Studies on Cadmium-induced carcinogenesis

by

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STATEMENT

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CONTENTS

ABSTRACT

Cadmium is a potent carcinogen in rodents and has recently been accepted by the International Agency for Research on Cancer as a category 1 (human) carcinogen, but the molecular mechanism of its action remains largely unclear. It has however been suggested that cadmium-induced carcinogenesis may involve either direct or indirect interaction of Cd^{2+} with DNA. In this study, it is found that when Cd^{2+} is allowed to interact with adenine and guanine, there is a marked change in the HPLC retention time for adenine but not for guanine. Since Cd^{2+} is believed to bind covalently to adenine and guanine, the changes in retention time but absence of any cadmium in the peak fraction point to the following: (1) lability of cadmium-nucleobase adducts, (2) introduction of some kind of chemical modification in adenine but not in guanine as a result of covalent binding. This result is different from that for Ni^{2+} in which case a change in retention time was observed for guanine but not for adenine. It is believed that the interaction with Cd^{2+} causes a shift in tautomeric equilibrium of adenine and adenine nucleotides towards the imino form followed by hydroxylation of the dominant form. This would provide an explanation as to why cadmium is mutagenic but would not explain why cadmium is comutagenic.

Cd^{2+} is believed to bind covalently with N7 centres of adenine and guanine. At low concentrations (≤ 50 mM) Cd^{2+} is found to react with plasmid DNA to produce a mixture of Form I and Form II bands whereas higher concentrations (≥ 100 mM) of Cd^{2+} cause extensive damage to DNA at solution pH of 5.8 for cadmium(II) nitrate. Within the range 0 to 100 mM (when pH is adjusted to 7.4 by adding NaOH) an increase in concentration of Cd^{2+} is found to cause a decrease in the gel mobility rate of plasmid and an increase in the intensity of the Form II band. When plasmid DNA is digested with BamH1, only the Form III band is observed both in the presence and absence of Cd^{2+}. However, the mobility of the band is found to decrease with the increase in the concentration of Cd^{2+}. When the enzyme Ssp1, which cuts plasmid DNA at the AT sites, is used instead of BamH1, two bands are observed in the presence of cadmium as against one band in the absence of cadmium. These results either suggest that Cd^{2+} binds covalently with DNA

(possibly at G, A and T centres) and can form intrastrand bifunctional AT adducts but not the GG adducts or it may be that neither GG nor AT adducts are formed and yet Ssp1 digestion is prevented because of a structural modification introduced in adenine by its interaction with Cd^{2+}. In the presence of antioxidants such as cysteine, glutathione and ascorbate (especially cysteine and ascorbate), DNA damage is found to be greater than that expected for the combined effects of the antioxidant and Cd^{2+}. The increased DNA damage is believed to be due to the formation of reactive oxygen species (ROS). It is found that Cd^{2+} binds with ascorbate to form 1:1 adduct in solution in 0.10 M $NaNO_3$, forming chelates in which ascorbate acts as a polydentate ligand. It is believed that it is this binding with Cd^{2+} that activates ascorbate so that it is more susceptible to attack by molecular oxygen. Reactive oxygen species that are produced, then damage DNA. If the results of the present *in vitro* experiments are also applicable to in *in vivo* situations, it would mean that when there is a sufficient body burden of cadmium, ascorbate which normally prevents damage to DNA by acting as a free radical scavenger, can promote damage to DNA by producing free radicals.

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CHAPTER 1

INTRODUCTION

Cadmium is a potent carcinogen in rodents (Waalkes, *et al.*, 1992) and has recently been designated by the International Agency for Research on Cancer and the US National Toxicology Program as a category 1 (human) carcinogen (IARC, 1993 and Ninth report on carcinogens, National Toxicology Program (Waalkes, *et al.*, 1992)). It is a very potent metallic toxicant of continuing environmental and occupational concern with a wide variety of adverse effects (Waalkes, 2000). Cadmium has an extremely long biological half-life that essentially makes it a cumulative toxin (Goering, *et al.*, 1994). Because of the central role of DNA in cellular replication and because it offers many binding sites for Cd^{2+} ion, it has been suggested that cadmium-induced carcinogenicity may involve direct interaction of cadmium with DNA (Coogan, *et al.*, 1992). However, the exact molecular mechanism of cadmium-induced carcinogenicity remains largely unclear.

1.1 Characteristics of metal carcinogens

Waalkes *et al.* (Waalkes, *et al.*, 1992) point out that there are several unusual characteristics of metallic carcinogens that differ in their physical and chemical properties. For example, some metal ions can easily undergo oxidation and reduction reactions while others do not. Also, some carcinogenic metal ions may mimic essential metal ions in some of their properties. It is generally thought that metals do not require metabolic activation to produce reactive species. However, this is not true for redox active carcinogenic metal ions such as those of chromium. Since it is well known that whereas Cr^{3+} is essential, higher oxidation states of the metal namely Cr^{5+} and Cr^{6+} are carcinogenic (Norseth, 1988, Wetterhahn Jennette, 1981).

Often the carcinogenicity due to metals is highly specific for particular tissues or glands where they seem to accumulate. According to Waalkes *et al.* specificity of metallic carcinogens may be dependent on tissue variability in biological defense mechanisms, creating situations in which tissue may be exposed to very little of the metallic carcinogen and yet a high rate of tumour formation may take place in the tissue (Waalkes, *et al.*, 1990, Waalkes, *et al.*, 1991).

It was stated earlier that some metal ions are similar in properties to essential metal ions. For example, the properties of Cd^{2+} are in some respects similar to those of Ca^{2+}, Mg^{2+} and Zn^{2+}. It is thought that a carcinogenic metal ion, which mimics an essential metal ion, may follow the same metabolic pathway. If that is so, they can disrupt the metabolism and function of the essential metals. It was reported that many carcinogenic metals are carried into the cell by mechanisms that are designed to carry essential metals (Wetterhahn Jennette, 1981). Also, it was observed that binding preferences of the carcinogenic metals and the essential metals are often similar (Wetterhahn Jennette, 1981, Clarkson, 1986). It should be pointed out that biological similarities of the metals do not necessarily conform to the chemical similarities (Wetterhahn Jennette, 1981, Clarkson, 1986).

Essential metals can also become carcinogenic. An example of that was provided earlier namely chromium is considered essential in one oxidation state whereas it is carcinogenic in a different oxidation state (Norseth, 1988, Wetterhahn Jennette, 1981). Other examples are provided by zinc and copper. Although both Zn^{2+} and Cu^{2+} are essential metal ions, direct injection in certain tissues may produce local carcinoma (Sunderman, 1979). Compounds of iron if injected in combination with sugar, may produce injection-site tumours (Sunderman, 1979).

Although biological systems have mechanisms by which metal ions can be effectively stored and utilised, the capacity of the systems is limited. Once this is exceeded, even essential metal ions can be toxic and/or carcinogenic.

There are several problems and confounding factors that make it difficult the assessment of the carcinogenic risk of metals to humans. Frequently, only a small group of populations are exposed to a high level of carcinogenic metals, where the nature of exposure can vary in both rate and duration (that may be brought about by changing technology and industrial hygiene practices over a period of time). Often we get exposed to more than one metal at a time (or more than one form of the same metal) and therefore it is difficult to quantify the hazard contributed by a single metal (or metal ion).

Thus, to define carcinogenicity due to a metal, speciation of the metal in the environment needs to be taken into account.

There are many reasons why animal studies that have been carried out fail to provide precise knowledge and understanding on the carcinogenic potential of metals to humans. For example, data from animal studies relating to carcinogenic potential of metals are often obtained from unrealistic routes of exposure such as subcutaneous or intramuscular routes, in which metals are often highly effective at producing injection-site tumours (Sunderman, 1979, Sunderman, 1978). This may however illustrate solid-state or foreign body carcinogenesis, in which a chronically irritating implant can produce a tumour locally. Waalkes *et al.* point out that intramuscular or subcutaneous injections of metals can cause a local accumulation of metals, which induces chronic inflammation and calcification. Solid-state carcinogenesis produced by such local accumulation is believed to be dependent more on the physical nature of the implant rather than its chemical nature (Waalkes, *et al.*, 1992). Thus, the formation of tumours at the injection site is difficult to explain precisely. On the other hand, the formation of tumours in tissues away from subcutaneous or intramuscular injection site can be supportive of carcinogenic potential.

But it is difficult to determine actual risk of carcinogenicity because of bolus injection used for producing tumour at the target site.

The two most common ways by which we get exposed to metals are the oral and inhalation routes. To date, however, surprisingly few animal studies have used these routes. This is perhaps because of the inherent difficulties and expense of such chronic studies. Intratracheal injections have sometimes been used to overcome at least some of those problems. Alternative routes such as injections of metal ions into a subcutaneous pocket or thigh muscles of animals have been used in animal studies in order to assess human risk and mechanism of action. In addition, direct injection into organs like kidney, prostate, bone and liver also have been used. The formation of a tumour in a tissue distant from the parenteral injection site is perhaps more relevant, although it may not still quite represent human exposure situations. It is also found that parenteral bolus injections of metal compounds result into vastly different toxicokinetics and toxicodynamics than gradual exposures by mouth and inhalation.

Dose response analyses (including statistical significance) are important factors that need to be considered in determining carcinogenic potential of metals. But in most of the earlier studies dose response analyses were not carried out.

Often, metals, which are carcinogenic, are found to be highly toxic (as well) to specific sites so that toxicity may abolish a carcinogenic response at higher doses.

Sunderman *et al.* pointed out that metals depending on dose or time frame could have both carcinogenic and anticarcinogenic effects (Sunderman, 1979, Waalkes, *et al.*, 1988). The efficacy of certain platinum compounds used as cancer chemotherapeutic agents may be cited in support of the statement (Sunderman, 1979). In fact, metal-based anticancer drugs such as cisplatin and carboplatin can themselves cause cancer in the long run. Recent evidence indicates that cadmium, a potent carcinogen in some tissues (Waalkes, *et* *al.*, 1990), can specifically inhibit liver and lung tumour formation in mice (Waalkes, *et al.*, 1991).

Summary

Carcinogenic metals differ widely in their physical and chemical properties. Hence the molecular mechanisms of carcinogenicity of different metal ions are likely to be different. Some carcinogenic metal ions mimic essential metal ions in some of their properties. Often carcinogenicity due to a metal is highly specific to a particular tissue.

1.2 Evidence of metal carcinogenesis in humans

There are various exposure situations to metals that have been associated with induction of tumours in humans. Processes involved in metal refining and smelting have led to a large number of reports associating metal exposure and human carcinogenesis (Sunderman, 1979, Kazantzis, 1981, Sunderman, 1978). Some previous medicinal uses of metals (eg archaic medicinal preparations based on arsenic) also have led to tumour development. They are clearly known as human carcinogens (Hutchinson, 1988, Ishinishi, *et al.*, 1986). Some metal salts including those of chromium, used for pigment production have been reported to be associated with the formation of tumours in humans (Sunderman, 1979, Sunderman, 1978). Individuals involved in certain mining processes may be also at greater risk for metal-induced carcinogenesis (Kazantzis, *et al.*, 1986).

1.3 Common target sites of carcinogenic metals with special reference to cadmium

The earliest report of a metal as a carcinogen was published in 1888 and it was found that arsenic therapy used for various skin disorders (Hutchinson, 1988) actually induced skin tumours in humans.

Inhalation exposure to compounds of arsenic, cadmium, chromium and nickel has been associated with pulmonary carcinomas (Sunderman, 1979, Waalkes, *et al.*, 1990, Sunderman, 1978). Some evidence indicates that exposure to cadmium either orally or by inhalation may cause prostate tumours (Waalkes, *et al.*, 1990).

It was stated earlier that animal studies in general do not provide reliable information on tumour formation in humans. However, it was also observed that the formation of a tumour at a distant site away from the site of injection might provide information that is better representative of the situation in humans. Recent studies involving rats show that parenteral administration or oral exposure to cadmium compounds can produce tumour in prostate (Waalkes, *et al.*, 1988, Waalkes, *et al.*, 1989, Waalkes, *et al.*, 1992), which is a clear target site in humans as well. It has also been reported that pulmonary carcinomas are produced in rodents when they are exposed to compounds of cadmium, chromium, beryllium and nickel either by inhalation or by intratracheal exposure (Sunderman, 1979, Waalkes, *et al.*, 1990, Sunderman, 1978). Intramuscular injection-site sarcomas seen with various metals including cadmium, cobalt, chromium, manganese, nickel, and titanium (Sunderman, 1979, Kazantzis, 1981, Sunderman, 1978) are found to be limited to rodent models. Some metallic prosthetic devices that have undergone corrosion have been found to develop sarcomas in dogs and humans around implanted region (Sunderman, 1989). Injection of copper, zinc, nickel and cadmium in testes has been found to produce malignant tumours, primarily teratomas (Sunderman, 1979, Sunderman, 1978). Systemic exposure to cadmium can result in testicular tumours other than teratomas (Waalkes, *et al.*, 1990). Intravenous injection of beryllium or nickel compounds can produce tumours at various sites including bone (Sunderman, 1979, Sunderman, 1978). Oral exposure of lead has been associated with the formation of tumours at the rodent kidney (Kasprzak, *et al.*, 1985) while the exposure to cadmium has been linked to tumours of the haematopoetic system in rats (Waalkes, *et al.*, 1992). It is also observed that subcutaneous exposure to cadmium can inhibit such tumours (Waalkes, *et al.*, 1991).

However, the target sites of metal carcinogens appear to be rather specific to the metals with the possible exception of the lung.

Summary

Arsenic therapy used for skin disorders actually induced skin tumours in humans. Inhalation exposure to compounds of arsenic, cadmium, chromium, and nickel has been associated with pulmonary carcinomas. Prostate is a clear target-site for cadmium-induced tumour. Some metallic prosthetic devices that have undergone corrosion have been found to develop sarcomas in dogs and humans around implanted region. Oral exposure of lead has been associated with the formation of tumours at the rodent kidney while the exposure to cadmium has been linked to tumours of the haematopoetic system in rats. It is also observed that subcutaneous exposure to cadmium can inhibit such tumours. The target sites of metal carcinogens appear to be rather specific to the metals with the possible exception of the lung.

1.3.1 Site specificity in metal carcinogenesis

Metals, as carcinogens appear to be highly tissue specific. There are several factors that influence this specificity: (1) proximity to high local concentration (2) direct exposure eg inhalation. At subcutaneous or intramuscular sites, an irritating deposit may induce biochemical reactions that can help dissolution and local release of the metal ions. In a similar way, after inhalation of metal-containing particulate, very high quantity of metal could be released locally in the lung. Specific disposition or uptake of metal within a tissue also may be an important factor in determining specificity of the target sites. For example, tumour produced in kidney due to lead is found to be associated with the formation of specific lead compounds (Goyer, 1991).

The difference in susceptibility also may dictate a target tissue. Various factors including the absence or suppression of defense mechanisms, presence of unique or specific carrier molecules may be involved in the process. For instance, it is found that the gene responsible for synthesising the metal-binding protein metallothionein (a key element of the normal cellular defense system against cadmium) is not responsive to metal stimulation in the rodent testicular interstitium (Waalkes, *et al.*, 1991, De, *et al.*, 1991) or the ventral prostate (Waalkes, *et al.*, 1991, Waalkes, *et al.*, 1992) both being target sites of cadmium-induced carcinogenesis (Waalkes, *et al.*, 1988, Waalkes, *et al.*, 1989, Waalkes, *et al.*, 1992). Thus the inactivity of a defense system specific to certain tissue may contribute to cadmium-induced carcinogenesis. Some endogenous secretion including that in testes and prostate may be associated with the formation of tumour in certain sites. Waalkes, *et al.* reported that testes and prostate after being initiated by cadmium appeared to require leutinising hormone (LH) and testosterone input respectively for the formation of tumours (Waalkes, *et al.*, 1990, Waalkes, *et al.*, 1988, Waalkes, *et al.*, 1989, Waalkes, *et al.*, 1992). Lead also induces tumour in the kidney by causing repeated tissue damage and subsequent proliferative cellular repair of the damaged tissue (Goyer, 1991).

Summary

Metals, as carcinogens appear to be highly site-specific. The difference in susceptibility, proximity to high local concentration and direct exposure may dictate a target site.

1.4 Factors modifying metal carcinogenesis

Several factors can modify metal-induced carcinogenesis. Perhaps the most important one is metal-metal interaction, in which exposure to one metal reduces or increases carcinogenicity of another. Species and strain of animals and metabolic fate may also contribute to carcinogenic response. Metal-metal interactions attribute much in determining carcinogenic potentiality and efficacy of a given metal (Nordberg, *et al.*, 1981, Furst, 1987, Kasprzak, 1990). It was evident from early studies that pretreatment with zinc could prevent cadmium carcinogenesis at the subcutaneous injection site and in the testes of rodents (Gunn, *et al.*, 1963, Gunn, *et al.*, 1964). Later studies have indicated that cadmium-induced injection-site tumour can be alleviated by magnesium treatment, whereas testicular tumours remain unaffected (Poirier, *et al.*, 1983). Zinc also can prevent cadmium-induced pulmonary tumours (Oberdorster, *et al.*, 1990); magnesium and manganese are effective against formation of tumour in rat muscles (Sunderman, *et al.*, 1975, Kasprzak, *et al.*, 1985). Likewise, it is also evident that concomitant exposure to another metal favours tumour enhancement due to exposure to the Cancer-inducing metal. For instance, exposure to calcium can promote lead-induced renal tumours in rats (Kasprzak, *et al.*, 1985) while zinc administration increases the incidence of cadmium-induced prostatic tumours in the same species. Deficiencies of essential elements also can modify response to carcinogenic metals (Waalkes, *et al.*, 1992, Waalkes, *et al.*, 1991). Strain and species differences may also contribute to carcinogenesis, although only limited data are available in this area.

Summary

Metal-metal interactions appear to be the most important factor modifying metal-induced carcinogenesis.

1.5. Animal models

Metal-metal interactions

It was stated earlier that metal-metal interaction could result in a reduction of carcinogenesis. Study of the several workers (Waalkes, *et al.*, 1992) showed that the presence of excess zinc ions could reduce cadmium-induced carcinogenesis in many target tissues. It was reported that zinc when administered subcutaneously to rats before, during, and after cadmium treatment, prevents testicular tumours (Gunn, *et al.*, 1963, Gunn, *et al.*, 1964, Waalkes, *et al.*, 1989) in a dose related fashion (Waalkes, *et al.*, 1989). It was also reported that parenteral administration of zinc prevents the carcinogenic effects of cadmium at the injection site as well (Gunn, *et al.*, 1963, Gunn, *et* *al.*, 1964, Waalkes, *et al.*, 1989) even when given by using a totally different route (Waalkes, *et al.*, 1989). Zinc is also found to resist cadmium-induced carcinogenesis in lung (Oberdorster, *et al.*, 1990, Oldiges, *et al.*, 1989, Waalkes, *et al.*, 1990). The preventive effect of zinc in so many different target tissues may point to the involvement of zinc in the basic mechanism of cadmium-induced carcinogenesis.

It is also possible that metal-metal interaction also can result in increased incidence of tumours. For example, although zinc prevents formation of testicular tumour due to cadmium, when administered subcutaneously (with increasing dosage) to rats before, during, and after cadmium, it increases the incidence of cadmium-induced prostate tumours (Waalkes, *et al.*, 1989). It is believed that this phenomenon may be due, at least in part, to an increased accumulation of cadmium in the prostate.

It is believed that the deficiency of an essential element can also modify carcinogenesis due to a metal. For example, dietary zinc deficiency has tissue-specific effects on cadmium-induced carcinogenesis. Waalkes *et al.* reported that dietary zinc deficiency enhances the progression of cadmium-induced testicular interstitial cell tumours (Waalkes, *et al.*, 1991). It was found that the dose of the metal could make a profound difference in the eventual incidence of specific tumours. It is not always the case with metallic carcinogens that higher doses will produce more tumors.

Summary

Presence of excess zinc ions can reduce cadmium-induced carcinogenesis in many target tissues. Metal-metal interactions can also cause increased incidence of tumour. For example, although zinc prevents formation of testicular tumour due to cadmium, when administered subcutaneously (with increasing dosage) to rats before, during, and after cadmium, it increases the incidence of cadmium-induced prostate tumors.

1.6 Sources of cadmium

Like lead, cadmium is an underground mineral that did not enter air, food and water in significant amount until it was mined as a part of zinc deposits. Now there is widespread environmental contamination due to cadmium. Cadmium enters the environment naturally mainly from gradual phenomena, such as rock erosion and abrasion, and singular occurrences like volcanic eruptions. Since cadmium has a variety of industrial uses, there has been an increase in cadmium level in our environment worldwide during the last several decades.

Cadmium is primarily used for electroplating metals such as iron. Cadmium-plated automobile parts are more resistant than zinc coated (galvanised) ones (Friberg, *et al.*, 1986). Cadmium compounds are also used as pigments (Aylett, 1979). These range in colour from yellow to maroon, have high thermal stability, and are used in paints, enamels, and plastics. Cadmium stearate is used as a stabiliser in plastics. Cadmium is found in silver solder, welding electrodes and as a hardening agent for copper. In addition, cadmium is used as an electrode component in nickel-cadmium batteries. Its special neutron-absorbing properties are used to advantage in the cadmium control rods of nuclear reactors.

Apart from localised high concentration of cadmium in the atmosphere near an appropriate industrial plant, the most serious source of cadmium intake by the general population is thought to be food. Cadmium has been found in a wide range of fresh and tinned foods, especially seafood (Chmielnicka, *et al.*, 1986). There are other sources from which our environment and our bodies can be contaminated with cadmium. Cigarette smoke, refined foods, water pipes, coffee and tea, burning of coal and fossil fuel and shell fishes are all definite sources of cadmium. From food grains we may have a significant amount of cadmium. During the growth of grains such as wheat and rice, cadmium (from the soil) is concentrated in the core of the kernel, while zinc is found in the germ and bran coverings. With refinement, zinc is lost, increasing the cadmium to zinc ratio. Refined rice flours and sugar all have relatively higher ratios of cadmium to zinc than do the whole foods. Water pipe can be a source of cadmium. As stated earlier, cadmium is often used to protect corrosion. Pipes galvanised with zinc usually contain some cadmium, as does the solder used to hold them together. Once soft or acid water flows through the pipe, it causes the metals in the pipes to break down, releasing cadmium and other minerals from them. On the other hand, hard water (containing calcium and magnesium salt) actually coats the pipes and prevents leaching of cadmium and other minerals.

Summary

There has been an increase in cadmium level in the environment worldwide during the last several decades because of increased industrial activity. Cadmium is used in the electroplating of metals and as an electrode component in nickel-cadmium batteries. Cigarette smoke, refined foods, water pipes, coffee and tea, burning of coal and fossil fuel and shell fishes are all definite sources of cadmium.

