100 mM, under these conditions because of the speed of the reduction the presence of oxygen has no effect on the rate of reduction or the effect is not detectable. On the other hand, Perez-Benito *et al.* (1994b) estimated $k_2$ at 0.078 $M^{-1}$ $s^{-1}$, which is of the same order of magnitude than the $k_2$ obtained in our studies under aerobic conditions ($k_2$ = 0.087 $M^{-1}$ $s^{-1}$). In their studies, GSH concentrations similar to ours were used; at these concentrations the effect of molecular oxygen on the reduction rate becomes apparent and explains the lower value of $k_2$ obtained compared to studies by Connett and Wetterhahn (1985) and O'Brien *et al.* (1992).

Also in agreement with our results (no effect of molecular oxygen in the initial part of the reduction process) the values of K, $k_1$ and $k_{-1}$ are similar in all the studies independently of the range of GSH concentrations investigated. Our estimations are consistent with the rate constants reported in the literature and support the idea that oxygen has a role in the reduction of chromate by GSH during the second phase of the reaction when the redox processes become important.

**Table 6-5**: Summary of the main kinetic studies on the reduction of chromate by GSH at neutral pH

| Research group | Experimental conditions | Rate constants |
|---|---|---|
| Connett and Wetterhahn, 1985 | Tris-HCl pH 7.4 (10 - 140 mM GSH, 0.37 mM $CrO_4^{2-}$) | K = 21<br>$k_2 = 0.2\ M^{-1}\ s^{-1}$<br>$k_1 = 0.6\ M^{-1}\ s^{-1}$ |
| O'Brien *et al.*, 1992 | no buffer pH 7 (20 - 100 mM GSH) | $k_2 = 0.32\ M^{-1}\ s^{-1}$<br>K = 50 |
| | HEPES | $k_2 = 0.358\ M^{-1}\ s^{-1}$<br>K = 131 |
| | $NaClO_4$ | $k_2 = 0.33\ M^{-1}\ s^{-1}$<br>K = 91 |
| Perez-Benito *et al.*, 1994b | Citrate buffer pH 6.2 (4.82 mM GSH, 0.16 mM $CrO_4^{2-}$) | $k_1 = 0.41\ M^{-1}\ s^{-1}$<br>$k_{-1} = 1.78 \times 10^{-3}\ M^{-1}\ s^{-1}$<br>K = 231<br>$k_2 = 0.078\ M^{-1}\ s^{-1}$ |
| These studies, 1995 | Phosphate buffer pH 6.8 (3 - 20 mM GSH, 0.175 mM $CrO_4^{2-}$) | *aerobic**<br>$k_1 = 0.52\ M^{-1}\ s^{-1}$<br>$k_{-1} = 8 \times 10^{-3}\ M^{-1}\ s^{-1}$<br>$K \approx 70$<br>$k_2 = 0.087\ M^{-1}\ s^{-1}$<br>*anoxic**<br>$k_1 = 0.61\ M^{-1}\ s^{-1}$<br>$k_{-1} = 7.9 \times 10^{-3}\ M^{-1}\ s^{-1}$<br>$K \approx 77$<br>$k_2 = 0.18\ M^{-1}\ s^{-1}$ |

* Means of the rate constants estimated from measurements at 372 and 433 nm, table 6-3.

The question arises as to the causes of the apparent delay of the reduction rate in the presence of oxygen. A side reaction occurring concomitantly with the reduction of chromate by GSH may be the cause of this effect.

A possible reaction that may take place at the same time as the reduction process under aerobic conditions is the autoxidation of GSH. Stoichiometry studies showed that 3 moles of GSH were consumed per mole of Cr(VI) under anoxic conditions, however it was not possible to determine the stoichiometry under aerobic conditions and neutral pH due to GSH autoxidation, even in solutions free of residual metal ions such as iron and copper. This observation seems to suggest that chromium can catalyse the autoxidation of GSH, probably in a way similar to iron or copper. If that is the case, then the autoxidation of GSH, proceeding in parallel with the chromate reduction, could be a possible explanation for the decrease in the rate of reduction observed under aerobic conditions.

The mechanism of thiol oxidation by metals such as copper is well described (Misra, 1974; Saez *et al.*, 1982). The thiol is initially oxidised by a redox metal to a thiyl radical (equation 1), which can then react with oxygen and propagate the reaction (3-8). On the other hand, the metal can be re-oxidised by molecular oxygen affording a continuous supply of RS· (equation 2). The reaction can be terminated by various mechanisms (9-11).

*Initiation:*

1. $RSH + M^{n+} \rightarrow RS\cdot + M^{(n-1)+} + H^+$
2. $M^{(n-1)+} + O_2 \rightarrow M^{n+} + O_2\cdot^-$

*Propagation:*

3. $RS\cdot + O_2 \rightarrow RSOO\cdot$
4. $RSOO\cdot + RSH \rightarrow RSOOH + RS\cdot$
5. $RS\cdot + RSH \rightarrow RSSR^{\cdot-} + H^+$
6. $RSSR^{\cdot-} + O_2 \rightarrow RSSR + O_2\cdot^-$
7. $O_2 + RSH \rightarrow H_2O_2 + RS\cdot^-$

8. $RSSR^{\cdot-} + H_2O_2 \rightarrow RSSR + OH^- + \cdot OH$

*Termination:*

9. $2RS\cdot \rightarrow RSSR$

10. $RS\cdot + RSSR^{\cdot-} \rightarrow RSSR + RS^-$

11. $O_2\cdot^- + 2H^+ \rightarrow H_2O_2 + O_2$

The autoxidation of GSH would interfere with the reduction of chromium(VI) by GSH by reducing the amount of GSH available for this reaction and by engaging a chromium species in a cycle reaction $M^{n+}/M^{(n-1)+}$ (equation 1, 2). At low GSH concentrations, this side pathway would directly compete with the reduction process and a depletion in the level of GSH would have marked effect on the reduction of chromium(VI). However at high concentrations of GSH (> 20 mM) the relative importance of the GSH autoxidation would decrease in relation to the chromate reduction pathway due to the vast excess of GSH and the speed of the reduction. Under anaerobic conditions the GSH autoxidation pathway cannot take place and the reduction of chromium(VI) by GSH would be the only reaction occurring.

Considering that no other pathway involving molecular oxygen has, to date, been described for the reaction of Cr(VI) with GSH, one can speculate that the reactive species responsible for the induction of DNA lesions by chromate and GSH, originates from the autoxidation of GSH.

Of the various intermediates thought to be formed during the autoxidation of thiols (equation 3-8), the following can be ruled out as participating in the formation of SSB and AP-sites: RS·, $RSSR^{\cdot-}$ and RSSR do not require the presence of oxygen to be formed, and no evidence for the formation of ·OH during the reduction of chromate by GSH has been obtained (chapter 3, Aiyar *et al.*, 1989), which would leave $O_2\cdot^-$, $H_2O_2$ and RSOO· as possible candidates. However, neither superoxide or peroxide anions can on their own lead to the formation of DNA damage (Imlay and Linn, 1988). Thiol-peroxy radicals (RSOO·) have been proposed to enhance DNA inactivation in irradiated

solutions, but their effect is far from clear (Lafleur and Retèl, 1993), and there is no direct evidence that such species are formed during the reduction of chromate by GSH. Moreover, it is not known if the oxidative potential of RSOO· would be high enough to initiate the abstraction of a hydrogen atom from the sugar moiety of DNA (von Sonntag, 1987 pp. 365-370). Although RSOO· or some reactive species derived from this radical cannot, at present, be ruled out, there is the possibility that the DNA damaging species arises from the reaction of chromium with one of the intermediates of the GSH autoxidation.

Molecular oxygen, $O_2$, and its two reduced species superoxide and peroxide ($O_2\cdot^-$ and $O_2^{2-}$) can act as ligands to transition metals (Cotton and Wilkinson, 1988). It is therefore reasonable to envisage a mechanism by which either $H_2O_2$ or $O_2\cdot^-$, formed during the autoxidation of GSH, react with a chromium species leading to the formation of a superoxo or peroxo complex with chromium in oxidation state IV or V. Such species would be highly oxidising, and could account for the proposed mechanism by which SSB and AP-sites arise from hydrogen abstraction of the deoxyribose (see chapter 2).