1.7 Chemistry of cadmium

The physical and chemical properties of cadmium will undoubtedly play a key role in the mechanisms by which it enters biological systems and interacts with molecules such as DNA. The melting point (321°C) and boiling point (765°C) of cadmium are low compared with those of most other metals; vapours are formed when the metal is heated. In the periodic table of elements, the congeners zinc, cadmium and mercury form Group IIB; the inner electron shells are filled, and the outermost shell has 2 electrons. The common oxidation state for zinc and cadmium is +2 whereas mercury commonly exists in +1 and +2 states. The *s-p* energy separation for cadmium is less than that of zinc, suggesting that excitation to the valence state followed by formation of two collinear covalent bonds may be easier for cadmium than for zinc (Aylett, 1979). Separation energies for *d-s* and *d-p* are similar for both. As stated earlier, although both cadmium and zinc exist mainly in the +2 oxidation state, cadmium (I) compounds have been isolated; but these are generally regarded as a short-lived unstable species (Aylett, 1979). From the solubility standpoint, sulfide, carbonate and oxide of cadmium are slightly soluble whereas nitrate or chloride is readily soluble in water. Organic cadmium compounds (R_2Cd and RCdCl, where R is an aliphatic hydrocarbon chain) are unstable when exposed to air or water.

From a biological standpoint, zinc appears to play an important role in the toxicity and carcinogenicity due to cadmium. This may be due to a similarity in their chemical nature and interactions in biological systems.

Cadmium is generally classified as a "soft" metal that is more likely to form covalent linkages with electron-donating ligands; whereas zinc is considered to be intermediate in "softness"(Jacobson, *et al.*, 1980). Several characteristics of cadmium are important in analysing interactions of the element with biomolecules. First, since cadmium has a high affinity for sulfur, it is expected to bind strongly with sulfur donor centres in amino acids and proteins. In fact, some toxic effects of cadmium are the result of its binding with sulfhydryl groups in proteins. Second, cadmium being located directly below zinc in the periodic table, it has chemical properties similar to those of zinc. Although both cadmium and zinc have an affinity for sulfur ligands, the affinity of cadmium is greater than that of zinc and this is consistent with their difference in "softness"(Jacobson, *et al.*, 1980). Cadmium is not mined as an element and is mostly found isomorphously replacing zinc in zinc minerals. Thus it is isolated as a by-product of the zinc metallurgical process (Cotton, *et al.*, 1999). The similar chemistry of cadmium and zinc and the greater affinity of cadmium for certain ligands may allow cadmium to replace zinc in a number of biological processes. For example, cadmium may act by replacing zinc in a number of zinc containing enzymes and alter their activity. A number of the enzymes involved in the synthesis and function of nucleic acids are zinc-containing enzymes and these may be a target of cadmium toxicity (Wu, *et al.*, 1987). The ability of cadmium to affect the properties of zinc-containing enzymes is well known, although the detailed mechanisms are largely unclear. Third, Cd^{2+} and Ca^{2+} have identical charges and almost the same physical sizes. Thus one may expect certain similarities in the behavior of these ions, although they differ in other properties. Cadmium has been found to replace calcium from certain calcium-containing proteins. Finally, the role of cadmium in altering the structure of nucleic acids has relevance since there are several binding configurations for metal-nucleotide complexes that are sufficiently stable to exist under normal physiological conditions (Jacobson, *et al.*, 1980). Cd^{2+} is found to react more with nucleobases than with phosphates in DNA and RNA, (Jacobson, *et al.*, 1980) although the binding with phosphate has also been observed (Aoki, *et al.*, 1984).

Cadmium can have coordination numbers from 2 to 8, but the most common ones are four and six. Tetrahedral (4) coordination is preferred by the softer ligands (eg S-donors), while octahedral (6) coordination is preferred by the harder ligands (eg O-and N-donors) (Aylett, 1979).

Summary

Like zinc, the common oxidation state for cadmium is +2. However, cadmium is considered to be a "soft" metal which is more likely to form covalent bonds with electron-rich centres in proteins (eg sulfhydryl groups) and other biomolecules (eg nucleobases in DNA). From a biological standpoint, zinc appears to play an important role in the toxicity and carcinogenicity due to cadmium. This may be due to a similarity in their chemical nature and interactions in biological systems.

1.7.1 Nucleic acids interaction sites for metal ions

The regular structures of the nucleic acids and oligonucleotides permit certain generalisations to be made about likely interaction sites for different metal ions. In terms of hardness and softness scale, the phosphate residues represent negatively charged hard sites preferring electrostatic binding. Electron-rich sites on nucleobases on the other hand interact with soft metal ions more likely forming covalent bonds. Hard metal ions such as Na^+, Mg^{2+}, and Ca^{2+}, tend to bind almost exclusively with the phosphate groups. Indeed such ions are essential for stabilisation of the Watson-Crick double helix of DNA (Zubay, *et al.*, 1958).

Ionic strength can drive borderline interactions one way or the other. For example, at low concentrations Zn^{2+} binds primarily with the phosphates in DNA and at higher concentrations it interacts more with the nucleobases (Weser, 1968).

Soft metal ions such as Cd^{2+} prefer to bind to electron-rich centres in nucleobases. In adenine, these are N7 and N1 positions and in guanine N3 and N7 positions are the preferred binding sites. The NH_2 group of both also has a high electron density and is attracted by soft metal ions. In cytosine, the preferred binding sites are the N1 and NH_2 positions, and in thymine and uracil these are O4 and N1 positions. Steric factors can also influence binding to any of these positions. Some sites are not available for binding with metal ions in DNA as they are involved in interbase H bonding between two polynucleotide chains. Even a site that is not involved in H bonding may not be available for binding with a metal ion (discussed more fully in Chapter 2).

Unhindered sites such as N7 centres of purines, can be accommodated in octahedral complexes of a metal ions such as cadmium whereas more sterically hindered sites such as N1 position of cytosine, prefer to form tetrahedral or square-planar complexes with metal ions (Davydova, 1979, Gellert, *et al.*, 1979).

Sissoeff *et al.* (Sissoeff, *et al.*, 1976) classified metal ions according to their effects on DNA structure. It was stated earlier that hard ions Na^+, Mg^{2+}, and Ca^{2+} are required to stabilise the double helix structure of DNA. They act as counter ions to reduce the unwinding tendency of the DNA strands due to electrostatic repulsion between the negatively charged phosphate groups on adjacent nucleotides. On the other hand, soft metal ions such as Cd^{2+} and Hg^{2+} destabilise DNA presumably because of their strong interaction with the nucleobases. It should be noted that the binding of metal ions to DNA is also affected by changes in pH, temperature in addition to changes in ionic strength of the medium.

Although Cd^{2+} ion prefers to binding to nucleobases (than phosphates) in DNA, its affinity for proteins and amino acids is much greater. Cd^{2+} has the highest affinity for cysteine and histidine as compared to other amino acids. Harder metal ion such as Zn^{2+} has a similar selectivity for cysteine but its affinity for this amino acid greatly exceeds that of Cd^{2+}. This phenomenon is not consistent with the predictions based on softness-hardness scale; it may relate to other factors such as sterical arrangement. As applied to the binding with Cd^{2+}, it is found that an increase in the number of cysteine residues in peptides and proteins causes an increase in its tendency to bind with the metal ion.

Among the sulfur rich proteins, metallothionein that found mainly in liver and kidney is important because of its role in cellular defense mechanism. Cd^{2+} is known to bind strongly with metallothionein. It has a low molecular weight of approximately 6-8 kilo Dalton. It contains no histidine or aromatic amino acids but it is rich in cysteine (one third of its 60 amino acid residues are cysteines). (Jacobson, *et al.*, 1980). The apoprotein thionein has an absorption maximum at 250 nm. When Cd^{2+} interacts with metallothionein to produce Cd^{2+}-thionein complex, the absorbance at 250 nm is found to increase. This is the result of binding of Cd^{2+} with cysteine residues of metallothionein.

Summary

Soft metal ions such as Cd^{2+} prefer to bind to electron-rich centres in nucleobases. These are N7 and N1 positions in adenine and N3 and N7 positions in guanine. In cytosine, the preferred binding sites are the N1 and NH_2 position whereas in thymine and uracil these are O4 and N1 positions.

1.8 Pathobiology of cadmium

There is no known useful biological function of cadmium in the cell except that of competing with zinc for binding sites. Therefore, it interferes with most of the essential functions of zinc including enzyme reactions and utilisation of nutrients by cells.

Cadmium is toxic to a wide range of tissues and organ systems, with the lung, kidney, liver and testes being clear target organs in rodents (Waalkes, *et al.*, 1990, Waalkes, *et al.*, 1994). The toxicity of cadmium is dependent on the concentration of cadmium in the organ, although there is wide variation with respect to tissues. In situations of high dose, (for short term exposures to cadmium) the lung is most likely the critical organ after inhalation while the gastric tract is the most affected tissue after acute high oral exposure at a high dose (Waalkes, *et al.*, 1992). Kidney appears to be the critical organ in a low chronic exposure situation irrespective of routes (Friberg, *et al.*, 1974, Foulkes, 1990, Waalkes, 1992). Chronic respiratory disease has been associated with occupational exposure to cadmium fumes and dust (Elinder, 1986). Chronic bronchitis, progressive fibrosis of the lower air ways and alveolar damage resulting in emphysema are the characteristics of the disease (Orberdorster, 1986). Chronic exposure to cadmium, regardless of route, results in renal dysfunction. This is predominantly apparent in cortex and is characterised as proximal tubule necrosis and chronic nephritis (Goyer, 1991, Goyer, 1986). It is likely that the toxic agent producing nephropathy may not be cadmium itself but rather the cadmium-metallothionein complex. As a result of this nephropathy, an increase proteinuria, aminoaciduria and glucosuria is observed (Goyer, 1991, Goyer, 1986). Rodent testes are extremely susceptible to cadmium-induced necrosis. Low doses of cadmium produce haemorrhagic necrosis despite the fact that relatively little of it distributes to the testes (Gunn, *et al.*, 1970). Cadmium appears to be acutely toxic to blood vessels. The interstitial and germinal elements of the gonad are destroyed by its acute toxicity. The formation of interstitial cell tumours is observed in rats due to high incidence of cadmium treatment (Gunn, *et al.*, 1970, Waalkes, *et al.*, 1990, Waalkes, *et* *al.*, 1988, Waalkes, *et al.*, 1989). Cadmium intoxication can affect calcium homeostasis and metabolism (Waalkes, *et al.*, 1992). This can lead to an increase in calcium excretion and the formation of renal calculi. This increased excretion can also lead to a condition in postmenopausal women termed "Itai-Itai" disease which was first observed in a group of Japanese women exposed to cadmium and is characterised by severe osteomalacia and osteoporosis (Nomiyama, 1980).

Hypertension that has been associated with chronic cadmium exposure in rodents (Schroeder, *et al.*, 1961, Perry, *et al.*, 1974), could be secondary to renal nephropathy, increased vasoconstriction, or increased cardiac output. An association between cadmium exposure and development of lesions due to atherosclerosis has been observed in rodents (Waalkes, 1992). An increased mortality from cardiovascular disease has been observed in humans from cadmium-polluted areas (Nagawa, *et al.*, 1979).

Cadmium appears to depress some immune functions mainly by reducing host resistance to bacteria and viruses. It may also increase cancer risk, possibly for the lungs and prostate. Cadmium toxicity has been implicated in generating prostate enlargement, possibly by interfering with zinc support.

Summary

Cadmium is toxic to a wide range of tissues and organ systems, with the lung, kidney, liver and testis being clear target organs in rodents. Kidney appears to be the critical organ in a low chronic exposure situation irrespective of routes. Chronic respiratory disease has been associated with occupational exposure to cadmium fumes and dust. Chronic exposure to cadmium, regardless of route, results in renal dysfunction.

1.9 Metabolism

Cadmium can enter the human body by a number of routes namely inhalation, ingestion, and absorption through the skin. Inhaled cadmium is more readily absorbed into the body than ingested cadmium. In occupational settings, cadmium is inhaled in the form of either small particles of fume or larger particles of dust. The extent of deposition depends on the particle size. It has been reported that small-sized particles deposit better than large particles. Twenty to twenty five percent of the particles deposited are systemically absorbed. It should be noted that high rate of absorption of inhaled cadmium was found in the case of smokers (10 % to 50% of the total inhaled cadmium). Thus, smoking habit and personal hygiene are of great importance as a source of indirect exposure in a cadmium-contaminated environment (Report from the U. S. Department of Labour 1986)

Many of the effects of cadmium are systemic. After initial exposure, inhalation, and absorption, cadmium is transported by the blood plasma, although the majority of the cadmium is bound to the blood cells particularly erythrocytes (Clarkson, 1986).

Cross-sectional studies of workers who had varying duration of exposure at varying levels of exposure to cadmium have yielded little definitive information about the kinetics of blood cadmium and the distribution of cadmium between the plasma and blood cells (Report from the U. S. Department of Labour 1986). Cadmium in blood increases rapidly during the first three to four months of high exposure and then reaches an apparent steady level, which is likely to reflect the average exposure during those months. When high exposures cease, blood levels of cadmium decrease with two distinct half-life components. One component has a half-life of a few months and probably reflects the turnover rate of red blood cells. The half-life of the remaining cadmium is several years long and is likely to reflect body burden (De Silva, 1986). Therefore, the transport of absorbed cadmium in blood is of crucial importance.

Following absorption cadmium is transported primarily to the liver, where it is bound to metallothionein (Fox, 1976). Metallothionein is thought to be composed of three protein subunits that strongly binds with cadmium and one protein subunit that loosely binds zinc. After cadmium is sequestered by the liver, a small amount of metallothionein-bound cadmium appears in the plasma where it is cleared efficiently by the kidney. In the kidney, the cadmium-metallothionein complex passes through the glomeruli and is reabsorbed by the proximal tubules. This complex can then be broken down by lysosomes, releasing unbound cadmium, which can induce renal synthesis of metallothionein. While accumulating in kidney cadmium may interfere with zinc-containing enzymes (eg leucine-aminopeptidase, which is believed to play a role in renal handling of protein). The increased renal cadmium accumulation may cause decreased catabolism or reabsorption of protein, resulting in tubular proteinuria. When this occurs, cadmium excretion will increase because less cadmium-bound metallothionein will be reabsorbed (Friberg, 1971). In workers with only short-term exposures to low levels of cadmium, it will be bound in the kidney to the locally produced metallothionein providing a protective effect from cadmium. However, after prolonged exposure, the binding sites in the kidney become saturated leading to an increase in unbound cadmium, which can result in toxic effects (Goyer, 1990). When cadmium exposure is moderate, renal concentration of cadmium rises slowly to about 200 μg per gram of tissue. At this concentration renal tubular injury may occur. Cadmium-exposed workers usually have a higher concentration of β-2 microglobulin in the urine. The higher concentration of the protein in urine and higher plasma metallothionein levels suggests a renal damage (Nordberg, *et al.*, 1982).

Cadmium metabolism is also known to affect the metabolic fate of calcium, but the metabolic antagonism of cadmium with calcium may not be a mimicking phenomenon. Excess loss of calcium in the urine through renal injury due to cadmium can lead to calcium mobilisation from skeletal stores to maintain serum calcium levels, which can lead to osteomalacia. Pregnant women are more susceptible to this condition since they need an increased calcium requirement during pregnancy and thus an inadequate supply of dietary calcium may aggravate the problem. Therefore, the pregnant women in cadmium-polluted zones are more prone to develop osteomalacia than in cadmium free zones. In the case of pregnant women in chronic cadmium exposure, pregnancy may induce a high risk of cadmium-induced nephrotoxicity. Pregnancy can easily mobilise the hepatic cadmium to the kidney through the blood plasma (Chan, *et al.*, 1993). Cadmium may also have a direct effect on bone but further studies are needed to confirm this (Bryce-Smith, 1982). Cadmium can concentrate in the testes and prostate during heavy exposure and cause a decrease in testosterone synthesis. Excess exposure may also interfere with a zinc/hormone relationship in the prostate.

It was stated earlier that unlike other heavy metals (such as mercury and lead) cadmium occurs mainly in only one oxidation state (namely +2) and does not form stable organometallic complexes of known toxicological significance (Casarett, 1986). Thus, it is Cd^{2+} that is responsible for toxic effects.

According to Roels and Ellis (Roels, *et al.*, 1986), regardless of the route of absorption or the type of compound, approximately one half to one third of the body burden of cadmium is found in the kidneys after chronic low-level exposure, with the highest concentrations being found in the renal cortex. After a long-term exposure, one sixth and one fifth of the body burden are found in the liver and muscles respectively. As the exposure level increases, a greater proportion of the body burden of cadmium will be found in the liver relative to that in the kidney. Also, upon the onset of renal dysfunction, the level of cadmium in the kidney will decrease (Friberg, 1990). The half-life of cadmium in the liver, kidney and muscles is respectively 5 to 15, 10 to 30 and more than 30 years (Report from the U. S. Department of Labour 1986).

Summary

Cadmium can enter the human body by a number of routes namely inhalation, ingestion, and absorption through the skin. Inhaled cadmium is more readily absorbed into the body than ingested cadmium. Following absorption, cadmium is transported primarily to the liver, where it is bound to metallothionein. Metallothionein is thought to be composed of three protein subunits that strongly bind with cadmium and one protein subunit that loosely binds zinc. After cadmium is sequestered by the liver, a small amount of metallothionein-bound cadmium appears in the plasma where it is cleared efficiently by the kidney. In the kidney, the cadmium-metallothionein complex passes through the glomeruli and is reabsorbed by the proximal tubules. While accumulating in the kidney cadmium may interfere with zinc-containing enzymes.

1.10 Human exposure to cadmium

It was stated earlier that increased industrial uses of cadmium have given rise to an increase in the contamination of soil, air and water. Cadmium enters the food chain through environmental contamination and can concentrate within organisms due to its relatively long half-life. Contamination of the food chain probably contributes most to human exposure, at least for those individuals who do not smoke tobacco or do not work in cadmium industries. Chmielnicka and Cherian (Chmielnicka, *et al.*, 1986) reviewed the environmental contribution of cadmium via food chain. The potential of cadmium exposure associated with foodstuffs can vary among cultures, regions of the world as well as with dietary habit and the level of cadmium. In general, meat products such as liver and kidney can be significant sources, in addition to shellfish and other seafood (Friberg, *et al.*, 1986, Chmielnicka, *et al.*, 1986). The contribution of cadmium directly from water is minor in most cases (Chmielnicka, *et al.*, 1986), and that from low level of cadmium in air in non-polluted regions is also found to be low. In general, people living in industrialised regions are more prone to cadmium exposure than in rural areas.

Occupational exposure to cadmium provides another important source of human exposure. As many as 1.5 million workers potentially have been exposed to cadmium at the work place (Waalkes, *et al.*, 1990, Current intelligence bulletin 42 on Cadmium, 1984), but this figure has decreased with gradual diminution of smelting process in the U. S. Cadmium-containing particles can be formed due to fumes generated by combustion of materials or by dust generated during mechanical operations. Industries that utilise large quantities of cadmium also represent sources of high exposure. For instance, large quantities of cadmium are used in the production of nickel-cadmium batteries. In addition, welders, particularly those using cadmium-based solders are exposed to a high level of the metal.

Tobacco smoke appears to be an important source of exposure to cadmium for many individuals due to the ready absorption of cadmium by tobacco plants. As stated earlier, the absorption of inhaled cadmium is much greater (40-60%) than that of ingested cadmium (2-4%). A single cigarette contains 1 to 2 μg of cadmium, and therefore, smoking can significantly increase the body burden of cadmium. Smoking two packs per day for 20 years can result in additional body burden of 15 mg cadmium (Lewis, *et al.*, 1972). Higher concentrations of cadmium in the blood of smokers as compared to non-smokers have been reported (Ulander, *et al.*, 1974, Friberg, *et al.*, 1983). In addition, cadmium levels in maternal and cord blood and placental tissue of pregnant women who were habituated to smoking was found to be greater than the levels found in those who did not smoke (Lauwerys, *et al.*, 1978, Kuhnert, *et al.*, 1982).

Summary

Contamination of the food chain probably contributes most to human exposure, at least for those individuals who do not smoke tobacco or do not work in cadmium industries. Tobacco smoke appears to be an important source of exposure to cadmium for many individuals due to the ready absorption of cadmium by tobacco plants. Occupational exposure to cadmium provides another important source of human exposure.

1.10.1 Evidence from human epidemiological studies

Evidence for cadmium as a human carcinogen is based mainly on epidemiological studies relating to workers who are occupationally exposed to cadmium. As stated earlier, in most animal studies it was found that exposure to cadmium caused a higher incidence of tumours in the lung and prostate and to a lesser extent that in kidney and stomach. However, the occurrence of tumours in humans was not always found to be consistent. Varying factors, such as exposure to different forms of cadmium (CdO dust, fumes or salts of Cd), concomitant exposure to other metals or metalloids, and difference in control groups probably contributed to difference in the results. In many of the available epidemiological studies, accompanying exposure to arsenic, a known human carcinogen is a serious confounding factor.

Occupational exposure to cadmium fumes, dusts, or aerosols produces acute pulmonary irritation and chronic pulmonary disorders such as bronchitis and emphysema (Waalkes, *et al.*, 1992, Elinder, 1986). An apparent increased risk of lung or respiratory tumours in cadmium workers was observed in an initial report (Potts, 1965), which was confirmed by later studies (Lemen, *et al.*, 1976, Kjellstrom, *et al.*, 1979). However, in these assessments no corrections were made for confounding factors such as exposure to other metals and smoking habits.

Subsequent studies have attempted to discount the effect of confounding factors and put an emphasis on the duration and intensity of cadmium exposure. An increase in lung tumours was observed in workers exposed to a moderate or low level of cadmium (Sorahan, *et al.*, 1983, Armstrong, *et al.*, 1983). Armstrong and Kazantzis (Armstrong, *et al.*, 1985) observed an increase in pulmonary disease (bronchitis and emphysema) due to an increase in exposure to cadmium. Increased lung cancer mortality was also observed in workers exposed to cadmium at a non-ferrous smelter. However, Ades *et al.* reported that some factors other than cadmium exposure are involved in the mortality ratio of lung cancer (Ades, *et al.*, 1988). Other studies (Sorahan, *et al.*, 1983, Armstrong, *et al.*, 1985, Kazantzis, *et al.*, 1988, Sorahan, 1987) also indicated an increased lung cancer incidence in cadmium-exposed workers that was found to be related to the intensity and duration of exposure. A recent working group has pointed out that the evidence for cadmium causation of lung cancer in humans has increased (Carcinogenecity of cadmium, 1986).

The role of cadmium as human prostatic carcinogen has been examined comprehensively following the initial reports linking occupational cadmium exposure to prostatic cancer (Potts, 1965, Kipling, 1967). Subsequent studies of workers occupationally exposed to cadmium in smelting, battery manufacture, or refining industries have been hampered by the same limitations as those addressing lung tumours (exposure to different forms of cadmium, co-exposure to other metals and varying lifestyle factors). Furthermore, prostatic cancer is a very complex form of human cancer and the exact etiology of this disease is elusive. The association of a single causative agent with prostatic cancer may be extremely difficult to establish (Piscator, 1981). Following the initial reports relating prostatic cancer and occupational exposure (Potts, 1965, Kipling, 1967), several other studies indicated tentative positive correlation. Non significant increase in the incidence of cancer of the prostate were observed in several groups of cadmium workers (Lemen, *et al.*, 1976, Kjellstrom, *et al.*, 1979, Armstrong, 1985, Andersson, *et al.*, 1985).

Holden (Holden, 1980) found that workers in areas of high exposure to cadmium in a cadmium-copper alloy production department had no increase in prostatic cancer mortality, while " vicinity workers" showed a significantly increased rate.

Although occupational exposure to cadmium does not appear to greatly increase the risk for prostatic cancer, some evidence suggests that environmental exposure to cadmium may lead to increased risk. Cadmium contents were measured in water, soil, and food stocks in distinct geographical regions of Alberta, Canada and were compared with the incidence of prostatic cancer (Bako, *et al.*, 1982). Bako, *et al.* found that the environmental cadmium burden was greater in areas where prostate cancer rates were significantly higher than the regional means, while lower rates were associated with lower environmental burden (Bako, *et al.*, 1982). Recently, the combination of high dietary intake, occupation and smoking as sources of cadmium has been linked to an increased risk of prostatic cancer in humans, particularly with active tumours (Abd Elghany, *et al.*, 1990). Additionally, in a case control study in Utah of older men (68 to 74 years), high dietary intake of cadmium (> 61 μg Cd/day) was associated with increased risk of prostatic cancer as compared to categorically matched controls (West, *et al.*, 1991). Single reports have linked cadmium to cancers of kidney and stomach (Kazantzis, *et al.*, 1988, Kolonel, 1976).

Summary

Evidence for cadmium as a human carcinogen is based mainly on epidemiological studies relating to workers who are occupationally exposed to cadmium. Occupational exposure to cadmium fumes, dusts, or aerosols produces acute pulmonary irritation and chronic pulmonary disorders such as bronchitis and emphysema. An increased incidence of lung cancer was reported in cadmium-exposed workers.

1.11 Evidence from animal studies

Cadmium was established as a potent carcinogen in rodents in the early 1960s (Gunn, *et al.*, 1963, Gunn, *et al.*, 1964, Roe, *et al.*, 1964). Cadmium was first shown to form tumour at the site of intramuscular or subcutaneous injection, in which it produced sarcomas. Cadmium also proved to be very effective at producing a high incidence of testicular interstitial cell tumours in rodents. Earlier studies for the mechanisms of cadmium carcinogenesis of the testes revealed a remarkable ability of zinc to protect against necrotizing and carcinogenic effects of cadmium (Gunn, *et al.*, 1963, Gunn, *et al.*, 1964).

Recent work on cadmium has focused it to be a prostatic and pulmonary carcinogen in rodents. In the case of rats, the lung was indicated as a target site of cadmium-induced carcinogenesis. Rats chronically exposed to cadmium aerosols (50 $\mu g/m^3$ $CdCl_2$) had over a 70% incidence of lung carcinoma (Takenaka, *et al.*, 1983). Various other forms of cadmium, including the oxide, have now been tested positively as pulmonary carcinogens (Oberdorster, *et al.*, 1990, Oldiges, *et al.*, 1989, Glaser, *et al.*, 1990).

In the rat prostate, two studies (Waalkes, *et al.*, 1988, Waalkes, *et al.*, 1989) indicate that systemic exposure results in adenomas. It appears that cadmium produces prostatic tumours if testicular support for tumour formation is maintained. The tumours appear only when the doses of cadmium come down below the threshold for induction of testicular degeneration and dysfunction (< 5.0 µmol/ Kg, sc) or when such degeneration is prevented by zinc. The maintenance and function of the prostate are highly androgen-dependent, and the tissue atrophies with cadmium-induced testicular degeneration. Recent work also indicates that oral exposure to cadmium can induce preneoplastic and neoplastic lesions in the rat prostate (Waalkes, 1992). Direct injection of cadmium in the rat prostate has produced carcinomas, the histomorphology and ultrastructural pathology, which show similarity to human prostatic carcinomas (Hoffmann, *et al.*, 1985, Hoffmann, *et al.*, 1988). The finding of prostatic tumours in cadmium-treated rats likewise supports the possible role of cadmium in forming human prostatic neoplasia.

The testes are well-defined target site of cadmium-induced carcinogenesis in the rat and mouse and a single dose of cadmium (> 20 µmol/Kg, sc) can result in a high incidence (> 80%) of testicular interstitial cell tumours (Waalkes, *et al.*, 1988, Waalkes *et al.*, 1989, Gunn, *et al.*, 1963, Gunn, *et al.*, 1964, Roe, *et al.*, 1964, Lucis, *et al.*, 1972). It is believed that such tumours are formed as a result of the secondary effect of cadmium-induced testicular degeneration and dysfunction, and not due to any transforming effect of cadmium. However, recent work (Waalkes, *et al.*, 1992) shows that oral exposure to cadmium also can result in the formation of testicular tumours in the absence of obvious testicular degeneration, perhaps indicating direct effect of cadmium on the cells of testes. The findings of Bomhard *et al.* (Bomhard, *et al.*, 1987) indicate that oral or parenteral exposure to cadmium can induce malignant transformation of interstitial cells, as assessed by local invasiveness. The occurrence of interstitial cell tumours after cadmium treatment in mice (a species that typically shows a very low spontaneous incidence) could also indicate that the carcinogenic events are due to cadmium itself and not from an augmentation of spontaneously occurring lesions (Cherian, *et al.*, 1976). Rare tumours of the rat testes that have occurred with systemic cadmium exposure include seminomas, rete testes adenocarcinomas and mixed Sertoli-Leydig cell tumours (Rehm, *et al.*, 1988, Boorman, *et al.*, 1987). It was reported that cadmium when injected directly into fowl testes induced teratomas (Guthrie, 1964).

Cadmium can affect tumours of the hematopoietic system in rodents. In mice infected with murine lymphocytic leukemia virus, it was reported that cadmium given orally to the infected mice, increased incidence of leukemia by over 30% (Blakely, 1986). Cadmium-impaired immunosurveillance was thought to allow emergence of the leukemia virus. In Wistar-Furth rats, oral cadmium induced a dose-related sixfold increase of leukemia (Waalkes, *et al.*, 1992).
One study showed that early injections of cadmium had a marked synergistic effect on the later development of diethylnitrosamine-induced renal and hepatic tumours in rats (Wade, *et al.*, 1987). Cadmium was thought to enhance initiation of tumours by diethylnitrosamine (Wade, *et al.*, 1987). On the other hand, oral cadmium when given chronically can inhibit diethylnitrosamine-induced liver tumours in the mouse (Waalkes, *et al.*, 1991).

So far evidence suggests that cadmium is a potent carcinogen in experimental animals. Development of tumours in several tissues in rodent models under cadmium exposure indicates marked human relevance.

Summary

Cadmium is an established potent carcinogen in rodents. The testes are well-defined target site of cadmium-induced carcinogenesis in the rat and the mouse. In the case of rat, the lung was indicated as a target site of cadmium-induced carcinogenesis.

1.12 Dose-response relationships in cadmium carcinogenesis

One of the major problems in the area of metal carcinogenesis is the lack of adequate data concerning dose-response. Generally, most available data are either from high-level exposures in humans or single high dose studies in rodents. Excessive exposures, which may give rise to clear effects, are seldom seen today (Nordberg, 1988). On the other hand, chronic low-level metal exposures probably occur in both the work place and the general environment (Nordberg, 1988).

The lack of clear dose-response data makes the extrapolation to effects at chronic low-level exposures difficult, in turn making the assessment of risk problematic.

1.12.1 Human studies

Data from workers occupationally exposed to cadmium suggest that a dose-response relationship may exist with respect to lung cancer (Elinder, *et al.*, 1985). It is also found that a dose-response relationship exists between cadmium exposure and development of chronic respiratory diseases, particularly emphysema and bronchitis (Armstrong, *et al.*, 1983, Armstrong, *et al.*, 1985). Increased incidence of these respiratory ailments occurred when the intensity and duration of exposure to cadmium fumes was increased. A strong dose-response relationship has also been demonstrated between cumulative cadmium exposure and respiratory cancer by Thun *et al.*, (Thun, *et al.*, 1985). The authors grouped workers according to a time-weighted cumulative exposure to cadmium and determined standard mortality ratios (SMR) for lung cancer. With exposure levels < 40 $\mu g/m^3$, the

SMR was 100; with cadmium levels between 41 and 200 $\mu g/m^3$, the SMR was 152; and with levels > 200 $\mu g/m^3$, it was 280. In a similar manner, nickel-cadmium battery workers working in areas of high or moderate exposure to cadmium showed an increasing risk of mortality from lung cancers (Sorahan, 1987). The increased risk of lung cancer from this study was observed only in workers employed from 1923 to 1946, (highest exposure period) but not in workers employed after 1947 because the working conditions were improved after the period. Kazantzis *et al.* (Kazantzis, *et al.*, 1988) also observed similar results. Thus, it appears that lung cancer in humans occupationally exposed to cadmium is related to both the intensity and duration of exposure.

Dose-response relationships between cadmium and prostatic tumours may be more subtle than that observed with lung tumours. According to Piscator (Piscator, 1981), factors such as lifestyle, race, and diet play an influential role in the development of prostatic tumours. Limited data concerning dose-response relationships are available on the development of human prostatic cancer due to cadmium. Bako *et al.* (Bako, *et al.*, 1982) found that a high local environmental cadmium burden was associated with a high rate of prostatic cancer.

1.12.2 Experimental dose-related studies on animal models

Chronic inhalation of cadmium, specifically the chloride salt of cadmium was observed to induce dose-related increases in pulmonary carcinomas in rats (Takeneka, *et al.*, 1983). The effect of cadmium was clearly found to be dose related over the range: 0, 12.5, 25, and 50 $\mu g\ Cd/m^3$. The tumours were predominantly adenocarcinomas and occurred in up to 71% of the animals belonging to the highest exposure group. These findings have now been repeated in several studies using various cadmium compounds in rats (Oldiges, *et al.*, 1989, Glaser, *et al.*, 1990). The rat seems to be one of the most sensitive rodent species to the pulmonary carcinogenic effect of cadmium. In a study on chronic inhalation of several doses (0, 30, 90, 270, 1000 $\mu g\ Cd/m^3$) of various cadmium compounds ($CdCl_2$, CdO, CdS, $CdSO_4$), neither the mouse nor the hamster showed overt neoplastic changes in the lung following inhalation of cadmium fumes. However, a dose related increase appeared to occur in mice (Heinrich, *et al.*, 1989).

The carcinogenic effects of cadmium on the testes are believed to be due to rather remarkable acute toxic effects of cadmium in this tissue (Gunn, *et al.*, 1970), including severe hemorrhage necrosis and loss of seminiferous tubules followed by fibrosis (Gunn, *et al.*, 1970). With sufficient time after cadmium exposure, surviving interstitial cells may regenerate and tumours develop at a high rate of incidence (Gunn *et al.*, 1970). As regard to dose-response effects of cadmium, the final incidence of testicular tumours is found to be positively correlated to the extent of testicular degeneration (induced by cadmium), at least when the metal is given parenterally (Waalkes, *et al.*, 1988, Waalkes, *et al.*, 1989). In the testes, however, it appears that a threshold exists for parenteral cadmium-induced necrosis of the rodent testes, which is about 5 μmol Cd/kg body weight or less (Gunn, *et al.*, 1970, Gunn, *et al.*, 1964). At doses above this threshold, there is a dose-related increase in the incidence of cadmium-induced interstitial cell tumours, while tumours do not appear to be induced below this dose (Waalkes, *et al.*, 1990, Waalkes, *et al.*, 1988, Waalkes, *et al.*, 1991). When given orally, cadmium (at 0, 25, 50, 100, and 200 ppm in drinking water) produces an increase in interstitial cell tumours related to dose in the absence of testicular degeneration (Waalkes, *et al.*, 1992).

Recent evidence indicates that parenteral cadmium exposure of rats can result in tumours of the prostate (Waalkes, *et al.*, 1988, Waalkes, *et al.*, 1989). This is found to be highly dose-dependent and also related to effects of cadmium on other tissues, particularly the testes (Waalkes, *et al.*, 1988, Waalkes, *et al.*, 1989, Waalkes, *et al.*, 1992). A single-injection analysis of cadmium-induced carcinogenicity over two years in rats using a wide range of doses (1 to 40 μmol Cd/kg, sc), prostatic tumours were elevated (maximum 31%) over control levels (11%) only at doses below which a significant testicular toxicity occurred (approximately 5.0 μmol Cd/kg, sc) (Waalkes, *et al.*, 1988). At these lower doses (0, 1, and 2.5 μmol Cd/Kg, sc), there were dose-related increase in prostatic adenomas. Multiplicity of adenomatous foci had a similar dose relationship, being elevated only at doses of $\leq$ 2.5 μmol Cd/kg (Waalkes, *et al.*, 1988). Multiplicity preneoplastic (hyperplastic) foci were elevated with increasing dose throughout most of the range (0 to 20 μmol Cd/kg), indicating that at doses > 2.5 μmol Cd/kg, cadmium induced initiating events (Waalkes, *et al.*, 1988). Testicular function, specifically production of androgens, is essential for the growth and maintenance of the prostate (Coffey, *et al.*, 1981). Evidence indicates that prostate tumours are often testosterone-dependent, and in rodents, testosterone is a clear promoter in two-stage systems of prostatic cancer produced by organic compounds. Hence, the effects of cadmium on the testes may well be responsible for the lack of prostate tumours at doses that result in loss of testicular integrity and testosterone production (Waalkes, *et al.*, 1988). Thus, toxic effects in other tissues can dictate tumour formation in this case.

The results of a dose-response assessment for the inhibitory effect of zinc (0 to 3000 μmol/kg, sc) on the carcinogenicity of cadmium (30 μmol Cd/kg, sc) have shown that, when zinc is given in sufficient doses to prevent cadmium-induced chronic degeneration and tumour formation in the testes, prostatic tumours occur at an elevated incidence 30% compared to control 9.6% (Waalkes, *et al.*, 1989).

As stated earlier, cadmium is able to induce tumour formation at the site of injection (Waalkes, *et al.*, 1990, Waalkes, *et al.*, 1988, Waalkes, *et al.*, 1989, Waalkes, *et al.*, 1991, Gunn, *et al.*, 1963, Gunn, *et al.*, 1964, Poirier, *et al.*, 1983, Waalkes, *et al.*, 1991 Waalkes, *et al.*, 1990, Gunn, *et al.*, 1967, Haddow, *et al.*, 1964, Levy, *et al.*, 1973) when injected subcutaneously, intramuscularly and subperiosteally (Gunn, *et al.*, 1967). The tumours produced at these sites are typically sarcomas. Injection-site sarcomas induced by cadmium appear to be related strictly to total accumulated dosage at the site, and several studies have shown a dose-related increase in sarcoma formation with cadmium (Waalkes, *et al.*, 1988, Haddow, *et al.*, 1964, Levy, *et al.*, 1973). There are also indications that the injection-site sarcomas are dose dependent, with higher accumulated doses inducing a more rapid appearance whereas lower doses cause a latency of the tumour (Haddow, *et al.*, 1964). Although the tumours produced by cadmium at the site of subcutaneous and intramuscular injections are found to be malignant, recent evidence indicates that the level of malignancy can be modified by accumulated dosage (Waalkes, *et al.*, 1988). Repeated injections of cadmium result in more malignant sarcomas, as assessed by metastases to the lung and lymph nodes, comparing to those from a single cadmium injection, which are usually do not cause metastases (Waalkes, *et al.*, 1988).

In a study using Wister rats, lifetime oral exposure to cadmium in the diet (0, 25, 50, and 100 ppm) induced dose-related increases in the incidence of leukemia. This was true both for animals receiving diets deficient (7 ppm) or adequate in zinc (Waalkes, *et al.*, 1992). Hence, cadmium appears to have a possible role in the induction of tumours of the haematopoietic system.

Cadmium is found to have a complex effect on the occurrence of tumours of the pancreas in rats. The rat pancreas often will show a fair incidence of spontaneous tumours depending on strain. Cadmium, when given over a wide range of subcutaneous dosages (0, 1, 2.5, 5, 10, 20, and 40 μmol/kg, sc) has been shown to induce a dose related reduction of pancreatic tumours, (both acinar cell and islet cell) in Wistar rats (Waalkes, *et al.*, 1988). In contrast, cadmium, when given subcutaneously concurrently with calcium, induced an elevated incidence of islet cell tumours of the rat pancreas (Poirier, *et al.*, 1983). Thus, cadmium is found to have variable effects on the incidence of tumours in the rat pancreas depending on the conditions of exposure. Recent work indicates that multiple subcutaneous injections of cadmium in rats result in pancreatic cell trans-differentiation into haepatocytes (Konishi, *et al.*, 1990). The role of these metaplastic lesions in the induction or reduction of pancreatic neoplasia by cadmium is unknown (Konishi, *et al.*, 1990).

1.13 Factors modifying cadmium-induced carcinogenesis

Human model

As stated earlier, unlike well-controlled animal studies, humans are almost never exposed to a single toxicant. The resulting effect of multiple exposures on cadmium-induced carcinogenesis could potentially be reductive, additive, or synergistic. Such factors should be considered in evaluating the carcinogenic potential of cadmium. However, very little work has been done in this area. It is believed that simultaneous occupational exposures to cadmium along with other metals, such as lead or arsenic, may lead to interactions between such suspected carcinogens (Kazantzis, *et al.*, 1988). In one epidemiological study, it was found that a marked synergism occurred between cadmium and cigarette smoking for renal tumour formation (Kolonel, *et al.*, 1976). However, this is the only study linking cadmium to renal carcinomas in humans. An increase in leukocytic chromosomal abnormalities has been shown in workers exposed to cadmium in combination with lead or cadmium in combination with lead and zinc as opposed to cadmium alone (Nordberg, *et al.*, 1981). The exact mechanism of this enhancement is not known, but it may involve, at least in part, a greater disposition of cadmium (Waalkes, 1986). Zinc-deficient diets were also found to increase the formation of injection site sarcomas (Waalkes, *et al.*, 1991).

On the other hand, a reduction of metal-induced cancer can also occur during essential element deficiency. For example, a deficiency in dietary zinc has been found to reduce the effects of cadmium in the prostate (Waalkes, *et al.*, 1992). According to the authors, zinc deficiency is associated with prostatic atrophy, which may be due to a reduction in testicular function and loss of androgen support for prostatic tissue tumours (Waalkes, *et al.*, 1992).

Tolerance systems

The mechanism by which zinc prevents carcinogenesis due to cadmium remains unclear. Zinc is a very effective inducer of the metal-binding protein, metallothionein, in various rodent tissues including the liver and kidney (Waalkes, *et al.*, 1985, Onosaka, *et al.*, 1982). Cadmium has a higher binding affinity for metallothionein than zinc and hence it can displace zinc from the protein (Webb, 1979). It is believed that the binding with metallothionein is a basic mechanism of detoxification operating within a cell as applied to cadmium (Webb, 1979, Goering, *et al.*, 1984, Goering, *et al.*, 1984). Therefore, it is logical to assume that the preventive effect of zinc on cadmium-induced carcinogenesis may involve metallothionein. However, accumulating evidence from several laboratories indicates that target sites of cadmium-induced carcinogenesis may actually be deficient in metallothionein (Waalkes, *et al.*, 1991, Waalkes, *et al.*, 1992, Waalkes, *et al.*, 1984, Waalkes, *et al.*, 1984, Waalkes, *et al.*, 1986, Deagen, *et al.*, 1985, Waalkes, *et al.*, 1988, Waalkes, *et al.*, 1988, Waalkes, *et al.*, 1989) and/or the metallothionein gene is not sensitive to metal stimulation (Waalkes, *et al.*, 1991, Waalkes, *et al.*, 1992 Waalkes, *et al.*, 1988, Waalkes, *et al.*, 1989). This appears to be the case with the rodent testes, at least within the interstitium (De, *et al.*, 1991), and the rat ventral prostate (Waalkes, *et al.*, 1992), which are the sites of cadmium-induced tumours (Waalkes, *et al.*, 1988, Waalkes, *et al.*, 1989, Waalkes, *et al.*, 1992). Thus, the metallothionein system, although it clearly plays a role in tolerance to cadmium in several tissues, may not play a major role in the preventive effect of zinc on cadmium-induced carcinogenesis. Alternative mechanisms of tolerance, such as enhanced efflux of cadmium resulting from zinc pretreatment (Waalkes, *et al.*, 1992), may be involved.

Species and strain

It has been stated earlier that rats are most vulnerable to carcinogenicity due to cadmium. Mice and hamsters are found to be more resistant (Oberdorster, *et al.*, 1990, Takenaka, *et al.*, 1983, Heinrich, *et al.*, 1989, Oldiges, *et al.*, 1989, Glaser, 1990). Clear strain-dependent differences in incidence and latency of cadmium-induced injection site sarcomas in rats have been observed (Waalkes, *et al.*, 1991). Differences in distribution of cadmium had a little bearing on this observation, which as Waalkes points out possibly indicates a genetic basis for susceptibility. The acute toxic testicular effects of cadmium are highly dependent on strain (Waalkes, *et al.*, 1990, Waalkes, *et al.*, 1991 Gunn, *et al.*, 1963, Gunn, *et al.*, 1964, Gunn, *et al.*, 1970, Waalkes, *et al.*, 1988), and in some mouse strains the testes are totally refractory to cadmium. The potential for cadmium-induced carcinogenesis appears to be lower in such resistant strains (Waalkes, *et al.*, 1991) than in susceptible strains (Gunn, *et al.*, 1963, Gunn, *et al.*, 1964) but this has not been directly tested. Further work is warranted to determine the effect of strain or species on cadmium carcinogenesis. Once determined, this could provide more insight into the molecular mechanism of cadmium-induced carcinogenesis.