In line with this suggestion, it has been shown using ESR studies that Cr(V) species generated during the reduction of chromate by GSH are formed independently of the presence of oxygen (Kortenkamp *et al.*, 1995). This would suggest that either Cr(V) intermediates are not responsible for the generation of SSB and AP-sites (these DNA lesions were not observed under anoxic conditions), or that they require the presence of oxygen to be activated. It is possible, that Cr(IV) species (Bose *et al.*, 1992; Kortenkamp *et al.*, 1995), in a way similar to Cr(V), may require molecular oxygen for its activation. At present there is no data available to support such a suggestion. Chromium-superoxo species can be formed during the reduction of chromate by GSH. Recently, Perez-Benito *et al.* (1994a) demonstrated the formation of a superoxochromium (III), which was taken as evidence for the presence of a Cr(II) intermediate ($Cr(II) + O_2 \rightarrow Cr(III)(O_2)^{\cdot}$ ) formed via a 2 electron reduction of a Cr(IV) species.

The suggestion that hydrogen peroxide or a peroxo anion may have an important role in the generation of DNA lesions is supported by the fact that in the presence of catalase

the formation of SSB and AP-sites during the reduction of chromate by GSH was completely inhibited (see section 3.3).

At present, all these suggestions are speculative. In future kinetic studies it would be of interest to accurately measure the amounts of molecular oxygen being consumed during the reduction process, which may enable to derive more firm conclusions about the reactions involving oxygen as most of the rate constants for the different steps of the reaction mechanism (equations 1-11) are known (Saez et al., 1982; von Sonntag, 1987, p. 366). The main challenge however, is the identification of the different reactive species formed during the reduction of chromate by GSH. Studies combining ESR and pulse radiolysis techniques have been carried out for the identification of sulphur radicals species such as RS·, $RSSR^{\cdot}$ and RSOO·, however such studies are difficult to carry out and their interpretations are complicated (von Sonntag, 1987, pp. 353-374; Asmus, 1990; Grierson *et al.*, 1992). Simple assays have been described for the determination of oxygen reactive species: 2'-7'-dichlorofluorescin is rapidly oxidised in the presence of $H_2O_2$ to form a highly fluorescent compound (LeBel *et al.*, 1992), and superoxide radicals can react with nitroblue tetrazolium to form formazan which is easily monitored spectrophotometrically (Misra, 1974). However, the specificity of these assays is unclear.

# *CHAPTER 7*

# CONCLUSIONS

One of the main goals in the study of the mechanisms mediating chromate genotoxicity is to establish which mutagenic DNA lesions are induced during the reduction of chromium(VI), the mechanism by which they arise and the nature of the species responsible for the damage. With these objectives in mind, the present studies were undertaken in order to investigate if the alkaline labile sites observed upon treatment of cultured mammalian cells with chromate (Cantoni and Costa, 1984) could be the consequence of the presence of AP-sites in the DNA phosphate backbone. Although AP-sites are well established mutagenic lesions, their possible formation during the reduction of chromate had not been assessed.

Using a simple and well defined *in vitro* system, we have been able to demonstrate that aldehydic AP-sites are generated in PM2 DNA during the reduction of chromate by GSH. A reactive intermediate was shown to be responsible for the damage. Furthermore, the formation of both AP-sites and SSB was dependent on molecular oxygen. It was observed that AP-sites and SSB were formed with equal probability and with a similar temporal pattern, which suggests that both DNA lesions originated from a common precursor lesion induced by a single reactive species. A mechanism of reaction involving attack at the C-4' of the deoxyribose moiety by an oxidising species was postulated to account for these observations. Abstraction of hydrogen from C-4' can lead to the formation of SSB and 4'-hydroxylated AP-sites. This suggestion was further substantiated by the observation that MDA-like products were released from DNA during the reduction of chromate by GSH, which is expected if SSB are formed as a consequence of hydrogen abstraction from C-4'. Moreover, kinetic and anaerobic studies provided evidence that an oxidising intermediate species was responsible for the damage. Although it was not possible to identify the intermediate species, it was

established that ·OHs were not formed during the reaction of chromium(VI) and GSH. It was speculated that the intermediate was a complex of chromium with an activated oxygen species, with chromium in oxidation state IV or V, however such a proposal requires further investigation.

Recently, evidence was obtained that AP-sites were also generated in isolated DNA upon reduction of chromate by AsA (da Cruz Fresco *et al.*, 1995). Given that GSH and AsA are the most important cytosolic reductants of chromium(VI) it is reasonable to assume that the alkaline-labile sites observed by Cantoni and Costa (1984) in calcium chromate-treated cells are indeed AP-sites and are caused by the intracellular reduction of chromium(VI) by GSH and AsA.

Autopsies of workers engaged in handling chromium(VI) have shown that after inhalative exposure the bulk of chromium(VI) compounds is deposited in the lungs where it stays for a very long time (Cohen *et al.*, 1993). Chromium(VI) containing particles are then slowly solubilised in the lung leading to a continuous exposure of epithelial cells to chromate anions. Once taken up by the cells, chromate is likely to be first reduced by AsA since it is a faster reducer of chromium(VI) than GSH. The process is likely to lead to a depletion of intracellular AsA levels, which cannot be replenished. As a result, reduction by GSH becomes increasingly important. The question arises whether the intermediate(s) responsible for the formation of AP-sites *in vitro* are able to interact with the nucleus of exposed cells before decaying or being scavenged. It has been shown that the half-lives of chromium(V) species inside cells are in the range of 10 - 15 minutes (Standeven and Wetterhahn, 1991a). GSH can act to stabilise chromium(V) species thereby enabling sufficiently large quantities to reach the cell nucleus. Although not causative in the generation of AP-sites and SSB, chromium(V) species, once near or inside the nucleus, can mediate the generation of the ultimately DNA damaging species.

Considering that the reduction of chromium(VI) by both GSH and AsA can lead to the formation of AP-sites *in vitro,* it is reasonable to assume that the cellular repair machinery which already has to cope with up to $1 \times 10^4$ spontaneously arising

depurinations per cell per day ((Loeb and Preston, 1986) can be overloaded. Unrepaired AP-sites may be the result. Moreover, chromium/GSH induced AP-sites are likely to be structurally different from spontaneously arising AP-sites and may therefore be difficult to be recognised by DNA repair enzymes such as AP-endonucleases.

Taking into consideration the potential mutagenicity of AP-sites, this kind of DNA damage which is induced during the reduction of chromium(VI) by GSH *in vitro* can indeed be of importance in the mediation of chromium genotoxicity. However, in order to firmly establish the role of AP-sites in the process of Cr(VI) genotoxicity, the formation of abasic sites in intact cells upon treatment with chromate would need to be demonstrated. The detection of AP-sites in *ex vivo*-isolated DNA is a difficult task, not only because the assays need to be highly sensitive to detect AP-sites at the femtomolar level, but also because the DNA isolation procedures can affect the AP-sites. In view of the mutagenicity of AP-sites new assays are currently being developed which could overcome these difficulties. These include $^{32}$P-post-labelling, modification of abasic sites with $^{14}$C-methoxyamine or O-(nitrobenzyl)hydroxylamine which is then detected by antibodies or with a biotinylated probe that can be analysed using ELISA techniques (Weinfeld *et al.*, 1990; Ide *et al.*, 1993).

Not only AP-sites, but also a variety of other DNA lesions arising from chromium(VI) upon reduction can be pre-mutagenic. Such lesions include chromium-DNA adducts and DNA-amino acids or protein cross-links. The potential mutagenicity of DNA lesions can be investigated by using plasmid DNA carrying a marker gene. The DNA is treated *in vitro* with chromate in the presence of a reductant and then inserted in bacterial cells. The possible mutations can be screened by cultivation of the transformed bacteria in selective medium in which the marker gene may or may not be expressed. In order to unambiguously establish that the mutations observed are caused by AP-sites, it would be necessary to compare the location of AP-sites in a particular DNA fragment with the mutation sites in the same DNA fragment. If a correlation existed between the two analysis that would be firm prove of the mutagenicity of chromium-induced AP-sites.

As more work is being carried out in the chromium field, the complexity of the mechanisms which may be contributing to the genotoxicity of chromium compounds becomes apparent. It is clear that the type and extent of the DNA damage in the *in vivo* situation will be a function of different factors, including the amount of chromium internalised by cells, the relative concentration of the various reducing agents in a particular cell and the amount of chromium or other reactive species which are able to reach the nucleus. Not one single, but various mechanisms are likely to be of importance in the formation of DNA lesions and mutations. Modelling the intracellular environment and predicting the various pathways of reaction by using *in vitro* systems may provide a suitable way forward in the unravelling of the mechanisms leading to chromium genotoxicity.