Synergism and antagonism

Although little work has been done in this area, there are a few specific examples of synergism in carcinogenic response applying to cadmium. For example, Harrison and Heath (Harrison, *et al.*, 1986) found that the combined exposure to cadmium with nitrosamine and asbestos increased lung tumour incidence in rats. Another study indicated that injections of cadmium within the first week after exposure to diethylnitrosamine had a marked synergistic effect on development of renal and haepatic tumours in rats (Wade, *et al.*, 1987). In contrast, chronic oral cadmium exposure starting 2 weeks after diethylnitrosamine injection markedly reduced the rate of development of liver tumour in the mouse (Waalkes, *et al.*, 1991). Clearly, the impact of such interactions can be quite dramatic but are as yet poorly understood.

1.14 Mechanistic implication of metal-induced carcinogenesis with special reference to cadmium

The evidence for cancer induction by occupational or iatrogenic exposure to compounds of metals such as arsenic, beryllium, chromium and nickel is convincing and well documented. These metal compounds are among several dozen environmental agents, including tobacco products. Asbestos and ionising solar radiation, for which there is sufficient evidence for human carcinogenecity (IARC, 1980, IARC, 1987, IARC, 1990). As stated earlier, cadmium is a known carcinogen in animals and is a category 1 carcinogen in humans. The mechanism of cancer induction by metallic carcinogens is not well understood. In most cases, it is not known whether the compounds of metals act as tumour initiators or tumour promoters, or both. Some metal compounds such as those of chromium (Wolf, *et al.*, 1989), nickel (Ciccarelli, *et al.*, 1985) and cadmium (Koizumi, *et al.*, 1990) can interact directly with nucleic acids and promote genotoxic damage or mutagenesis. Some other compounds of metals may also alter gene expression (Chin, *et al.*, 1990, Cosma, *et al.*, 1991, Hamilton, *et al.*, 1989). They may act alone or in synergy with other agents. Compounds of nickel (Schubert, *et al.*, 1987, Smialowicz, *et al.*, 1987) and chromium (Kumar *et al.*, 1991) together stimulate cellular immune responses, while nickel and cadmium together can promote the release of reactive oxygen species (Zhong, *et al.*, 1990).

It can be said that experimental evidence for metal-induced carcinogenesis is abundant but often conflicting. Carcinogenic metal ions such as cadmium, arsenic, chromium and nickel vary widely their chemical properties so that metal-induced carcinogenesis is likely to be complex and that for some metals more than one mechanism may operate. For example, cadmium ions are likely to bind covalently with DNA whereas chromium ions are likely to cause oxidative damage to DNA. Antioxidants such as ascorbate, cysteine and glutathione, which normally protect DNA and other biomolecules from oxidative damage, may directly or indirectly be involved in metal-induced carcinogenesis. In the present study, it has been found that in presence of cadmium there is a pronounced damage to DNA due to antioxidants.

There are four overlapping stages of carcinogenesis namely initiation, promotion, progression and metastasis suggested. Metal ions may be involved at each and all stages of the carcinogenic process.

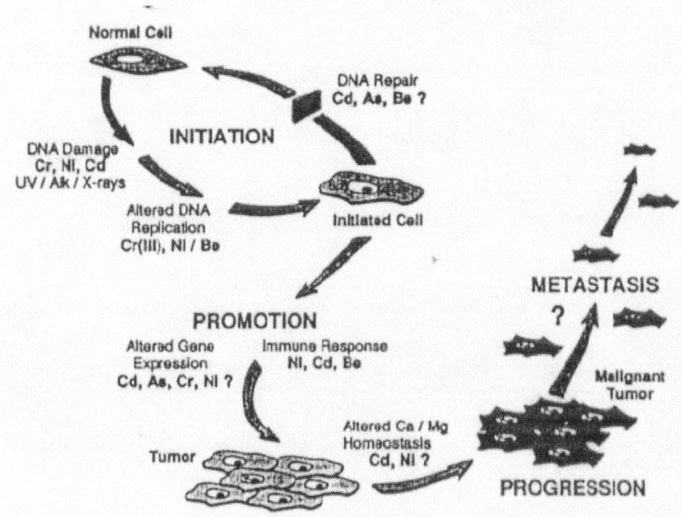


FIGURE 1.1: Shows the role of metals in carcinogenesis: Carcinogenesis comprises a multistage process involving initiation, promotion, progression and metastasis. Compounds of metal can be involved at many different stages of carcinogenesis and each type of metal is likely to be involved at more than one step and also via different mechanisms (Snow, 1992).

Initiation

In this process, chemical carcinogens including metal ions need be modified to a reactive electrophilic forms either by metabolic process or any other chemical means so that these could attack any nucleophilic sites in molecules such as proteins and nucleic acids to form covalent adducts. Since initiation is a non-enzymatic process so it is likely that the reaction is nonspecific. Because of this, electrophilic forms of chemical carcinogens can react at numerous sites within the cell to produce mutagenic damage in the cell (Tannock, *et al.*, 1998).

Metallic carcinogenic compounds have been shown to produce both bacterial and mammalian cell mutagenesis *in vitro*. Chromate salts, in particular, tend to give positive responses in most short-term genotoxicity assays (De Flora, *et al.*, 1989).

Unlike many organic carcinogens, metal compounds do not produce known types of direct mutagenic DNA damage or adducts. They can however produce general oxidative DNA damage (Rodriguez, *et al.*, 1991, Standeven, *et al.*, 1991a) or DNA crosslinks, which may occur directly or as a by-product of metal metabolism within a cell (Wedrychowski, *et al.*, 1985). Oxidative DNA damage induced by redox cycling of specific metal ions in the presence of H_2O_2 has been shown to be mutagenic in bacterial cells (Mc Bride, *et al.*, 1991, Tkeshelashvili, *et al.*, 1991). However, the actual mutagenic lesions produced by these processes are not yet known. It is also unknown whether carcinogenic metals that involve redox cycling *in vitro* (eg chromium, nickel) can induce production of active oxygen species by phagocytic cells *in vivo* (eg nickel) can also produce mutagenic DNA damage in target cells *in vivo* (Zhong, *et al.*, 1990).

Promotion

Promotion may be defined as the process in which initiated cells develop into tumour cells under the stimulation of an agent that itself may be incapable of inducing neoplastic transformation. Initiation is a rapid and irreversible process whereas promotion usually takes place over a prolonged period of time and is reversible up to the development of the first autonomous tumour cell. In contrast to initiators, most of the promoters do not form electrophilic species.

Metal salts can promote altered gene expression (Flamigini, *et al.*, 1989) or modify intra- or inter-cellular communication (Mikalsen, 1990). These and other alterations in signal transduction are generally considered to be related to tumour promotion rather than initiation. Tumour promotion is also associated with the production of oxidation bursts by phagocytic cells and cellular recruitment. Phagocytosis of particulate metal compounds has been shown to promote these processes *in vitro* (Zhong, *et al.*, 1990) and *in vivo* (Knight, *et al.*, 1991), Sunderman, *et al.*, 1989, Wiernik, *et al.*, 1983).

Progression

Tumour progression is a cumulative process, which can be studied independently of promotion by examining the characteristics of a developing tumour and the cells within it. Tumour promotion can be believed of as a series of events that lead to tumour progression. Progression is associated with altered tumour cell metabolism, the acquisition of additional phenotypic characteristics and multiple genotypic changes (Kaden, *et al.*, 1989, Lehman, *et al.*, 1991, Loeb, 1991). As the tumour progresses, the cells within the tumour may acquire the ability to produce increased amounts of active oxygen species, in turn, may induce additional somatic mutations within both the oxidative metabolism of the cells and by changes in immune status of the host. Carcinogenic metals such as nickel (Judde, *et al.*, 1987, Smialowicz, *et al.*, 1987) and chromium (Kumar, *et al.*, 1991) can interfere with the redox status of mammalian cells in vitro and have strong effects on the cellular immune system (De flora, *et al.*, 1989, Rodriguez, *et al.*, 1991, Shainkink-estenbaum, *et al.*, 1991, Standeven, *et al.*, 1991a). Having already achieved an altered metabolic profile, those tumour cells with increased growth potential, a more rapid growth rate, or any other advantage, will undergo selection such that the characteristics of the cells within the tumour undergo gradual change.

Metastasis

The ability of the cancer cells to disseminate and form new foci of growth at non-contiguous sites is known as metastasis or more generally the final stage of malignant tumourigenesis is metastasis. This occurs when the cells within the tumour mass acquire the ability to travel and colonise at sites distant from the initial cancer.

One potential role for exogenous metals in this process may be related to alterations of calcium regulation. Calcium plays an essential role in intra and inter cellular communication and interference with calcium binding or regulation. It may be pivotal in both tumour progression and metastatic potential (Whitfield, 1990).

Transformation *in vitro*

Morphological transformation *in vitro* is an experimental endpoint, which has been associated with changes in oncogene activation and alterations in signal transduction (Landolph, 1990). All known carcinogenic metals have been shown to induce morphological transformations of cells in culture in one or more assay system. (Biedermann, *et al.*, 1987, Dipaolo, *et al.*, 1979, Landolph, 1990). Even metal salts, which have not been shown to be mutagenic to mammalian cells, in vitro, such as chromium(III) chloride (Biedermann, *et al.*, 1987) and sodium arsenite have been positive in cell transformation assays. It is likely that cellular transformation or anchorage independence may occur by mechanisms such as alterations in gene expression or signal transduction that are independent of the gene mutation and inactivation process which are typically assayed in mammalian cell mutagenesis protocols. It is intriguing that different valence forms of the same metal, such as chromium(VI) and chromium(III) may be differentially active in one or both of these assays. These findings suggest that uptake and metabolic processing of certain metal salts may give rise to different genotoxic endpoints and that cell transformation (a more general endpoint) can occur by different process than mammalian cell mutagenesis. Cell Transformation *in vitro* displays characteristics of both carcinogenic initiation and promotion.

Summary

There are four overlapping stages of carcinogenesis namely initiation, promotion, progression and metastasis suggested. It is believed that cadmium may be involved more than one of these stages.

1.15 Genotoxicity and mutagenesis of cadmium

As stated earlier, a number of metals have been shown to be genotoxic, and cadmium is no exception. A variety of mechanisms exist by which metals can exert their genotoxic potential which are summarised in the following table.

TABLE 1.1

Genotoxicity associated with several metals of known carcinogenic potential (Waalkes, *et al.*, 1992).

Metal	Type of damage to the DNA molecule	Repair inhibition
Arsenic	**SS PC**	**Yes**
Beryllium	**--------**	**Yes**
Cadmium	**SS PC**	**Yes**
Chromium	**SS DS PC DC**	**Yes**
Lead	**PC**	**Yes**
Nickel	**SS PC DC**	**Yes**

Note: SS = single-strand DNA damage; DS = double-strand DNA damage; PC = DNA-protein crosslinks; DC = DNA-DNA crosslinks.

Genotoxic effect induced by metals mainly consists of single -and/or double-strand DNA scission, DNA-DNA crosslinking and/or base modification. In some situations, DNA may even be totally damaged by metal ions. Table 1.1 summarises the various ways by which carcinogenic metal ions are believed to induce genotoxicity. DNA-strand scission may be the effect of a direct interaction of metal ion on the phosphate backbone itself or may be due to the removal of modified or damaged bases by the repair process. As listed below, a number of other processes involving DNA may also be affected by metals and these may ultimately result in alteration in cellular function.

1. Changes in DNA conformation
2. Binding of metal ions to regulatory proteins
3. Changes in fidelity in DNA replication
4. Alterations in DNA synthesis
5. Modification of DNA repair mechanism

As Waalkes *et al.* (Waalkes, *et al.*, 1992) point out, the ultimate goal in determining genotoxicity is to measure effects at the DNA level that are meaningful at the whole cell level. The resultant DNA damage must not be lethal to the cell, whereby it should result in a modification that will have an impact on the cell (as for example, genotypic changes result in phenotypic changes). Cadmium(II) has been found to induce cytotoxicity, chromosomal aberrations and mutagenicity in mammalian cells (Ochi, *et al.*, 1983, Ochi, *et al.*, 1984, Howard, *et al.*, 1991). Cadmium-induced genotoxicity and carcinogenicity are rather complex because Cd^{2+} interacts with both DNA and proteins. Moreover, it has been reported that cadmium compounds can inhibit DNA replication by decreasing the fidelity of DNA polymerases (Zakour, *et al.*, 1984). Cadmium(II) ions can also inhibit DNA repair pathways such as O^6-alkyl guanine-DNA alkyltransferase and nucleotide-excision repair (Hartwig, 1994). Hartwig and Hartman also showed that cadmium ion interfered with the repair of DNA that was damaged by UV irradiation (Hartwig, 1995). Yang *et al.* reported that cadmium compounds disturbed DNA-protein interactions that are essential for the initiation of nucleotide excision repair, most likely by the displacement of essential metal ions (Yang, *et al.*, 1997). Cadmium has been shown to bind to DNA *in vitro* and can produce protein associated alkali-labile lesions in the DNA of cultured cells (Kasprzak, 1995, Maki, *et al.*, 1992, Hayakawa, *et al.*, 1995). Cadmium has been shown to cause lipid peroxidation in rat haepatocytes *in vitro* (Coogan, *et al.*, 1992), promoting an oxidative burst by pulmonary alveolar macrophages *in culture* (Ochi, *et al.*, 1983). Cadmium can also substitute for zinc in the zinc-finger motif of transcription factors (Predki, *et al.*, 1994, Bialkowski, *et al.*, 1999), alters calcium homeostasis and interferes with the expression of certain functional genes and signal transduction pathways (Beyersmann, *et al.*, 1997). Thus it is clear that cadmium plays either direct and/or indirect roles in genotoxicity and as stated earlier, metallothionein is the most important metal-binding protein that resists cadmium genotoxicity (Masters, *et al.*, 1994, Lazzo, *et al.*, 1995).

Cadmium was shown to induce DNA damage in cultured cells, and according to Waalkes, *et al.* this damage was caused by free radicals (Ochi, *et al.*, 1983, Ochi, *et al.*, 1983, Ochi, *et al.*, 1988, Burkart, *et al.*, 1986, Snyder, 1986, Biggart, *et al.*, 1988). However, the authors did not explain how free radicals were produced. Some workers also reported that cadmium chloride induced single-strand DNA damage in cultured Chinese hamster V-79 cells under aerobic condition (Ochi, *et al.*, 1983). When the experiments were repeated under anaerobic conditions, no damage to DNA was observed (Waalkes, *et al.*, 1992). From the above results we may conclude that oxygen may be playing a key role in the DNA damage induced by cadmium.

Intracellular reactive oxygen species (ROS) eg H_2O_2, superoxide anion, and hydroxyl radical are produced by cellular redox cycling systems that are important mediators of the cytotoxicity induced by many metals and other substances (Imlay, *et al.*, 1988, Sinha, *et al.*, 1990). Oxygen radicals can induce genotoxicity that include damage to DNA and gene mutations in cultured cells (Halliwell, *et al.*, 1991). Calvero *et al.* proposed the involvement of reactive oxygen species in the genotoxicity cadmium. They examined the effect of Cd^{2+} on the production of superoxide anion and DNA strand breaks in J744A. 1

cell culture and observed that an increase in DNA single strand breaks was caused as the concentration of Cd^{2+} was increased (Calvero, *et al.*, 1998).

In determining genotoxicity induced by metals or by any other substances the role of ROS scavengers needs to be taken into account. It has been observed that DNA strand breakage and chromosomal aberrations induced by cadmium can be suppressed by ROS scavengers. The suppression caused by ROS scavenger implies that ROS may mediate genotoxicity due to cadmium. In addition, other ROS scavengers (eg superoxide dismutase) were also found to reduce greatly the genotoxicity associated with cadmium exposure. Further studies also provided evidence of a prooxidant state induced by cadmium (Ochi, *et al.*, 1983). In this series of studies, it was also reported that the diffusible radical scavenger, butylated hydroxytolune (BHT) suppressed cadmium-induced inhibition of cell growth, thymidine incorporation and cadmium-induced clastogenesis. On the other hand, mannitol, a hydroxyl radical scavenger, was found to suppress cadmium-induced inhibition of cell growth and thymidine incorporation only (Waalkes, *et al.*, 1992). It was found that cadmium treatment had no effect on superoxide dismutase, catalase, glutathione peroxidase, or reductase levels although the glutathione levels were found to be reduced within the cells by about 40% after treatment. In studies where human diploid fibroblasts were treated with cadmium chloride, it was observed that hydroxyl radical scavengers, mannitol and potassium iodide decreased cadmium-induced DNA strand breakage (Waalkes, *et al.*, 1992). Snyder (Snyder, *et al.*, 1986) reported that superoxide dismutase did not provide any protection against cadmium-induced DNA damage but the addition of catalase was found to reduce such DNA damage. A study of Yang *et al.* (Yang, *et al*, 1996) showed that the colony-forming ability of Chinese Hamster Ovary (CHO) K-1 cells had been markedly decreased when the cells were exposed to cadmium acetate. Cadmium also induced mutation frequency in the hypoxanthine (guanine) phosphoribosyltransferase (*hprt)* gene. The mutation frequency of cadmium-treated cells was found to be approximately 20 times that of untreated cells. In the same study the author established the role of D-Mannitol, (a ROS scavenger) to protect cells against Cd(II)-induced cytotoxicity and mutagenicity. In a mutation assay study, Biggart and Murphy demonstrated that cadmium was able to induce frameshift mutations. They explained that these were caused by reactive radical intermediates produced by cadmium (Biggart, *et al.*, 1988). Investigations of Biggart and Murphy are especially important for cadmium-induced mutagenesis, because mutation assays for metal compounds are found to be negative except for compounds of chromium (Waalkes, *et al.*, 1992). Apart from providing evidence for the involvement of free radicals in cadmium-induced genotoxicity, the studies further illustrated the idea that metal-induced gentotoxicity is likely to be complex and that more than one mechanism may operate.

Another often used endpoint method to determine carcinogenic potential is to examine a metal's ability to induce cell transformation. In many ways, this endpoint method is found to be meaningful to demonstrate effects that are not lethal to the cell, and one can easily identify important cellular changes leading to transformation of the cell. Terracio and Nachtigal (Terracio, *et al.*, 1986) demonstrated that cadmium compounds are capable of transforming rat ventral prostate epithelial cells following *in vitro* exposure. The findings have toxicological relevance since the rat ventral prostate is a target site of cadmium-induced carcinogenesis (Waalkes, *et al.*, 1988, Waalkes, *et al.*, 1989, Waalkes, *et al.*, 1992). The tumourigenicity of the cells was demonstrated following injection of cadmium salts into newborn rats (Terracio, *et al.*, 1988). The two epithelial cell lines were tested after injection, which showed highly differentiated squamous cell carcinomas. In one of the cell lines tested, 20% of the animals developed lung and splenic metastasis. In addition, a fibroblastoid rat ventral prostate cell line was also found to be tumourigenic (yielding sarcomas) after treatment with cadmium. This tumourigenesis is due to cellular transformation. Although there would appear to be a lack of specificity for the transforming effect of cadmium, squamous metaplasia was observed during *in vitro* growth of the epithelial cells that were treated with cadmium compounds.

Investigation of genotoxicity *in vitro* can provide much valuable information, especially from a mechanistic standpoint. However, this only can provide an indication of what may occur *in vivo*. Numerous factors such as concentration, volume, and time of exposure need to be considered in *in vitro* studies. The choice of target cell or cell line is also a factor, because it can modulate genotoxicity produced by metal ions. Many cells differ in their intracellular levels of cytoprotective compounds, especially metallothionein, which (as stated earlier) is mostly related to cytotoxicity produced by cadmium. Perhaps the most important information lacking for cadmium genotoxicity is the demonstration of DNA damage that follows after *in vivo* exposure of cadmium. Realising the limitations of *in vitro* exposures, questions may arise about the role of different factors in causing genotoxicity produced by metal ions. One such question involves the role of metallothionein in cadmium-induced genotoxicity. Muller and co-workers (Muller, *et al.*, 1991) recently demonstrated that cadmium, when bound to metallothionein was able to induce DNA strand breaks in isolated supercoiled plasmid DNA. According to the authors, damage to plasmid DNA was caused by free radicals produced by cadmium-bound metallothionein complex. However, in another study it was observed that induction of metallothionein by pretreatment of cells with either zinc or low dose of cadmium resulted in decreases in cadmium-induced DNA damage (Waalkes, *et al.*, 1992). Although other effects can be associated with this pretreatment, the data suggest that metallothionein gene expression provides protection against cadmium-induced genotoxicity. If the binding of cadmium to metallothionein resulted into the formation of free radicals in a whole cell system, (as shown from the studies by Muller *et al.*) (Muller, *et al.*, 1991), then it is logical to assume that the binding of cadmium to metallothionein within the cytosol would also produce free radicals. If this is the case, there would be no protection for cadmium-induced toxicity. Although cadmium bound to metallothionein (cadmium-thionein) has been reported to cause DNA strand breaks *in vitro* (Muller, *et al.*, 1991), increased production of metallothionein *in vivo* was found to be protective against cadmium toxicity (Coogan, *et al.*, 1992) despite its increased cellular intake.

Waalkes *et al.* points out that a number of questions remain unanswered regarding cadmium genotoxicity. It was stated earlier that due to the similar chemistry of cadmium and zinc one may expect that cadmium replaces zinc in zinc-containing enzymes. A number of important enzymes involving DNA have been shown to require zinc for their activity (Wu, *et al.*, 1987). Replacement by cadmium could easily modify enzyme activity, as has been shown for other zinc-containing enzymes. Although ample evidence exists demonstrating that zinc provides protection against cellular toxicity, little information is available on the effect of zinc on cadmium-induced genotoxicity. There is strong evidence in support of the contention that expression of metallothionein genes plays an important role in the cellular defense against toxicity due to cadmium.

It was hypothesised by Sunderman *et al.* that the introduction of cadmium into zinc finger domains not only could alter the three dimensional structure of finger-loop domains, (altering their specificity or affinity for DNA binding sites) but also this could lead to the generation of reactive oxygen species (Sunderman, *et al.*, 1988). However, the authors did not explain how this could be possible. If it is true, it gives credence to the free radical mechanism of genotoxicity due to cadmium.

Summary

Metal-induced genotoxicity may be due to a number of causes. Cadmium-induced genotoxicity is believed to be associated with single strand DNA damage and DNA-protein cross-links. There is strong evidence in support of the contention that expression of metallothionein genes plays an important role in the cellular defense against toxicity due to cadmium.