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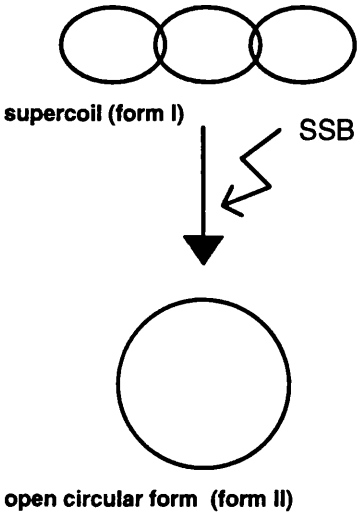
# APPENDICES

## *Appendix I : Quantification of the Level of Breaks in PM2 DNA*

PM2 DNA was loaded on to agarose gels and electrophoresed as described in section 2.2. The gels were then stained with ethidium bromide and photographed under UV light. An example of a typical photograph is shown in Figure 2.3-3 (page 54). The relative intensity of the two DNA bands, corresponding to the supercoiled and open circular forms, was analysed by scanning a digitalized image of a gel using the programme PC-Image Plus version 1.54 (a typical scan is shown below).

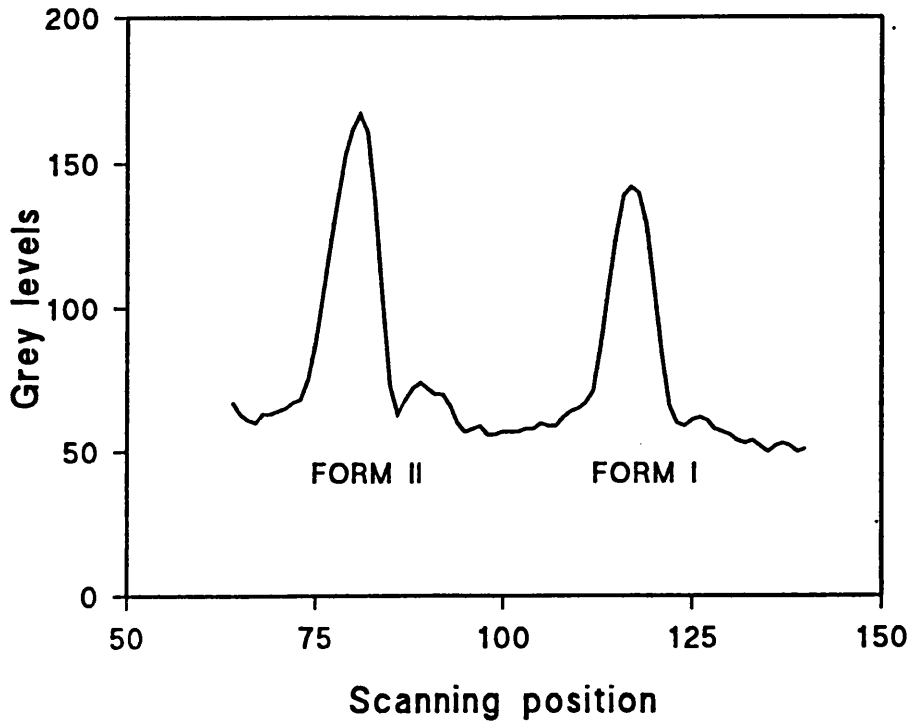



Subsequently the peaks were integrated by using the computer programme Fig. P. The example shown in the scan yields 973 for Form II (open circular) and 916 for Form I (supercoiled), arbitrary units.

The fraction $f_s$ of unbroken (supercoiled) DNA molecules was then determined using the formula

$$f_s = \text{form I} / (0.8\ \text{form II} + \text{form I}); \quad f_s = 916/(0.8 \times 973 + 916) = 0.54$$

The correction factor of 0.8 was used to account for the higher fluorescence of the open circular DNA (form II) when compared to intact supercoiled DNA (form I) (Kortenkamp *et al.*, 1989).