1.16 Possible mechanisms of cadmium-induced carcinogenesis

As stated by Waalkes and Misra cadmium can produce genotoxic and mutagenic events in the cell, and these generally require high doses of exposure (Waalkes, 1995, Waalkes, 1996, Misra, 1998). Since cadmium is not a redox active metal, it cannot undergo redox cycling producing free radicals. However, it may activate other biomolecules eg when bound to cadmium, they may be more susceptible to oxidation – a process in which free radicals may be generated. Non-genotoxic epigenetic or indirect genotoxic mechanisms may also be applicable to cadmium-induced carcinogenesis. Such mechanisms may include altered gene expression, which may result in stimulation of cell proliferation or blockage of apoptosis. According to Hartwig, cadmium can inhibit repair of DNA (Hartwig, 1998) and this could be an indirect source of mutational events. Another example of mutational events was provided by the study of Bialkowski, *et al.* relating to enzyme 8-oxo-dGTPase which protect DNA from misincorporation of 8-oxo-dGTP, (an oxidative product of dGTP) that causes AT→ CG mutation in DNA. They found that cadmium(II) strongly inhibited the enzyme system causing accumulation of 8-oxo-dGTP in the cell, which in turn caused mutation (Bialkowski, *et al.*, 1998). Thus, inhibition of DNA repair system is believed to be an alternative way by which cadmium manifests itself as a carcinogen. Beyersman reported that perturbation of DNA repair mechanism together with upregulation of mitogenic signaling, and the resulting indirect genotoxicity by cadmium could be key events in carcinogenesis induced by cadmium (Beyersman, *et al.*, 1997).

Activation and modulation of DNA transcription factor also may be involved in carcinogenic process. Cadmium can clearly modulate transcription factors that normally require zinc, such as MT gene (Goering, *et al.*, 1994, Klaassen, 1999). Abshire and Zheng demonstrated that cadmium compounds can activate some proto-oncogenes or genes associated with cell proliferation, such as c-*myc* or c-*jun*, in cells and in animals (Abshire, 1996, Zheng, 1996). It is possible that the activation process induced by cadmium generating oncogene may enhance proliferation in a cell population. Continuous cell proliferation could result in an expansion of damaged cell population. Hartwig further reported that the suppression of DNA repair system by cadmium (Hartwig, I998) would potentially add to the population of cells having damaged DNA. Apoptotic cell death is an ongoing normal event in the control of cell populations and will cause elimination of cells with damaged genetic material. Shimada reported that chemically induced apoptosis can be very effectively disrupted by cadmium (Shimada, 1998).

Thus for cadmium, disorders of cell accumulation, including enhanced proliferation and disrupted apopotosis may be crucial events in carcinogenesis induced by cadmium. Our working hypothesis after reviewing the existing literature is that cadmium-induced carcinogenicity is likely to be highly complex and that multiple mechanisms including indirect oxidative DNA damage, impairment of repair process due to the formation of DNA-protein and DNA-aminoacid crosslinks and selective damage in the DNA molecule due its direct interaction with cadmium ions may operate. As applied to the nature of interaction between Cd^{2+} and DNA and its constituents, a number of questions that remained partly or totally unanswered were:

(1) Does Cd^{2+} cause any damage to nucleobases and other parts of DNA? If it does, is it likely to be relevant in cadmium-induced cancinogenesis?

(2) Does Cd^{2+} bind to nucleobases and nucleotides in solution forming adducts other than the commonly assumed 1:1 adducts? Although, this information as such may not appear to be relevant to cadmium-induced carcinogenesis, it is considered to be an important information in understanding the nature of interaction between Cd^{2+} ion and DNA (eg the formation of intrastrand or interstrand bifunctional adducts).

(3) What is the exact effect of the presence of Cd^{2+} ions on the oxidative DNA damage or how does Cd^{2+} influence oxidative damage to DNA in presence of antioxidants? The answer to the question is considered to be highly significant in the understanding of DNA damage in presence of Cd^{2+}.

The general aim of the present research was to investigate the mechanism of cadmium-induced carcinogenesis. The specific objectives of the research were to investigate the nature of interaction of Cd^{2+} with DNA and its constituents using a number of chemical and biochemical techniques including UV-visible spectrophotometry, high performance liquid chromatography (HPLC), submarine gel electrophoresis, PCR, DNA sequencing and molecular modeling. Another objective of the project was to quantify the DNA damage due to antioxidants in presence of cadmium ions. This objective was formulated as the project progressed.

As stated earlier, cadmium is a soft metal that binds preferentially to sulfhydryl groups in proteins and to DNA bases more than DNA phosphates (Jacobson, *et al.*, 1980) although there is evidence for the binding of Cd^{2+} to phosphate in nucleotides as well.

When cadmium binds covalently with nucleobases in DNA there may result perturbation in the electron density in the heterocyclic ring as a result of which even the phosphodiester bonds may be weakened (Martin, 1996). Consequently the DNA may be damaged.

Although the interaction between Cd^{2+} and nucleobases, nucleosides and nucleotides has been studied by using UV-difference spectroscopy, there is still uncertainty in the exact nature of binding. For example, Cd^{2+} can bind to more than one nitrogen centre in nucleobases and can form adducts with different stoichiometries. However, only 1:1 adducts were considered to form in solution. For example, in interpreting UV-difference spectral results, Sorokin *et al.* (Sorokin, *et al.*, 1997) assumed the formation of only 1:1 adduct between nucleotides and metal ions. Also, in considering isomeric equilibria that exist in solution for complexes between metal ions and nucleotides, the formation of 1:1 adduct was only considered (Sigel, *et al.*, 1996). Likewise, in considering the binding of metal ions to N1 and N7 sites in purines, Martin only considered the formation of 1:1 adducts. It should however be mentioned that there is some evidence in the literature for the existence of 1:1 and 1:2 with adenine and also with cytosine (Munno, *et al.*, 1993) applying to the structures of crystals.

The results of UV difference spectra, changes in HPLC retention times and quantitation of HPLC fractions suggest that Cd^{2+} binds covalently with N7 centres of the nucleobases. In view of above, we also undertook to investigate the following: (1) to determine whether Cd^{2+} forms adducts other than commonly observed 1:1 adduct with nucleobases and nucleotides, (2) to investigate whether covalent binding between Cd^{2+} and nucleobases and nucleotides can introduce any chemical modification in the latter. We also carried out molecular modeling of the expected adducts to investigate the formation of any intramolecular H bonds due to interaction with Cd^{2+}. We further investigated the interaction between Cd^{2+} and salmon sperm DNA (ssDNA) by UV-visible difference spectrophotometry and HPLC. Retention time for incubated mixture of Cd^{2+} and adenine was changed but not guanine. Similar type of results was also observed with nucleotide AMP and 2'-dGMP.

Cadmium-plasmid (pBR322) DNA mixtures at different concentrations of Cd^{2+} were electrophoresed after incubation under conditions to identify qualitatively damage or change in the structure of DNA due to the binding of Cd^{2+} to plasmid DNA. Cd^{2+} was found to cause total damage to DNA at the pH of the reaction medium (5.70). However, at pH 7.4 no damage to DNA was occurred although the band mobility decreased as the concentration of Cd^{2+} increased. Restriction enzyme (hydrolysing guanine sites or adenine sites of DNA strand) digestion works were also carried out with Bam H1 (cuts GG sites) and Ssp1 (cuts AT sites) restriction enzymes to understand whether these enzymes are able to cut GG sites or AT sites of DNA strand binding with Cd^{2+} ions.

Further we aimed to investigate any changes in the DNA damage due to its interaction with Cd^{2+} ions in the presence of cysteine, glutathione or ascorbate.

Cysteine, glutathione and ascorbate are naturally occurring cellular antioxidants involved in metal detoxification and have affinity to bind soft metal ions such as Cd^{2+} A natural question arises whether the antioxidants prevent DNA damage due to cadmium or not. Thus, plasmid DNA was allowed to interact with cadmium salts in presence of antioxidants cysteine, glutathione and ascorbate under different conditions and the results observed were found to be different from what were expected. It was found that Cd^{2+} plays a catalytic role in the oxidation of antioxidants (especially cysteine and ascorbate), producing free reactive oxygen species, which in turn damage DNA.

To investigate whether Cd^{2+} binds directly with these antioxidants prior to interaction with DNA, we also carried out HPLC studies with Cd^{2+}-cysteine, and Cd^{2+}-glutathione adducts, and UV-spectral study for Cd^{2+}-ascorbate adducts. Change in retention time for Cd^{2+}-cysteine, and Cd^{2+}-glutathione adducts as compared to that of cysteine and glutathione indicated the binding between cadmium and cysteine or glutathione. UV-difference spectral studies also indicated 1:1 binding between cadmium and ascorbate.

To understand whether Cd^{2+}-cysteine, or Cd^{2+}-glutathione adducts bind with nucleobases or not, a series of HPLC experiments was undertaken on the interaction of Cd^{2+}-cysteine or Cd^{2+}-glutathione adducts with adenine and guanine. Changes in retention time indicated the binding between Cd^{2+}-cysteine and Cd^{2+}-glutathione adducts and nucleobases.

To compare the effect of binding between Cd^{2+} and different DNAs we carried out a series of electrophoretic binding assays in which salmon sperm DNA and calf thymus DNA were used.

Summary

Although cadmium is a known carcinogen in both animals and humans, the molecular mechanism of cadmium-induced carcinogenicity remains largely unclear. A number of authors suggested cadmium-induced DNA damage may be due to a Fenton type reaction in which cadmium undergoes redox cycling. Since cadmium is not redox-active metal, it is difficult to see how cadmium ion itself can undergo redox cycling similar to that experienced by ions of iron or chromium. Cadmium being a soft metal, cadmium ions are believed to bind strongly with nucleobases in DNA. Whether such binding causes any chemical modification in nucleobases as a result of electronic perturbation has not been established. It is commonly assumed that divalent metal ions including Cd^{2+} form 1:1 adducts with nucleobases and nucleotides in solution in water. However, no study has been carried out to establish the stoichiometry of different adducts formed between Cd^{2+} and nucleobases and nucleotides in solution in water.

It is well-known that antioxidants that normally protect DNA from oxidative damage by acting as scavengers of free radicals, can also promote DNA damage by producing free radicals. As stated earlier, cadmium is not a redox active metal, hence it cannot directly oxidize antioxidants such as ascorbate. A question that arises is that if there is a pronounced DNA damage in the presence of cadmium ions together with antioxidants, how can that be explained?

Hence the aims of the project were:

(1) To quantify the nature of interaction of cadmium ions with nucleobases and nucleotides in terms of adducts formed and any changes introduced in nucleobases as a result of binding.

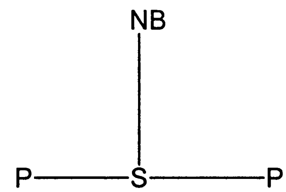
(2) To investigate the nature of binding of cadmium ions with DNA.

(3) To determine DNA damage due to cadmium ions both in the absence and presence of antioxidants.

CHAPTER 2

STRUCTURE OF DNA

The word DNA stands for deoxyribonucleic acid. It is made of a number of deoxy ribonucleotide units which in turn made up of three basic components: a nucleobase (NB), 2'-deoxy ribose, a member of the pentose sugar family (S), and one or more phosphate units joined together as illustrated below

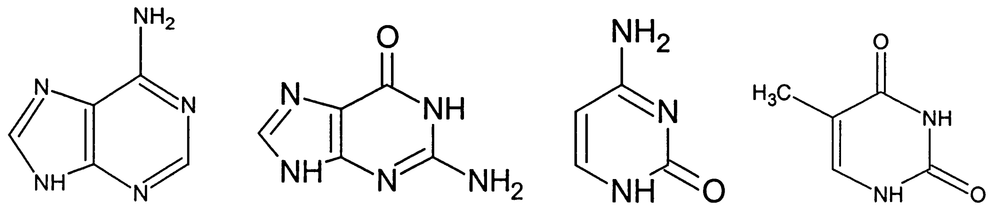


Representation of a nucleotide

FIGURE 2.1: Represents a nucleotide

The prefix 2'-deoxy denotes the ribose lacking the oxygen atom in second carbon.

Four different heterocyclic amines known as nucleobases are present in DNA. They are adenine (A) and guanine (G), which are bicyclic purines, cytosine (C) and thymine (T), which are monocyclic pyrimidines. The invariant part in the structure of DNA is the sugar-phosphate backbone and the variant part is the sequence of nucleobases.



Adenine Guanine Cytosine Thymine

FIGURE 2.2: Adenine, Guanine, Cytosine and Thymine

In the case of deoxyribonucleotide, the C1 of the ribose is linked to N1 of a pyrimidine base or N9 of a purine base. The primed number denotes an atom on the sugar whereas the unprimed one denotes an atom of the purine or pyrimidine base.

The repeating units of sugar and phosphate group are joined in such a way that each phosphate is joined to 3' carbon of one deoxyribose unit and 5' carbon of another sugar. This conformation enables the structural stability of DNA. The base is compared to the side chain of protein in activity and carries all the necessary information that need to synthesise a protein molecule.

One end of a DNA chain has 5'-OH group and the opposite one has 3'-OH group, which are not linked to any other nucleotide thus retaining the polarity of the whole molecule. The base sequence is indicated as 5'→3'

2.1 Shapes of nucleotides

Nucleotides have a compact structure in which there are several interactions taking place between non-bonded atoms. The molecular geometry of the nucleotides is so closely related that some argue that the helical structure is a consequence of the conformational preference of each individual nucleotide.

The shape of the nucleotide is based on four different parameters.

A) The sugar pucker
B) The syn-anti conformation of the glycosidic bond
C) The orientation of C4'-C5', and
D) The shape of phosphate ester bonds

2.1.1 Sugar pucker

The furanose rings are twisted out of plane in order to reduce non-bonded interactions between their substituents. The 'puckering' is described by identifying the major displacement of C2' and C3' from the median plane of C1'-O4'-C4'. There are two different types of puckering known as **endo** puckering and **exo** puckering. In endo pucker, the endo-displacement of C3' is observed and in the exo pucker the endo-displacement of C2' is less than the exo-displacement of C3'.

2.1.2 Syn-anti conformation

The nucleobase lies almost perpendicularly to the plane of sugar and it almost bisects the O4'-C1'-C2' angle. The free rotation of the base about the glycosidic bond enables it to occupy either of the two principal orientations, **syn** and **anti.** When the bulky part of the base is oriented towards the sugar it is called syn while the base oriented away from the sugar is called anti.

2.2 Double helix structure of DNA

The two right-handed polynucleotide chains are coiled in a helical fashion around the same axis, thus forming a double helix of DNA. The two chains or strands are antiparallel; i.e., their 3'5'-internucleotide phosphodiester bridges run in opposite directions. The coiling of the two chains is such that they cannot be separated except unwinding the coils. The purine and pyrimidine bases of each strand are stacked on the inside of the double helix, with their planes parallel to each other and perpendicular to the long axis of the double helix. The bases of one strand are paired in the same planes with the bases of the other strand. The pairing of the bases contributed by the two strands is such that only certain base pairs fit inside this structure in such a manner that they can hydrogen bond to each other. The allowed pairs are A-T and G-C, which are precisely the base pairs showing equivalence in

DNA. The disallowed purine pair A and G is rather large to fit inside a helix and the disallowed pyrimidine pair C and T would appear to be too far apart within helix to form stable hydrogen bonds with each other. The allowed pairs A-T and G-C are not only about the same size, they are also more strongly hydrogen-bonded than A-G and C-T pairs.

The double helical fashion of DNA structure leads to an element, namely, a mechanism by which genetic information can be accurately replicated. Since the two strands of double-helical DNA are structurally complementary to each other and thus contain complementary information, the replication of DNA during cell division was postulated to occur by replication of the two strands, so that each parent strand serves as the template specifying the base sequence of the new complementary strand. The end result of such a process is the formation of two daughter double-helical molecules of DNA, each identical to that of the parent DNA and each containing one strand from the parent.

2.3 Different forms of DNA

B-DNA is the most common form of DNA in which the phosphates are on the exterior to the helix and the bases are paired on the interior to the helix. In most of the cases B-DNA exists as hydrated crystalline and also in solution. When B-DNA is slightly dehydrated, another form of DNA is formed known as A-DNA. A-DNA differs from B-DNA only in base pair geometry i.e., the base pairs in A-DNA are not perpendicular to the plane as a result of puckering of the sugar unit.

A-DNA is slightly shorter than B-DNA hence the helix is broader. Another form of DNA called the Z-DNA having left handed conformation in structure usually exists in alternating purines and pyrimidines of guanine and cytosine residues in a row. Table 2.1 lists the characteristics of all the forms of DNA.

TABLE 2.1

The characteristics of various forms of DNA and those of RNA:

Helix	**Pitch (A)**	**Base pairs per turn**	**Translation (A)**	**Rotation (°) Per residue**	**Base Tilt**
A-DNA	28.15	11	2.55	32.7	20
B-DNA (Na)	34.6	10	3.46	36	0
B-DNA (Li)	33.7	10	3.37	36	2
C-DNA	31.0	9.3	3.32	39	6
Z-DNA	45	--	3.52 (G-C) 4.13 (C-G)	-51.3 (G-C) -8.5 (C-G)	7
RNA	29-30	12	2.9 (2.64)	--	--

2.4 Major and minor grooves of DNA

There are two different kinds of grooves that exist in B-DNA. They are called the major grooves and minor grooves (Figure 2.3). The grooves are so existed because the glycosidic bonds of a base pair are not diametrically opposite to each other. The minor groove contains the pyrimidine O2 and the purine N3 of the base pair and the slightly deeper major groove exists on the opposite side of the pair.

Each groove has several active hydrogen bond donor and acceptor atoms. N3 of adenine and guanine, O2 of thymine and cytosine, can act as hydrogen acceptors and the amino group attached to the C2 of guanine can act as hydrogen donor in the minor groove. N7 of guanine and adenine and also O4 of thymine and O6 of guanine are potential acceptors. The amino group attached to C6 of adenine and C4 of cytosine can act as hydrogen donors.

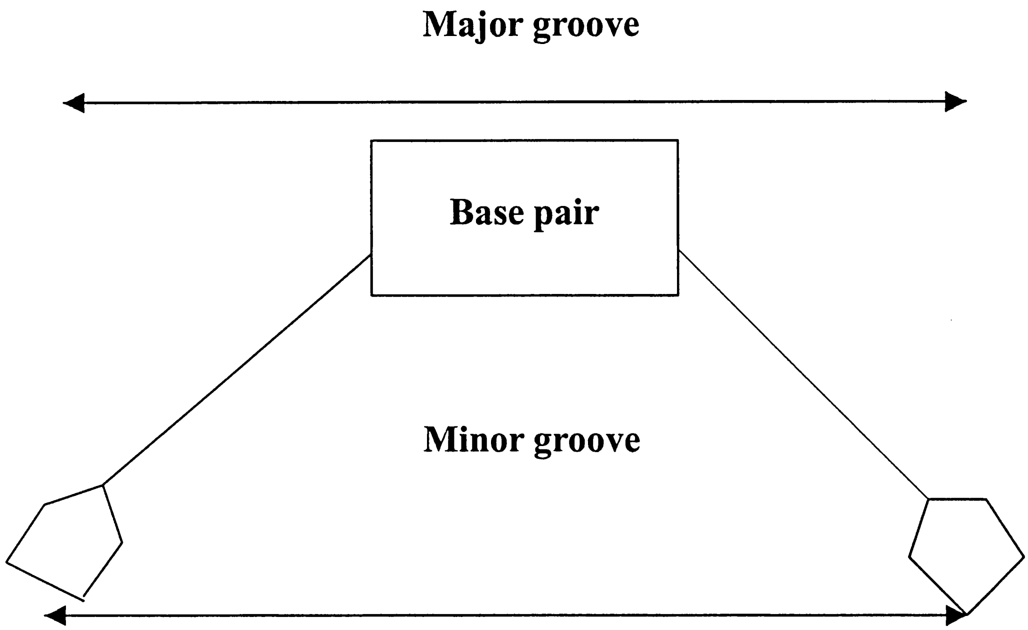


FIGURE 2.3: Represents major and minor grooves of DNA

The larger portion of the major groove makes it more accessible for the interactions with proteins and metal ions (Bettleheim, *et al.*, 1997).

CHAPTER 3

MOLECULAR MODELING

Molecular modeling using computers, also called computer aided molecular design (CAMD), is now identified as a mature discipline that is being increasingly used to design new molecules with desired characteristics. It allows the researcher to easily build, visualise, analyse and present molecular structures. Using molecular modeling, one can quickly compute spatial relationships between functional groups, explore conformational possibilities and compute interactions between ligands such as cadmium ions and target compounds such as nucleobases, nucleotides or DNA. In this study, molecular modeling based on molecular mechanics, semi-empirical and *ab intio* quantum mechanical calculations has been used to optimise the structures and to predict the molecular properties such as bond length, bond angles, presence of intramolecular hydrogen bonds.

Ab initio calculations have also been carried out to optimise some of the proposed structures. Molecular dynamics calculations have been carried out to search the conformational space in order to obtain the structure with the lowest energy. To simulate the behavior in aqueous solution, as in biological system, the designed molecules have been placed in a periodic box of TIP3P water molecules. This chapter gives a brief introduction to molecular modeling (Jorgensen, *et al.*, 1983).

3.1 Molecular Mechanics Calculations

Molecular mechanical force field uses the equation of classical mechanics to describe the potential energy surfaces and physical properties of molecules. Basically it treats a molecule as being composed of masses and bonds, which can be likened to springs. It uses the laws of classical mechanics to treat the various types of interactions that may occur in real molecules. This description of a molecule is called a force field. One component of the force field is the energy arising from the compression and stretching of a bond. If a diatomic molecule is approximated as a harmonic oscillator, the potential energy (V) of the compressed or stressed molecule is given by:

$$V \quad = \quad 1/2k_r(r - r_o)^2$$

where k_r and r_o are constants for a specific pair of atoms connected by a certain bond.

In this case the constants k_r and r_o are the force field parameters. The potential energy of a molecular system in a force field can be represented as a sum of terms associated with various types of molecular deformations (changes in bond length, valence or torsion angles) or atom-atom interactions.

$$V \quad = \quad V(r) + V(\theta) + V(\varphi) + V\,(nb) + (specific\ terms)$$

The key for successful predictions in molecular mechanics lies in the choice of the force field. Unlike quantum mechanics, molecular mechanics does not treat electrons explicitly and hence molecular mechanics calculations cannot describe bond formation, bond breaking or systems in which electronic delocalisation or molecular orbital interactions play a major role in determining geometry or properties. In this project molecular modeling has been carried out using HyperChem 5 molecular visualization and simulation program (HyperChem 5 1996).

Molecular mechanics has three important concepts, which are: (1) functional form, (2) atom type and (3) parameters sets. HyperChem 5 offers the following functional forms: MM+, AMBER, OPLS and BIO+. The force field equations for MM+, AMBER, BIO+ and OPLS are similar in types of the term they contain. However, there are some differences in the exact form of the equation they contain. BIO+ force field is the implementation of the Charmm force field developed by the group of Martin Karpulus at Harvard University (Brooks, *et al.*, 1983). Like AMBER and OPLS, it is primarily designed to explore large molecules. In MM+ force field both the bond length and bond angle terms can contain higher order terms than the standard quadratic. MM+ is based on MM2 developed by Allinger. The parameters distributed with HyperChem include the public domain values developed by Allinger (Allinger, 1977). MM+ provides cut offs for calculating non-bonded interactions and periodic boundary conditions.

3.1.1 More on the Components of force field

Bond Stretching

$$V(r) = \sum 1/2\, k_r (r - r_o)^2$$

As a bond is stretched or compressed from its standard equilibrium length, energy is stored in the bond. For small displacements from equilibrium, the bond stretching contribution is represented by a harmonic function that obeys Hooke's law
where the Σ (summation) is performed over all bonds

Angle Bending

This term is associated with the deformation of an angle from its normal value. For small displacements from equilibrium, the bond bending contribution is represented by a harmonic function.

$$V(\theta) = 1/2 k_\theta (\theta - \theta_o)^2$$

Dihedrals

This term is associated with the tendency of dihedral angles to have a certain n-fold symmetry and to have minimum energy for the cis-, gauche- and trans-conformation, etc. In molecular mechanics, the dihedral potential function is often implemented as a truncated Fourier series. HyperChem 5 uses the following periodic function for the torsion potential.

$$V_{dihedrals} = \Sigma_{dihedrals} V_n/2[1+\cos(n\phi-\phi_o)]$$

In the above equation, V_n is a dihedral force constant n is the periodicity of the Fourier term; ϕ_o is the phase angle and ϕ is the dihedral angle.

Van der Waals Interactions

To ensure the transferability of the force field from one molecule to the other, molecular mechanics takes into account explicitly the interactions between atoms, which are not bonded, to each other or to a common atom. These interactions are referred to as van der Waals interactions. HyperChem 5 uses a Lennard-Jones function to simulate van der Waals interaction in a force field.

$$V_{VDR} = \sum_{i<j} \left[\frac{A_{ij}}{R_{ij}^{12}} - \frac{B_{ij}}{R_{ij}^{6}} \right]$$

where R_{ij} is the nonbonded distance between two atoms, A_{ij} and B_{ij} are the van der Waals parameters for the interacting pair of atoms. The R^{-6} term describes the attractive interactions between two atoms corresponding to induced dipole interactions and the R^{-12} term describes the repulsive interactions caused by Pauli exclusion principle.

Hydrogen bonding

In HyperChem 5, the hydrogen bonding interaction is calculated using the function:

$$V_{H-bonded} = \sum_{ijH-bond} \left[\frac{C_{ij}}{R_{ij}^{12}} - \frac{D_{ij}}{R_{ij}^{10}} \right]$$

This function is used to model interaction between donor hydrogen atoms and a strong electronegative acceptor atom. Not all force fields in HyperChem add a specific hydrogen bonding term since, as evident from quantum mechanical calculations, hydrogen bonding interaction is accounted for by normal electrostatic interaction. In HyperChem only the AMBER force field employs an explicit hydrogen bonding term.

Electrostatic Potentials

The electrostatic potential at any position is defined as the amount of work done to bring a positive charge of one coulomb from infinity up to that point. The electrostatic potential is primarily responsible for long-range interaction between molecules. So that the results of electrostatic potential calculations can be used to rationalize interaction between molecules and molecular recognition process, electrostatic potential contours can be used to propose where electrophilic attack might occur. Electrophiles are often attracted to regions where the electrostatic potential has the most negative values. A typical function for electrostatic potential in molecular mechanics is given by:

$$V_{EEL} = \sum_{i<j} \frac{q_i q_j}{\varepsilon R_{ij}}$$

where V_{EEL} represents the electrostatic energy due to interactions between two atoms (i and j) with point charges q_i and q_j respectively.

3.2 Quantum Mechanical calculation

It was stated earlier that the molecular mechanics calculations depend on the concept of atom type and parameters associated with these atom types. Ideally each compound should have its own set of atom types, which means that the number of atom types would be very large for the universe of possible molecules. Therefore for a random new molecule it is quite likely that some parameters would be missing unless a force field had been developed from a molecule similar to the new molecule. Molecular mechanics then predicts how a new molecule will behave based on the behavior of known similar molecules. In quantum mechanical calculations generally each element would have one atom type, which means that the number of quantum mechanics parameters required for all possible molecules would be much smaller.

In quantum mechanical calculations, one starts with the given position of the nuclei and searches for the best repartition of electrons in the potential generated by the nuclei and the calculation can be continued with the modified positions of atoms to get the conformation of the lowest energy. In molecular mechanics, on the other hand, one studies the position of the nuclei in the field generated by the electrons. With some exceptions, electrons are not explicitly considered in methods used for molecular mechanical calculations, and the field they generate is not actually calculated, but represented by an "effective" potential treated according to classical mechanics.

Molecular modeling using quantum mechanical calculations is ultimately based on Schrodinger's wave equation, the solution of which consists of wave function for system of particles.

The Schrodinger's equation may be written as:

$$H\psi = E\psi$$

where H is the Hamiltonian Operator and ψ is the wave function.

To solve the Schrodinger's wave equation, it is necessary to find out the values of E and function ψ such that when the wave function is operated by the Hamiltonian, the result is the wave function multiplied by the energy. The wave function contains, in principal all the information needed to describe the properties of the system of interest. However, an exact solution of Schrodinger's equation is not possible for atoms containing more than one electron, that is, it cannot be solved exactly even for the helium atom. Thus any solution of the Schrodinger's equation for polyelectron atoms and molecules can only be an approximation. Hence approximate quantum mechanical methods of calculation have been developed. These include *ab initio* and semi-empirical calculations (Doucet, *et al.*, 1996). *Ab initio* quantum mechanical calculations take much longer time than semi-empirical quantum mechanical calculations, which in turn take much longer time than molecular mechanics calculations.

Semi-empirical methods may be defined as approximate procedures that rely on a set of empirical parameters used to calculate the wave functions of only the valence electrons. Thus, the methods are based on the use of approximate Hamiltonians acting on the valence space of the molecule, with inner shell electrons and the nucleus being treated as the unpolarisable core (Thiel, 1988).

Molecular Hamiltonian is composed of the operators: the kinetic energy of nuclei (N) and electrons (E), nuclear-nuclear repulsion (NN) and electron-electron repulsion (EE) and the attraction between electrons and nuclei (NE).

$$H = (\text{Kinetic Energy})_N + (\text{Kinetic Energy})_E + (\text{Repulsion})_{NN} + (\text{Repulsion})_{EE} + (\text{Attraction})_{NE}$$

Since nuclei have masses many times larger than the masses of electrons, during small period of time when the electrons are moving very fast the nuclei may be considered to be at rest. This leads to the approximation that the electron distribution is dependent only on the fixed position of the nuclei but not on their velocities. Thus the nuclear kinetic energy and the nuclear-nuclear repulsion energy may be considered to remain constant for a fixed configuration of atoms. When these simplifications are made molecular Hamiltonian becomes purely electronic:

$H_{electronic}$ = (Kinetic Energy)$_E$ + (Repulsion)$_{EE}$ + (Attraction)$_{NE}$

However after sorting the Schrodinger's equation we must add back a nuclear-nuclear repulsion term to calculate a potential energy surface.

The *ab initio* method considers molecular orbitals as being composed of a linear combination of atomic orbitals (LCAO). *Ab initio* calculations use parameters (contracted basic functions) associated with shells, such as an s shell, sp shell, etc, or atomic numbers.

Semi-empirical calculations use parameters associated with specific atom numbers. Semi-empirical quantum mechanical methods use a rigorous quantum mechanical formulation combined with the use of empirical parameters obtained from comparison with experiment. If parameters are available for the atoms of a given molecule, the *ab initio* and semi-empirical calculations have a priori aspect when compared with a molecular mechanics calculations, letting the experimenter to explore new molecular systems beyond those for which the parameters were generated originally.

Accurate *ab initio* calculations require enormous computation power and are only suitable for molecular systems of small or medium size. *Ab initio* calculations for very large molecules are beyond the realm of current computers. The inaccuracy of the approximation made in the semi-empirical method is partially compensated by recourse to experimental data in defining the parameters of the method.

Indeed, semi-empirical methods can sometimes be more accurate than some poorer *ab initio* methods, which require much longer computation times.

Independent electron approximation

As pointed out earlier, Schrodinger's equation cannot be solved exactly for many-electron atoms and molecules. However, simplifications can be obtained by valid approximations. One such approximation consists of seperation of nuclear motion from electronic motion, as suggested by Born and Oppenheimer. This is a reasonable approximation since the mass of the proton is larger than the mass of an electron by a factor of 1836. This means that the ratio of the mean velocity of the electrons to that of the nuclei is so large that the electrons adapt almost instantaneously their motion to the small changes in the configuration of the nuclei. Hence, it is reasonable to assume that the electrons move in the field generated by the nuclei. Thus when Born-Oppenheimer approximation is applied, the total wave function for the molecule can be written in the form:

$$\Psi\text{total(nuclei, electrons)} = \Psi\text{(electrons)}\ \Psi\text{(nuclei)}$$

The total energy of the molecule equals the sum the nuclear energy and the electronic energy. The nuclear energy comprises the electrostatic repulsion between the positively charged nuclei, whereas the electronic energy comprises the kinetic and potential energy of the electrons moving in the electrostatic field of the nuclei, together with electron-electron repulsion. Thus the total energy can be written as:

$$E(tot) = E(electrons) + E(nuclei)$$

As stated earlier, when the Born-Oppenheimer approximation is applied we can concentrate on electronic motions since the nuclei are considered to be fixed. Thus for each arrangement of the nuclei, the Schrodinger equation is solved for the electrons alone in the field of the nuclei. However, when the nuclear positions are changed it is necessary to add the nuclear repulsion to the electronic energy in order to calculate the total energy of the configuration. Thus electronic Schrodinger equation can be written as:

$$H_{elec}\psi_{elec} = E_{elec}\,\psi_{elec}$$

for ψ_{elec}, which describes the motion and position of electrons in a molecule.

The next simplification is to assume that the n electrons move independently of one another, which means that many-electron wave function

Ψ(electrons) = Ψ (1,2,…n) may be written as the product of one-electron wave functions Ψ_i, called spin orbitals:

$\Psi(1, 2, \ldots n) = \Psi_1(1)\,\Psi_2(2)\ldots\,\Psi_n(n)$

Molecular orbital

The one electron wave functions Ψ_i in the equation above are defined as the product of a spatial functions ϕ_i (x, y, z) and a spin function $\eta(\xi)$:

$\Psi_i(x, y, z, \xi) = \phi_i(x, y, z)\,\eta(\xi)$

where x, y, z are the coordinates of the electron and ξ its spin variable. The spatial functions ϕ_i are called the molecular orbitals of the system. There are only two possible spin functions $\alpha(\xi)$ and $\beta(\xi)$, corresponding to the commonly described as spin-up and spin-down notations. Thus, the spin orbitals are of the form $\phi_i(x, y, z)$ $\alpha(\xi)$ or $\phi_i(x, y, z)$ $\beta(\xi)$. This amounts to saying that a molecular orbital can accommodate a maximum of two electrons only - one with α spin and the other with β spin. So that the property of anti-symmetry as required by Pauli exclusion principle is obeyed (i.e., any n-electron wave function must change sign if we interchange the coordinates of any pair of electrons), the n-electron wave function is conveniently written as a Slater determinant built over the spin orbitals.

$$1, 2, \ldots n) = \frac{1}{\sqrt{n!}} \begin{vmatrix} \psi_1(1) & \psi_1(2) & \Lambda & \psi_1(n) \\ \psi_2(1) & \psi_2(2) & \Lambda & \psi_2(n) \\ \mathrm{M} & \mathrm{M} & \mathrm{M} & \mathrm{M} \\ \psi_n(1) & \psi_n(2) & \Lambda & \psi_n(n) \end{vmatrix}$$

where $\frac{1}{\sqrt{n!}}$ is the normalizing factor, calculated assuming that the spin orbitals are orthonormal, ensuring that:

$$\int \psi^*(1,2,\ldots n)\psi(1,2,\ldots n)d\tau_1 d\tau_2 \ldots d\tau_n = 1$$

where the * denotes the complex conjugate $d\tau = dxdydzd\xi$.

3.3 Hartree-Fock and Roothaan Equations: *Ab initio* Calculations

In molecular modeling, we are usually concerned with the ground state of molecules. We first consider a closed shell configuration, which is the case with most molecules. A closed shell means that every occupied orbital contains exactly two electrons. A restricted Hartree-Fock (RHF) description means that one spin-up electron and one spin-down electron occupy orbital. Conversely an unrestricted Hartree-Fock (UHF) description implies that there are two different sets of molecular orbitals, those molecular orbitals occupied by the electrons of the spin-up (alpha spin) and those molecular orbitals occupied by the electrons of the spin-down (beta spin). This may not be true for transition metal complexes some of which are paramagnetic.

For a closed shell system consisting of N nuclei and 2n electrons, all the occupied molecular orbitals (MOs) accommodate two electrons one with α spin and the other with β spin. For such a system the Slater determinant can be written as:

$$\psi(1,2,\ldots 2n)=\frac{1}{\sqrt{(2n)!}}\begin{vmatrix}\phi_1(1)\alpha(1) & \phi_1(2)\alpha(2) & \cdots & \phi_1(2n)\alpha(2n)\\ \phi_1(1)\beta(1) & \phi_1(2)\beta(2) & \cdots & \phi_1(2n)\beta(2n)\\ \phi_2(1)\alpha(1) & \phi_2(2)\alpha(2) & \cdots & \phi_2(2n)\alpha(2n)\\ \vdots & \vdots & \vdots & \vdots\\ \phi_n(1)\beta(1) & \phi_n(2)\beta(2) & \cdots & \phi_n(2n)\beta(2n)\end{vmatrix}$$

The value of normalizing factor for the above determinant has been calculated by assuming that the MOϕ_i values are orthonormal. For most electronic structural calculations we are usually trying to calculate the molecular orbitals. However, this is an insoluble problem since, as stated earlier, there is no correct solution of the Schrodinger's equation applying to many-body problems. We therefore require some means to decide whether one proposed wave function is a better approximation than the other. The variation theorem provides mechanism for answering this question. The theorem can be stated as follows. *The energy calculated from an approximation to the true wave function will always be greater than true energy.* Hence the better the wave function, the lower will be the energy. Thus the best function is obtained when the energy is minimum. At a minimum the first derivative of energy δE will be zero. The Hartree-Fock equations are obtained by imposing this condition on the expression of the energy subject to the constrain that the molecular orbitals remain orthonormal. The orthonormality condition is written in terms of the overlap integral, S_{ij} between two orbitals *i* and *j*.

$$s_{ij} = \int \chi_i \chi_j d\tau$$

Thus the two basic steps in obtaining Hartree-Fock equations are:

(1) Derivation of the total energy E using Ψ as the Slater determinant and H as the Hamiltonian

(2) The minimisation of the total energy under the imposed constraint.

This leads to the well-known Hartree-Fock one-electron equations:

$$F(1)\phi_i(1) = \varepsilon_i\phi_i(1)$$

Where ε_i is the energy of orbital ϕ_i and F, the Fock operator is, given by:

$$F(1) = h(1) + \sum_{j=1}^{n}\left[2J_j(1) - K_j(1)\right]$$

Hartree-Fock equations cannot be solved rigorously for molecules and hence one uses the method proposed independently by Hall and Roothan known as the linear combination of atomic orbitals (LCAO) approximation. In this method the MOϕ_i are expanded over a basis set of atomic orbitals $\{\chi_\mu\}$ of dimension m:

$$\phi_i(1) = \sum_{\mu=1}^{m} C_{\mu i}\chi_\mu(1)$$

The problem now is to determine the cooefficient $C_{\mu i}$ of our system for a given AO basis $\{\chi_\mu\}$. The matrix form of the Roothan equations can be written as:

FC = SCE

where F, C and S are *mxm* matrices with elements $F_{\mu v}$, $C_{\mu i}$ and $S_{\mu v}$ respectively, E being a diagonal matrix with elements ε_i.

Solving the Roothan equations leads to a set of MOs. The ground state configuration is then obtained when n lowest energy MOs each accommodates two electrons and the remaining m-n MOs remain empty. As stated earlier, the two major categories of quantum mechanical molecular orbital calculations are the *ab initio* and semi-empirical methods. *Ab initio* usually refers to calculations, which uses the full Hartree-Fock/Roothan Hall equations without ignoring any of the integral or any term in the Hamiltonian. Semi-empirical method simplifies the calculations using parameters for the some of the integrals/or ignoring some of the terms in Hamiltonian.

Any set of one-electron functions can be a basis set in the LCAO approximation. However, a well-defined basis set will predict electronic properties using fewer terms than a poorly defined basis set. Therefore the choice of a proper AO basis set in *ab initio* calculations is critical to the reliability and accuracy of the results. For practical reasons the atomic basic functions used in *ab initio* calculations are generally of Gaussian type, which means that they are characterised by a drop off as $\exp(-\zeta r^2)$, where ζ(zeta) is a constant called the orbital exponent. The maximum accuracy of the Roothan method is achieved with a basis set of infinite dimension. However, no computers are as yet available to solve matrix equation of such dimension. Thus depending upon the size of the molecule and the computational resources available the following three different types of AO basis set can be used in *ab initio* calculations.

Minimal basis sets, consisting of one contracted Gaussian function for each occupied AO in the separated-atom limit ground state of the molecule under study. For example the STO-3G basis set developed by Hehre *et al.* (Hehre, *et al.*, 1969), where each AO of the minimal basis set is made of a contraction of three individual Gaussian functions.

Multiple Zeta basis sets, which use two or more contracted Gaussian functions to describe the AOs. For example, the double-Zeta basis set comprises twice as many basic functions as the minimum one. Multiple Zeta basis sets are more flexible than minimal basis sets and generally lead a significant improvement in the description of the electronic structure of the molecular system. Since core electrons are little affected by the formation of the chemical bonds, in most cases it is not necessary to use a multiple zeta description of the AOs and a good compromise is found in split-valence set in which a single contracted function corresponds to core AO whereas double zeta functions are used for each valence AO. An example is 6-31G basis set developed by

(Hehre, *et al.*, 1972) where a contraction of six primitive Gaussian is used for core AOs and the valence AOs are described by contracted functions made of three and one Gaussian functions respectively.

Polarised basis set allows us to describe charge polarisation effect resulting from the internal molecular electric field. For example, the 6-31G* basis set of Hariharan and Pople (Hariharan *et al.*, 1972) is constructed by the addition of 6d-type Gaussian primitives expressed in Cartesian coordinates.

Pople-Nesbet Unrestricted equations

The Roothan equations are the basic equations for closed shell RHF molecular orbitals whereas the Pople-Nesbet equations are the basic equations for open shell UHF molecular orbitals. The latter ones are essentially the generalization of the former ones where the spatial functions ψ_i^{α} and ψi^{β} as shown previously are not defined to be identical but are solved independently.

Electron correlation

The most significant deficiency of Hartree-Fock theory is that it fails to adequately describe the correlation between motions of electrons. This deficiency is due to independent electron approximation. In the self-consistent field (SCF) method the electrons are assumed to be moving in an average potential of the other electrons such that the instantaneous position of an electron is not influenced by the presence of a neighbouring electron. In actual fact, the motions of electrons are correlated and they tend to "avoid each other" more than what is suggested by Hartree-Fock theory. The result is the lowering of the actual energy. The correlation energy is defined as the difference between Hartree-Fock energy and the exact energy. There is increasing evidence to suggest that accounting for electron correlation is warranted when quantitative information is required and it is crucial in the study of dispersive effect, which plays a major role in intermolecular interaction.