Assuming that the distribution of SSB and AP-sites among individual DNA molecules follows a Poisson distribution, the parameter $f_s$ can be used to calculate the average number of SSB and AP-sites per DNA molecule.

As $f_s$ expresses the probability P(0) of no SSB and AP-sites occurring, the mean of the corresponding Poisson distribution is

$$P(0) = f_s = e^{-m}$$

where m is the average number of SSB and AP-sites per DNA molecule. Thus

$$m = \text{SSB} + \text{AP-sites} = -\ln f_s,$$

yielding 0.61 as the number of breaks per DNA molecule in the above example. This data has to be corrected for the level of breaks in untreated controls, typically around 0.3 breaks per DNA molecule.

At the level of an individual DNA molecule, one break is sufficient to accomplish the relaxation of a supercoil to the open circular form, and an additional break will not lead to further conformational changes.

## *Appendix II : Quantification of DNA-Base Damage*

The yield of modified DNA bases in samples of calf-thymus DNA treated with chromate and GSH, as described in section 3.2, was estimated by determining the area under the peaks of the different DNA-base adducts in the SIM spectra. The retention times for the different peaks and the mass of each sililated DNA adduct are indicated in the table below. The response factor (K) was calculated from the slopes of calibration curves which were constructed using known concentrations of internal standards and synthesised base-adducts.

| Damage base product | Retention time (min) | Mass ion selected | Response factor (K) |
|---|---|---|---|
| 6-Azathymine (Int. Std.) | 2.75 | 256 | 1 |
| 5-Hydroxyuracil | 4.00 | 329 | 3.31 |
| 5-(Hydroxymethyl)uracil | 4.85 | 358 | 2.56 |
| 8-Azaadenine (Int. Std.) | 6.40 | 265 | 1 |
| FAPy-adenine | 6.85 | 354 | 3.38 |
| 8-Hydroxyadenine | 7.50 | 352 | 8.75 |
| FAPy-guanine | 8.05 | 442 | 0.99 |
| 8-Hydroxyguanine | 8.40 | 440 | 3.53 |

The yield of product (nmol/mg of DNA) was calculated for each sample according to the following equation:

$$\text{product yield} = (1/K) \times IS \times (A/A_{IS})$$

IS = concentration of internal standard (12.5 nmol.mg$^{-1}$ DNA)

A = peak area of product

$A_{IS}$ = peak area of internal standard

K = relative molar response factor

## *Appendix III : Deduction of the Rate Law for the Reaction of Chromate and GSH*

The rate law for the reduction of chromate by GSH is derived assuming that an initial rapid pre-equilibrium is established between chromium(VI) and a Cr(VI)-thioester, which is followed by a much slower reduction step.

$GSH + Cr(VI) \rightleftharpoons GSCr(VI)$ $\quad K = k_1/k_{-1}$

$GSCr(VI) + GSH \rightarrow Cr(IV) + GSSG$ $\quad k_2$

Considering that the redox reaction is the rate determining step the formation of products (P) with time is:

$$\frac{dP}{dt} = k_2[GSH][GSCr(VI)] \qquad (1)$$

Assuming a rapid pre-equilibrium, the equilibrium constant K can be define as:

$$K = \frac{[GSCr(VI)]}{[GSH][Cr(VI)]}$$

The total amount of chromium(VI) in the system is:

$$[Cr(VI)]_t = [GSCr(VI)] + [Cr(VI)]$$

Substituting [Cr(VI)] in the equilibrium constant yields:

$$K = \frac{[GSCr(VI)]}{[GSH]\{[Cr(VI)]_t - [GSCr(VI)]\}}$$

and resolving to [GSCr(VI)] results in:

$$[GSCr(VI)] = \frac{K[GSH][Cr(VI)]_t}{1 + K[GSH]}$$

Substituting [GSCr(VI)] in equation (**1**) yields:

$$\frac{dP}{dt} = \frac{k_2 K[GSH]^2}{1 + K[GSH]}[Cr(VI)]_t$$

# LIST OF PUBLICATIONS

## Papers

Casadevall, M. and Kortenkamp, A. (1994) The generation of apurinic/apyrimidinic sites and single strand breaks in isolated DNA during the reduction of chromate and glutathione, *Carcinogenesis*, **15**, 407-409.

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## Abstracts

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