There are a number of ways in which correlation effects can be incorporated in *ab initio* molecular orbital calculations. A commonly used approach is the configuration interaction (CI), in which excited stage is included in the description of electronic state. Thus the configuration interaction adds to the single determinant of Hartree-Fock wave function, a linear combination of determinants that play the role of atomic orbitals. This is similar to constructing molecular orbitals from a linear combination of atomic orbitals. Two types of CI calculations are implemented in HyperChem– singly excited CI and microstate CI. The singly excited CI is available for both *ab initio* and semi-empirical calculations. It may be used to generate UV-spectra. The microstate CI is available only for semi-empirical methods and it is used to improve wave function and energies including the electronic correlation. The CI calculations are used in HyperChem to do the following:

- Calculate UV spectra
- Calculate the energy of excited states
- Study the making or breaking of bonds, and change of spin coupling (e.g. dissociation of H_2)
- Capture the effects of London dispersion forces
- Describe a nearly degenerate state
- Study singlet-triplet splittings more accurately

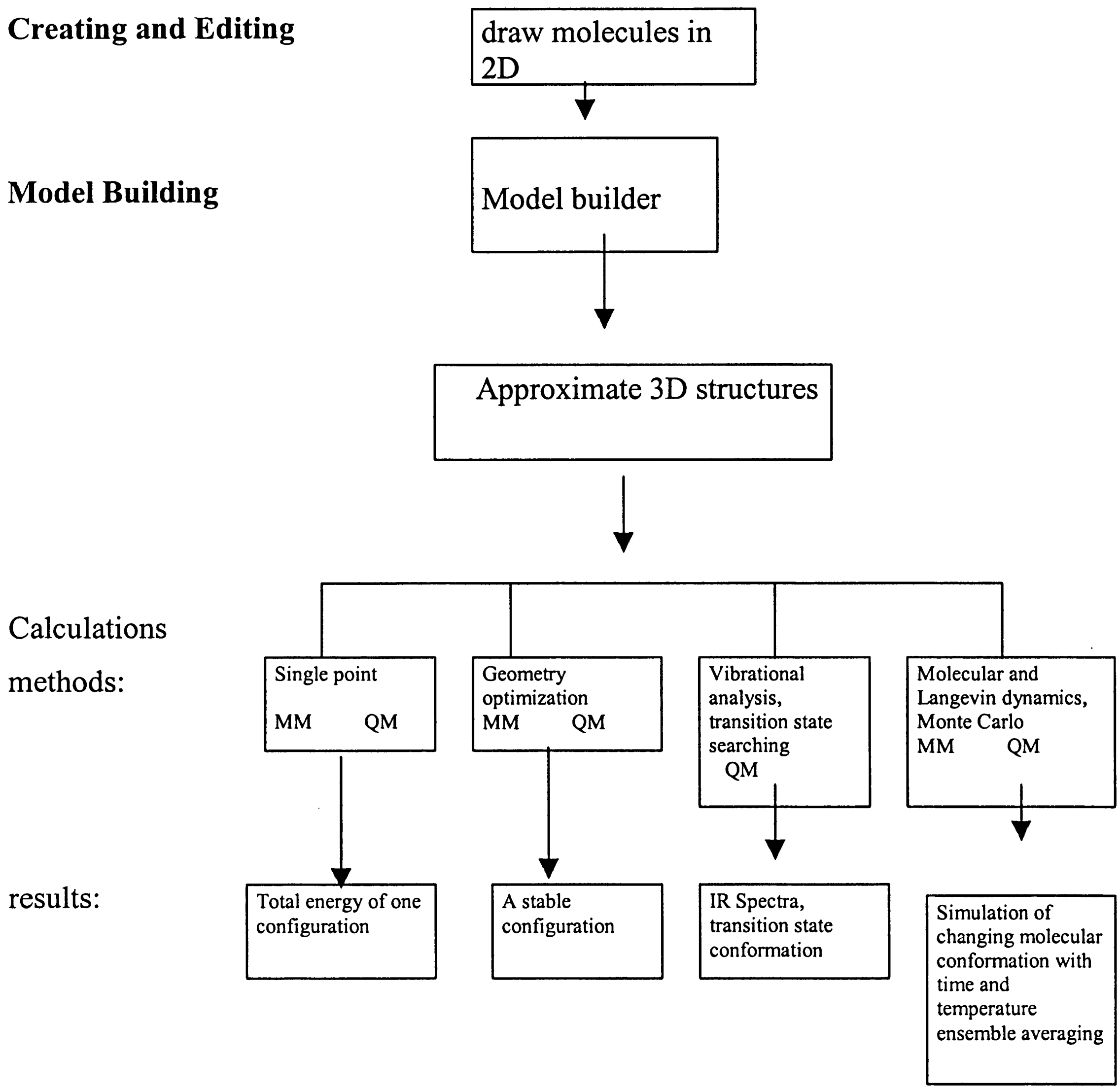
The Moller- Plasset perturbation theory provides a second approach to account for the correlation energy. In this approach the total Hamiltonian of a system is divided into two parts: a zero-order part, H_0 and a perturbation $V_{0;}$ the exact energy is then expressed as infinite sum of contributions of the increasing complexity. The total Hamiltonian can be written as: $H = H + \lambda V$ where the λ is the ordering parameter. The exact eigen functions and eigen values can then be expanded in Taylor series in λ

Types of calculations

Three types of calculations carried out in molecular modeling are (1) single point (2) geometry optimization (3) molecular dynamics. A single point calculation gives static properties of a molecule including potential energy, electrostatic potential and molecular orbital energies and the coefficient of molecular orbitals of ground or excited molecules. The single point calculation is usually carried on the optimised structure. As pointed out earlier, in HperChem single point calculations based on semi-empirical and *ab initio* calculations can be used to predict fundamental vibrational frequencies and infra red absorption intensities, and the frequencies and intensities of an electronic ultraviolet-visible spectrum. The calculation in addition to providing a value for total energy also provides a value for gradient of that energy. The gradient is the root-mean square of the derivative of energy with respect to Cartesian coordinates. At a minimum the force on atoms (i.e., the gradient) is zero. The size of the gradient thus can provide qualitative information to determine if a structure is close to a minimum.

To carry out geometry optimization HyperChem starts with a set of Cartesian coordinates for a molecule and tries to find a new set of coordinates with the minimal potential energy. Since minimization calculations cannot cross or penetrate potential energy barriers, the optimised structure found may be a local minimum rather than the global minimum. Molecular dynamic simulation samples the phase space i.e. the region defined by atomic positions and velocities by integrating numerically Newton's equations of motion. Unlike single point and geometry optimization, molecular dynamics calculations account for thermal motion. This means that the molecules may contain enough thermal energy to cross thermal barriers, and thus molecular dynamics calculations may allow a molecule to come out of a local minimum eventually to reach a global minimum. Also molecular dynamics calculations provide information about possible conformations, thermodynamic properties and dynamic behavior of molecules.

The following flow chart summarises the major functions of HyperChem



MM= molecular mechanics QM= Semi-empirical or *ab initio* quantum mechanics.

FIGURE: 3.1 Major functions of HyperChem

CHAPTER 4

POLYMERASE CHAIN REACTION (PCR)

PCR is a method for *in vitro* amplification of DNA. It is a multiple rounds of primer extension reactions in which complementary strands of a defined region of a DNA molecule are simultaneously synthesised by a thermostable DNA polymerase. During repeated rounds of these reactions the number of newly synthesised DNA strands increases exponentially so that after 20-30 reaction cycles, the initial template DNA will have been replicated several million-fold. The power to amplify DNA successfully, along with the low cost of materials and over all simplicity of the method, have made PCR an indispensable tool in current biological research. This technique can be used to identify with a very high-probability, disease-causing viruses and/or bacteria, a deceased person, or a criminal suspect.

The building block sequence of a gene is four different deoxyribonucleotide components, which are arranged, in an orderly fashion within a stretch of DNA. The components are: Adenine, Thymine, Guanine and Cytosine and, abbreviated as: A, T, G and C, respectively (a 4-letter alphabet). The orderly arrangement of the letters of this 4-letter alphabet makes a sentence, which is known as a gene sequence. The number of letters in the sentence may be relatively few, or relatively many, depending on the gene. If the sentence is 1000 letters-long, the sequence would be said to be 1 kilobase (1000 bases).

As an example:

ATATCGGGTTAACCCCGGTATGTACGCTA represents a part of one gene. DNA is double-stranded (except in some viruses), and the two strands pair with each other in a very precise way. Each letter in a strand will pair with only one kind of letter across from it in the opposing strand: A always pairs with T; and, G pairs with C across the two strands.

So: TTAACGGGGCCCTTTAAA........ TTTAAACCCGGGTTT would pair with: AATTGCCCCGGGAAATTT...AAATTTGGGCCCAAA.

Principles of PCR

First step: Denaturation: The DNA of interest is heated to 94°C causing the paired strands separated so that the single strands could be formed

Second step: Annealation: Large excess of primers relative to the amount of DNA being amplified is added and the reaction mixture is cooled to 45-55°C so that the double-strands could form again. Because of the large excess of primers, the two strands will always bind to the primers, in stead of binding with each other. At this step the primers are jiggling around template DNA caused by the Brownian motion. Ionic bonds are constantly formed and broken between the single stranded primer and the single stranded template. Stability of ionic bonds is occurred when the primers fit the sequence and the double stranded piece of DNA (single stranded template and primer) is synthesised. At this stage enzyme polymerase can attach and starts copying the template. In this way when a few bases are formed the ionic bond becomes so strong between the template and the primer, that it does not break any more.

Third step: Extension: The ideal working temperature for extension by polymerase is 72°C. As a few bases are formed the primers already have a stronger ionic attraction to the template than the forces those break this attraction. DNA polymerase is added to a mixture of all 4 individual deoxyribonucleotides (called dNTPs) which can read the opposing strand's corresponding deoxyribonucleotides and extend the primer's base sequences by hooking letters together so that they can pair across from one another – A:T and C:G. The polymerase enzyme that is used in PCR is known as Taq polymerase (originally isolated from a bacterium that can live in hot springs and can withstand the high temperature necessary for DNA-strand separation, and can be left in the reaction without being changed). Now, the enzyme will synthesise new DNA in opposite directions – but only the particular region of DNA, which has been primed.

After one cycle, more primers are added, dNTP mixtures are added, and the cycle is repeated. The primers will bind to the old sequences as well as to the newly synthesized sequences. The enzyme will again extend the sequences newly primed. Finally, plenty of DNA will be synthesised which will be copies of just the primed region.

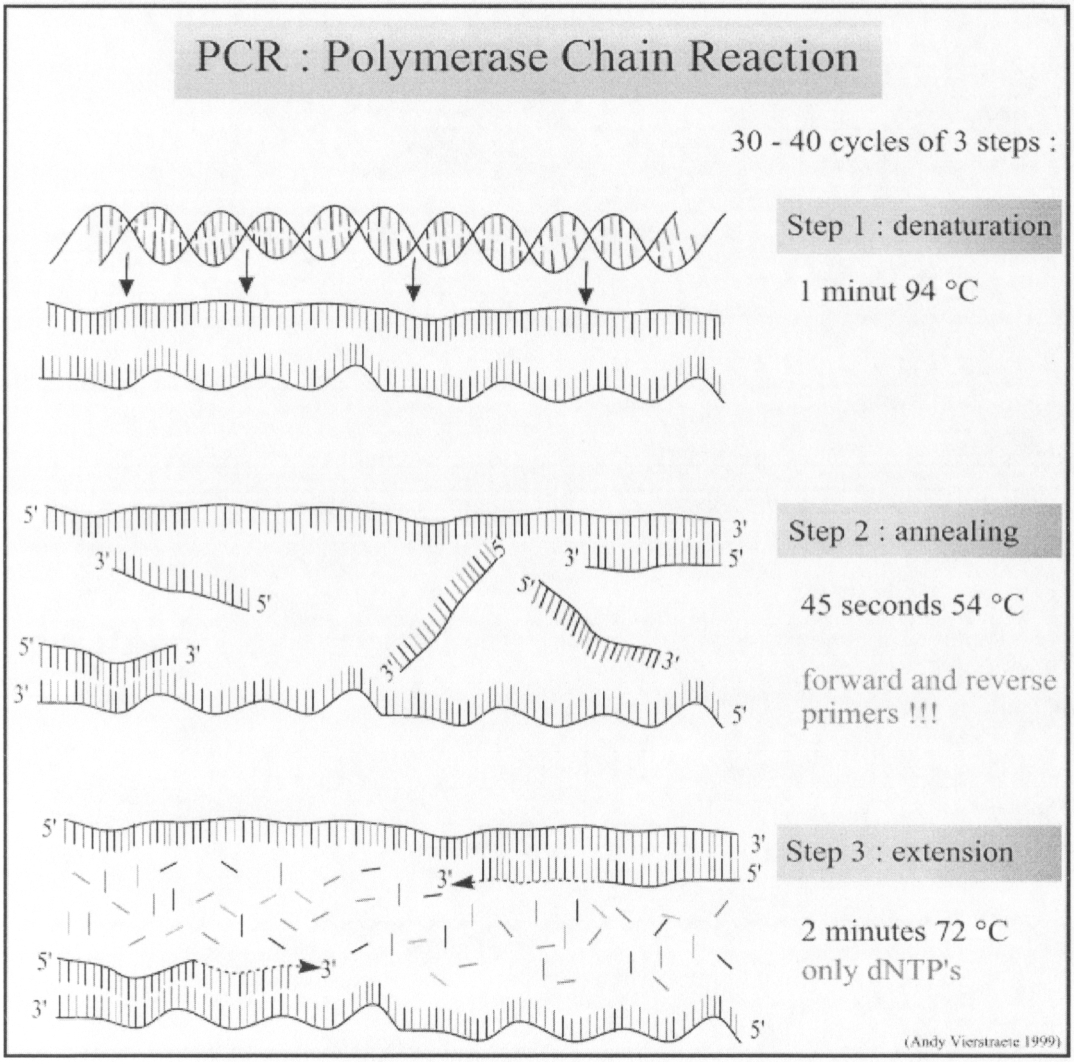


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FIGURE 4.1: Shows the steps of PCR reactions

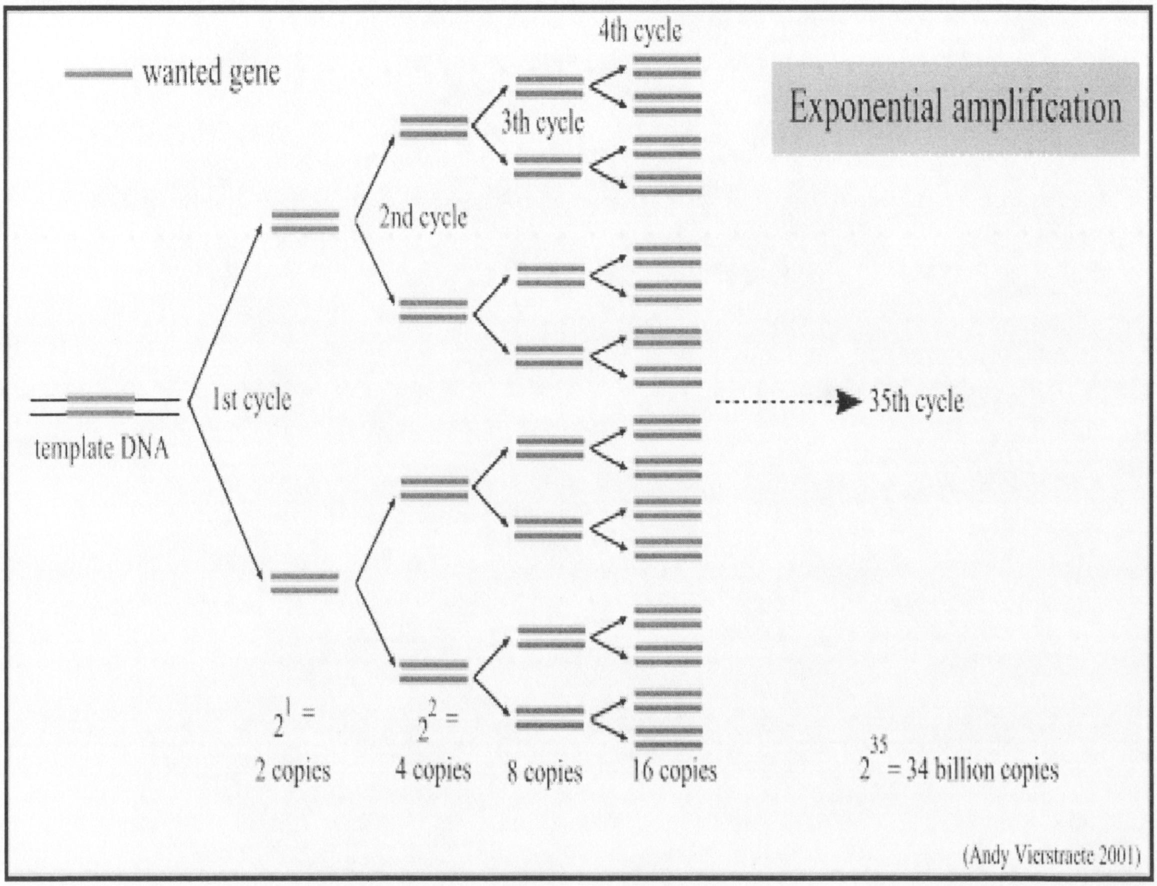

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FIGURE 4.2: Shows the exponential amplification of DNA

Parameters influencing the yield and specificity of PCR (Dieffenbach *et al.*, 1995, McPherson, *et al.*, 1991, McPherson, *et al.*, 1995)

DNA Polymerase

Many thermostable DNA polymerases are now commercially available, each with different characteristics of thermal stability, exonuclease activity, processivity, fidelity, and reverse transcriptase activity. Taq DNA polymerase, is the most widely used thermostable DNA polynerase because of its heat stability, high optimal temperature, and broad pH tolerance, The recommended concentration range for Taq DNA polymerase is between 1 and 1.25 units per 100 reactions. Enzyme requirements may vary with respect to individual target templates, primers and suppliers. Taq DNA polymerase from different suppliers may behave differently because of formulations, assay conditions, and unit definitions (especially for non-licensed products). If the enzyme concentration is too high (> 4 units/mL), nonspecific background products may accumulate, if it is too low an insufficient amount of desired product is made.

PCR Buffer

Each thermostable polymerase has unique buffer requirements for its optimal activity. The most requiring buffer is Tris-CL whose concentration should lie between 10 mM and 50 mM, and the pH should range from 8.3-9.0. A stabilizing protein such as BSA or gelatin, and a non-ionic detergent such as Tween 20, NP-40 or Triton X-100, may usually required for efficient reaction. The reaction buffers in concentrated form are normally provided by the supplier of the enzyme, but for certain applications other additives such as formamide or glycerol may be essential for an efficient reaction.

Magnesium concentration

Magnesium concentration has a significant effect on the efficiency and specificity of the PCR reaction. In addition to its direct influence on enzyme activity and fidelity, magnesium also affects the T_m (Temperature at which DNA melts in to single stranded forms) of the various hybrids that form during PCR cycling. These include primer-template hybrid, template-template hybrid, and primer-primer hybrid. It should be remembered that free nucleotides and any EDTA present in the reaction influence the effective magnesium concentration. If the magnesium concentration is too low, little or no product may be produced. If its is too high, there is a possibility of formation of artifactual products that result from non-specific priming of template DNA or from primer-dimers. For each new primer pair (forward and reverse) it is generally worthwhile to titrate the magnesium concentration in 0.5 mM steps over the range of 0.5 mM to 5 mM.

DNTPs

Total dNTP (a mixture of four individual dNTP) concentration should be taken in to account for successful amplification of DNA. A recommended total dNTP concentration for most PCR reactions should range between 80 mM and 800 mM (20mM to 200 mM each dNTP). It has been determined that 5 μg of PCR product can be synthesized from only 15 nmoles of dNTP. Concentrations higher than 800 mM increase the rate of reaction such that Taq DNA polymerase misincorporates nucleotides in the sequence. This effect can be exploited to generate mutants, but normally it is avoided.

Template

In some respects the quality of template DNA is not as critical for PCR as it is for certain other enzymatic manipulations. Evidence for this can be found in the wide variety of sources of DNA that can be successfully amplified, including "fossil DNA" entire bacterial colonies, paraffin-embedded tissue, and single human hairs. For some applications, however, the quality of the DNA template is a very important factor. In the case of amplifying of long pieces of DNA the template is a very important factor. For amplification of long pieces of DNA the template should be prepared in such a way that it can minimise single-strand nicks. Another example may be cited for a GC-rich template, in which case higher or prolonged denaturation steps and more quantity of thermostable polymerase are required.

An addition of 10^7 -fold excess of primer to template is a good rule for a standard PCR reaction. Because primer concentrations above about 0.5 nM can result in a detrimental amount of primer-dimer formation, the concentration of template DNA is limited to about 50 fm. For a 100 μL reaction, this concentration represents pg amounts of plasmid DNA, ng amounts of bacterial DNA, and μg amounts of human genomic DNA. Under specific reaction conditions, however, genomic DNA from a single cell can be amplified.

Cycle Parameters

Its is critical that complete strand separation occurs during the denaturing step. A temperature of 94 to 96°C for 1 minute is usually sufficient, but GC-rich templates may require a higher temperature. Due to excess of primer being added to template, the optimal annealing temperature is dependent on the rate of cooling from the denaturing temperature. The annealing temperature usually remains several degrees higher than the calculated T_m for the primer-template adducts. The extension reaction is fairly temperature-tolerant and 72°C is optimal for many thermostable polymerases. The duration of the extension step should be adjusted for the length of the amplified region, with 1 minute recommended for targets less than 500 bp (base pairs) in length and 2 to 3 minutes for targets greater than 500 bp. To reduce the chance of mispriming that may result from non-specific hybridization occurring during the addition of reaction components, so called hot-start PCR may be recommended in which case Taq DNA polymerase is added only after the other reactants being cocktailed are raised to a temperature of 70 to 80 °C.

Primer design and concentration

Primers from 10 to 30 nucleotides in length with a GC composition of 40 to 60% are used in most PCR reactions. It is obvious that, self-complementarity between primers should be avoided. Primers corresponding to regions of the template containing high degree of potential secondary structure should also be avoided. Specificity and efficiency of the reaction is in many cases improved if the 3' end of each primer terminates in two or more G or C residues. Due to the increased possibility of mispriming, however, it is best to avoid unusually high GC content at the 3' end of the primer. Many applications depend on the use of primers incorporating special features in their design, either to control specificity or to facilitate subsequent manipulations of the amplified product. These features may include modified nucleotides or non-complementary sequence.

Generally recommended primer concentrations are between 0.1 M and 0.5 mM. Higher primer concentrations may promote mispriming and accumulation of non-specific binding as well as may increase the probability of generating a template-independent artifact such as primer-dimer. These side products become the substrates for PCR and complete with the desired product for enzyme, dNTPs, and primers resulting in lower yield of desired PCR fragment.

CHAPTER 5

DNA SEQUENCING

The purpose of sequencing is to determine the order of the nucleotides of a gene. For sequencing we do not start from DNA template (like in PCR) but mostly either from PCR fragments or cloned genes

The 'dideoxy' chain termination DNA sequencing procedure of Sanger (Sanger *et al.*, 1997, Sanger *et al.*, 1978) and others (Smith, 1980) is considered one of the most simple, rapid and accurate methods of DNA sequencing. It makes use of the ability of DNA polymerase to faithfully synthesise a complementary copy of a single-stranded (ss) DNA template using a short DNA fragment as primer and to randomly incorporate an analog of each deoxyribonucleoside triphosphate; a 2', 3'-dideoxy-nucleside triphosphate (Figure 5.1). Once the analog is incorporated, the 3'-end is no longer a substrate for chain elongation and then growing of DNA chain is terminated.

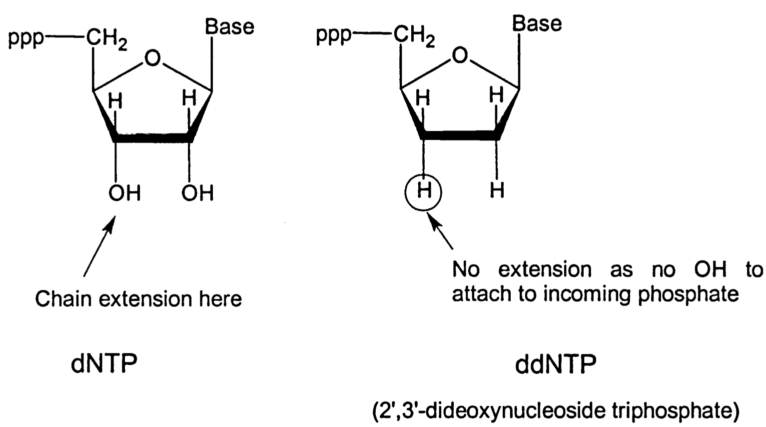


Figure 5.1: Structures of deoxy- and dideoxynucleoside triphosphates: There is chain termination when the ddXTP is incorporated.

The next figure (Figure 5.2) gives a diagrammatic representation of the mechanism of extension, by a DNA polymerase and the result of extension when the dideoxynucleoside triphosphate is incorporated.

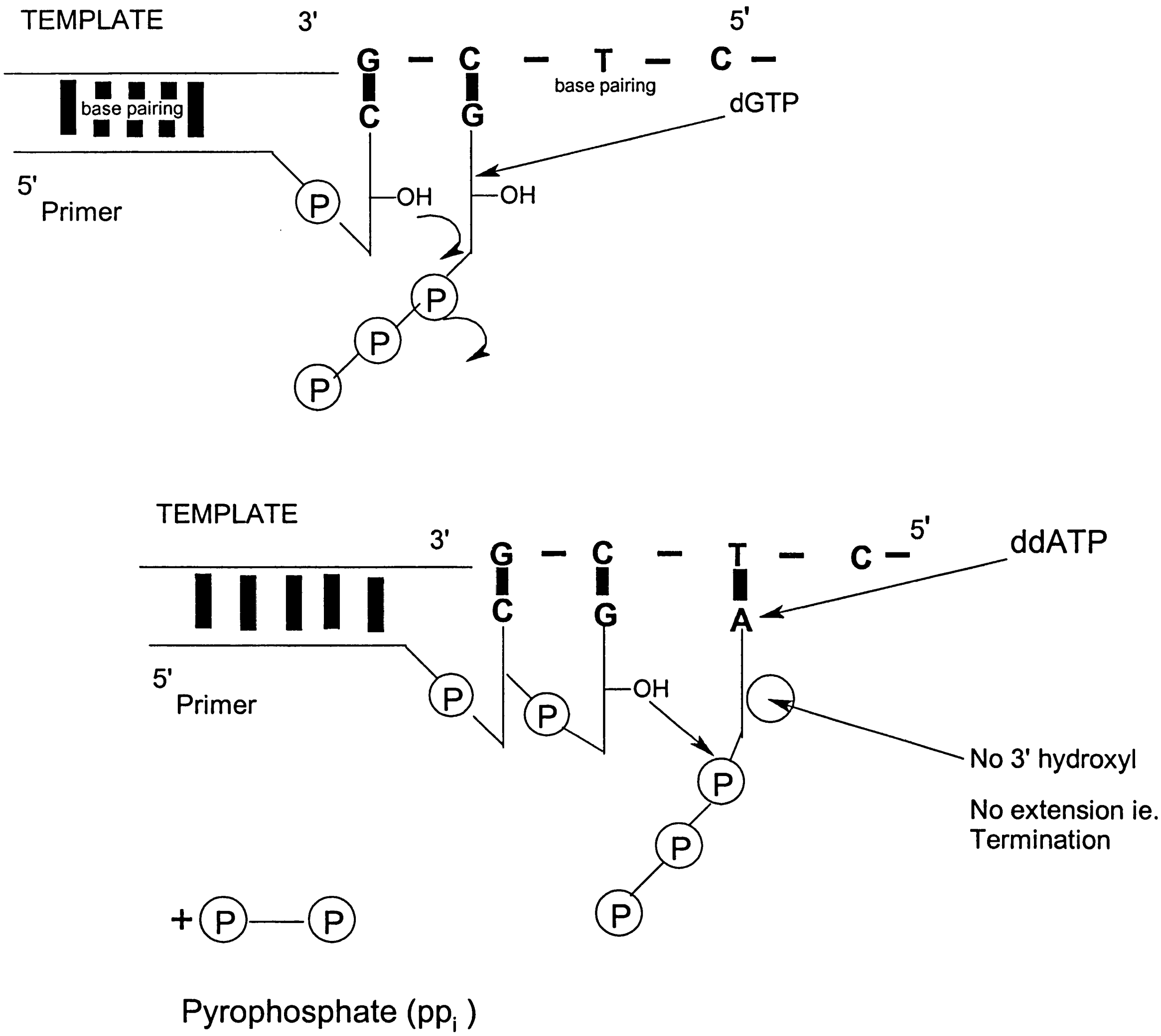


FIGURE 5.2: Representation of the mechanism of incorporation of a deoxynucleoside triphosphate (dGTP) and chain extension followed by the incorporation of a dideoxynucleoside triphosphate and chain termination.

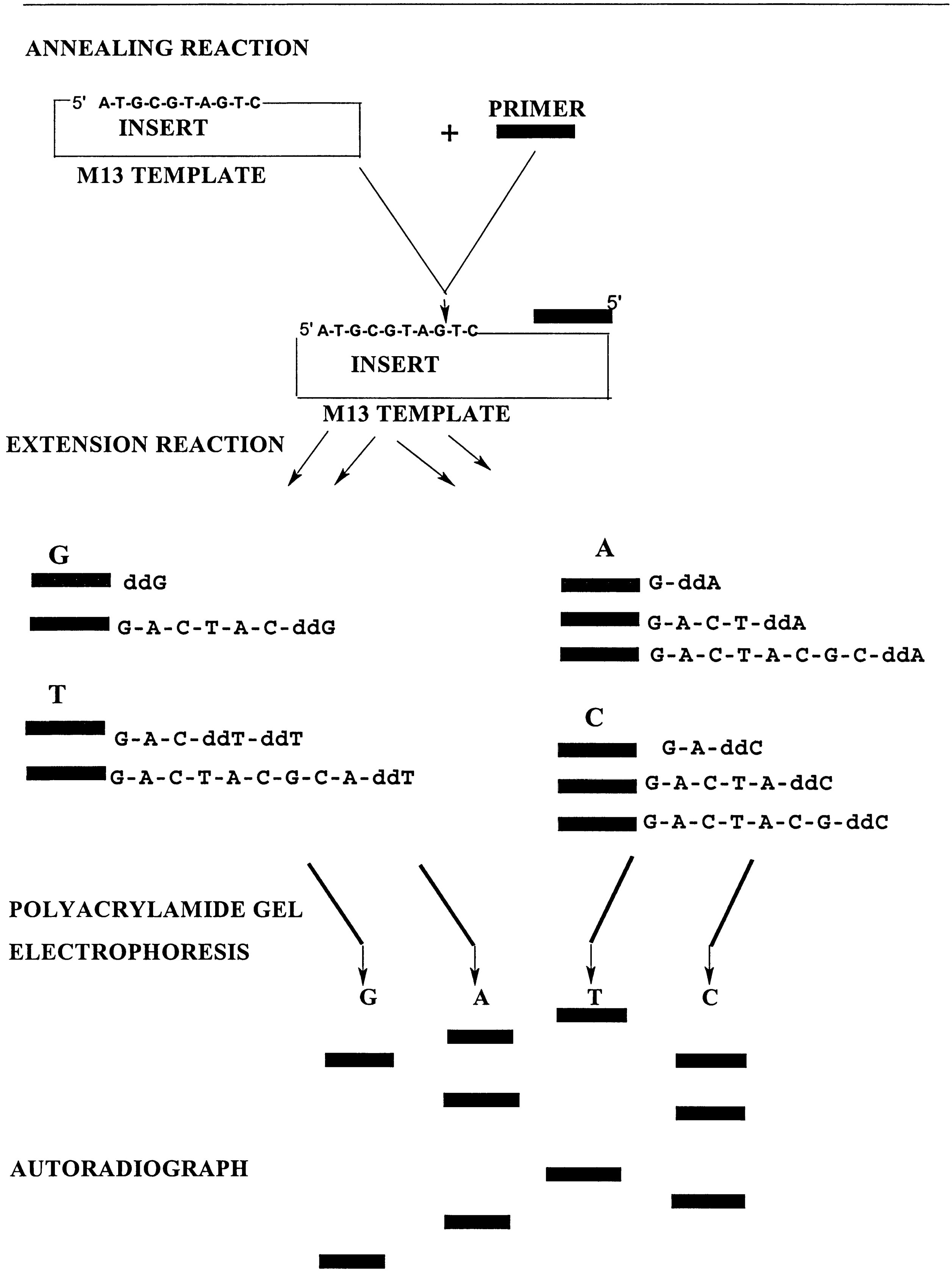


FIGURE 5.3: DNA Sequencing by the Chain Termination with ddNTP

Virtually all DNA sequencing is now performed using automated machines in which detection of products is done by fluorescent labelling. The principle is exactly the same as for manual sequencing.

The advantages of this method over other DNA sequencing techniques lies in the high degree of base-specificity (accuracy) obtained, in the easily controllable enzyme reaction employed. In addition, fewer manipulations are needed and there are less hazardous chemicals to deal with in the protocols as compared to the partial chemical degradation method of Maxam and Gilbert (Maxam, *et al.*, 1977)

Principle of Sequencing (Vierstraete, A. 1999)

Denaturation at 94 C:

During the denaturation, the double strand melts open to single stranded DNA, all enzymatic reactions stop.

Annealing at 50 C:

In sequencing reactions, only one primer is used, so there is only one strand copied whereas in PCR: two primers are used, so two strands are copied.

Extension at 60 degree C:

This is the ideal working temperature for the polymerase (normally it is 72 degree C, but because it has to incorporate ddNTP's which are chemically modified with a fluorescent label. The temperature is lowered to 60°C so that the enzyme could recognise the modified dNTP (ddDNTP) and has time to incorporate the same to the sequence.

When a ddNTP is incorporated, the extension reaction stops because a ddNTP contains H-atom on the third carbon atom in stead of OH- (dNTP's contain an OH on that position). Since the ddNTP's are fluorescently labeled, it is possible to detect the color of the last base of this fragment on an automated sequencer.

Because only one primer is used, only one strand is copied during sequencing, there is a linear increase of the number of copies of one strand of the gene. Therefore, there has to be a large amount of copies of the gene in the starting mixture of sequencing. Suppose there are 1000 original templates and 1000 complementary strands with each one fluorescent label on the last base, after two cycles, there will be 2000 complementary strands, three cycles will result in 3000 complementary strands and so on.

Separation of the molecules : After the sequencing reactions, the mixture of strands, all of different length and all ending on a fluorescently labeled ddNTP have to be separated; this is done on an acrylamide gel, which is capable of separating a molecule of 30 bases from on of 31 bases, but also a molecule of 750 bases from on of 751 bases. All this is done with gel electrophoresis. DNA has a negative charge and migrates to the positive side. Smaller fragments migrate faster, so the DNA molecules are separated on their size.

Detection on an automated sequencer:

The fluorescently labeled fragments that migrate through the gel are passing a laser beam at the bottom of the gel. The laser exits the fluorescent molecule, which sends out light of a distinct color. That light is collected and focused by lenses into a spectrograph. based on the wavelength, the spectrograph separates the light across a CCD camera (charge coupled device). Each base has its own colour, so the sequencer can detect the order of the bases in the sequenced gene.

Assembling of the sequenced parts of a gene:

For publication purposes, each sequence of a gene has to be confirmed in both directions. To accomplish this, the gene has to be sequenced with forward and reverse primers. Since it is only possible to sequence a part of 750 till 800 bases in one run, a gene of, for example 1800 bases, has to be sequenced with internal primers. When all these fragments are sequenced, a computer program tries to fit the different parts together and assembles the total gene sequence.

CHAPTER 6

EXPERIMENTAL

6.1 Materials

Analytical grade cadmium(II) nitrate tetra hydrate (CdN_2O_6. $4H_2O$), cadmium(II) acetate dihydrate ($C_4H_6CdO_4$. $2H_2O$) (obtained from Fluka, Switzerland, Aldrich Chemical Company, Inc. USA) were used as sources of Cd^{2+} ions. Salmon sperm DNA (ssDNA) was obtained from Fluka, Switzerland; HPLC grade methanol was obtained from Mallinckrodt, USA. All other chemicals including adenine, guanine, thymine cytosine, AMP, dGMP, cysteine, glutathione, ascorbic acid were obtained from Sigma, USA; pBR322, pUC8 plasmid DNA were obtained from ICN Australia and Sigma, USA respectively. The reagents for electrophoretic assay were all obtained from Sigma, USA except agarose gel, which was obtained from ICN, Australia. Restriction enzymes (Bam H1 and Ssp1) were also obtained from Sigma, USA. PCR reagents and consumables were obtained from QIAGEN, Australia and the primers from Sigma, USA.

6.2 Methods

6.2.1 Interaction between Cd^{2+} and nucleobases or nucleotides

UV-difference spectral analysis

In this series of experiment the interaction between cadmium (II) nitrate and adenine, guanine, thymine, cytosine, AMP, 2'-dGMP was investigated using UV-visible difference spectroscopy.

Solutions of cadmium(II) nitrate (1 mM) and nucleobases and nucleotides (1 mM) both made in mQ water except in the case of guanine which was made basic (pH 9.7) with 0.01 M NaOH and were mixed in a series in such a way that the concentration of both the reactants varied continuously from 0 to 1 mM. The mixtures were left standing at room temperature for 24 h. At the end of period, each solution was scanned to determine UV-visible absorbance (from 190 to 600 nm of wavelengths) using a Cary IE UV-visible spectrophotometer. The solution of the corresponding nucleobase or nucleotide at the same concentration was used as the blank. A scan rate of 200 nm/min with a slit bandwidth of 2 nm was used. The wavelengths corresponding to peaks and troughs (if any) in difference spectra (λ_{max}'s and λ_{min}'s) were noted. The absorbance values at λ_{max} or λ_{min} were plotted against added concentration of the reactants to determine the binding ratios of the reactants. The above experiments were repeated by dissolving the reactants in 0.10 M $NaNO_3$ (instead of mQ water) in order to keep the ionic strength of the solutions constant. It was found that guanine dissolved on sonication in 0.10 M $NaNO_3$ without any adjustment of pH, unlike that in mQ water, where the solution needed to be made basic (pH 9.7).

TABLE 6.1

Interaction between $Cd(NO_3)_2$ (1 mM) and nucleobase or nucleotide (NB) made in mQ water or in 0.10 M $NaNO_3$: Incubated for 24 h at room temperature

Absorbances taken against adenine blanks

Solution	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S
mLs of $Cd(NO_3)_2$	4.0	3.75	3.50	3.25	3.0	2.75	2.50	2.25	2.00	1.75	1.50	1.25	1.13	1.00	0.88	0.75	0.50	0.25	0.00
mLs of NB	0.0	0.25	0.50	0.75	1.0	1.25	1.50	1.75	2.00	2.25	2.50	2.75	2.88	3.00	3.13	3.25	3.50	3.75	4.00
$[Cd^{2+}]$ mM	1.0	0.94	0.88	0.81	0.75	0.69	0.63	0.56	0.50	0.44	0.38	0.31	0.28	0.25	0.22	0.19	0.13	0.06	0.00
[NB] mM	0.0	0.06	0.12	0.19	0.25	0.31	0.37	0.44	0.50	0.56	0.62	0.69	0.72	0.75	0.78	0.81	0.87	0.94	0.00

HPLC analysis

In this series of experiment solutions of cadmium(II) nitrate or cadmium(II) acetate and nucleobases, nucleotides (both 1 mM) were mixed at 1:1 molar ratio (except in the case of adenine where the molar ratio of the two reactants was varied). The pH was maintained close to 7.0 in all cases and the mixtures were left standing at room temperature for 24 h. In the case of adenine, a number of experiments were done where the period of incubation was varied. At the end of the period, a 50 μL aliquot was injected into Waters HPLC (high Performance liquid chromatography) system consisting of two Waters (Milford, MA, USA) 510 pumps and a 484 Waters absorption detector set at 260 nm. A WISP 712 (Waters) auto-injector, the Novapak Model 2110 Fraction Collector and Board and Baseline chromatography workstation were used. Reverse phase method with a Novapak C-18 column (8 mm × 100 mm, pore size 6 nm and 6 μm particle size) was used. Ammonium acetate (0.10 M at pH 5.50) was used as eluent A whilst methanol (HPLC grade) was used as eluent B. A gradient of 0 to 100% B over a period of 27 min was used with a flow rate of 1 mL/min. 0.50 mL/min fractions were collected. The retention times for reaction mixtures and the components were noted.

AAS analysis

The Cd^{2+} contents (if any) of the eluted fractions were determined using a Varian SpectrAA-20 plus Atomic Absorption Spectrophotometer (AAS) with a GTA-96 Graphite Furnace Tube Atomiser facility using the technique of standard addition. Tables 6.2-6.4 gave the AAS conditions used for the determination of cadmium.

TABLE 6.2

Conditions of AAS used for the determination of cadmium content

Instrument mode	Absorbance
Calibration mode	Standard addition
Lamp position	1
Lamp current (milli amp.)	4
Slit width	0.5
Wavelength	229 nm
Sample introduction	Sample automixing
Time constant	0.05
Measurement time (sec)	1
Replicates	2
Background correction	On

TABLE 6.3

Furnace parameters used for the determination of cadmium content

Step No	Temperature (°C)	Time (sec)	Gas flow (L/min)	Gas type	Read command
1	85	20	3	Normal	No
2	90	30	3	Normal	No
3	95	20	3	Normal	No
4	120	30	3	Normal	No
5	400	2	3	Normal	No
6	800	2	3	Normal	No
7	1000	5	3	Normal	No
8	1200	1	3	Normal	No
9	1200	2	0	Normal	No
10	2700	1.30	0	Normal	Yes

TABLE 6.4

Sample parameters used in AAS analysis

	Standard (μL)	Sample (μL)	Blank (μL)	Total volume (μL)	Concentration of Cd^{2+}
Blank	----		30	30	0.0
Addition 1	2	2	26	30	0.2
Addition 2	4	2	24	30	0.4
Addition 3	6	2	22	30	0.6
Addition 4	8	2	20	30	0.8
Addition 5	10	2	18	30	1.0
Sample	----	2	28	30	

Recalibration Rate 0

Multiple injection- No   Hot inject- No   Preinject- No

Determination of nucleobase, nucleotide contents by UV-visible spectrophotometry

The nucleobase or nucleotide content of a HPLC fraction was determined based on its UV absorbance at around 260 nm of wavelength using the following equation:

Nucleobase or nucleotide content in milli moles = $(A/\varepsilon) \times V$

Where A is the absorbance of the fraction, ε is the molar absorptivity of the nucleobase or nucleotide at the same wavelength where absorbance is occurred, V is the volume of the fraction in mL.

6.2.2 Interaction between Cd^{2+} and salmon sperm DNA (ssDNA)

As stated in the introduction, cadmium-induced carcinogenesis is likely to be highly complex and that more than one mechanism may operate in the process. It was also stated that one of the possible mechanisms by which Cd^{2+} could damage DNA was due to its direct binding with DNA or due to free radicals whose production was some how enhanced by Cd^{2+}. In order to investigate whether Cd^{2+} directly binds with the nucleobases of ssDNA (as did with free nucleobases and nucleotides), a set of experiments was carried out on the interaction between Cd^{2+} and salmon sperm DNA.

UV- difference spectral analysis

In this series of experiments, the interaction between Cd^{2+} and salmon sperm DNA was investigated using the technique of UV-difference spectroscopy. This technique offers a simple method to investigate direct binding between metal ions and DNA. Maxima or minima observed in the difference spectra can indicate the nature of interaction between Cd^{2+} and DNA. It has been suggested that in some way the method is superior to NMR, IR and circular dichroism spectroscopy (Sorokin, *et al.*, 1996) eg there will be less associative interaction at low concentration used in UV-difference spectral studies. The procedures used were as follows:
Solution of cadmium(II) nitrate (20 mM) and salmon sperm DNA (0.15 mM) solutions were prepared in a buffer consisting of 1 mM CH_3COONa and 0.10 M NaCl. The reactant mixtures were prepared in such a way that the concentration of Cd^{2+} varied from 0.005 to 20 mM whilst that of salmon sperm DNA kept constant (0.07 mM in term of phosphate). The mixtures were then incubated at 37°C for 24 h, at the end of which, UV-difference spectra of the incubated mixtures were recorded against corresponding DNA blanks.

HPLC analysis

In this series of experiments, solutions of cadmium(II) nitrate and salmon sperm DNA were mixed such that the concentration of Cd^{2+} varied from 0 to 50 mM but that of salmon sperm DNA kept constant at 0.07 mM in term of phosphate. The mixtures were incubated for 24 h at 37°C, at the end of which, 50 μL aliquot of incubated mixtures was injected to Waters HPLC system (as described before). The retention times were noted.

6.2.3 8-oxoguanine and 8-oxoadenine

It was stated in the introduction that 8-oxo-2'-deoxyguanosine5'-triphosphate (8-oxo-dGTP) (an oxidative product of dGTP) that can be misincorporated into DNA causing AT→CG mutations. Cells are protected against 8-oxo-dGTP by an enzyme 8-oxo-dGTPase that converts it into 8-oxo-dGMP. Inhibition of 8-oxo-dGTPase may lead to cancer. Cd^{2+} ion is found to inhibit to the activity of the enzyme in isolated bacterial (MutT) and human (MTH1) 8-oxo-dGTPases (Porter, *et al.*, 1997). Also metal ions can induce changes due to their direct binding with nucleobases. This may be reflected as a change in HPLC retention times. Indeed it has been found that when Cd^{2+} was allowed to interact with adenine and adenine nucleotides there was a change in retention time in each case (See chapter 7 section 7.3). To determine whether the change in retention time applying to adenine due to its interaction with Cd^{2+} was due to the formation of 8-oxoadenine, it was prepared and injected into HPLC. Although there was no change in retention time for guanine and guanine nucleotides, it was still prepared and injected into HPLC, to determine how its retention time compared with that of guanine.

Preparation of 8-oxoguanine and 8-oxoadenine

Ascorbic acid (100 mg) and guanine (21.76 mg) or adenine (19.46 mg) was dissolved in 40 mL of 20 mM phosphate buffer at pH 7.0 taken in a ground joint conical flask with stopper. Reaction was initiated by adding 0.50 mL of 30% H_2O_2 and the solution was left in the dark for 3 h. At the end of period, the reaction was terminated by adding 120 μg of enzyme catalase, followed by incubation at 37°C for 20 min with vigorous stirring. The reaction mixture was then filtered through a 0.20 μm nylon membrane vacuum filtration unit. The filtrate was subjected to HPLC analysis (Bialkowski, *et al.*, 1998).

50 μL aliquot of the filtrate was injected to HPLC and the retention times for the major peaks were noted. 50 μL of the corresponding blank guanine or adenine (0.06 mM) was also injected and the retention times were noted.

6.2.4 Interaction between Cd^{2+} and antioxidants

Cysteine, glutathione and ascorbate are known antioxidants involved in metal detoxification in the cell. Because of the high affinity of the thiol group for heavy metal ions such as Cd^{2+} it has been suggested that complexation of Cd^{2+} with glutathione and cysteine may have a significant effect on its binding with DNA in biological systems (Rabenstein, 1989). Based on differential pulse polarographic (DPP) data it was suggested that Cd^{2+} formed 1:2 complex with glutathione (Diaz-Cruz, *et al.*, 1997) in which Cd^{2+} joined to sulfhydryl group of cysteine residue.

The interaction between Cd^{2+} and cysteine, and glutathione was followed by HPLC to serve as a reference in studying the interaction between Cd^{2+} and antioxidants mixtures with nucleobases.

The interaction between Cd^{2+} and ascorbate was followed by UV-difference spectroscopy using the technique of continuous variation (See chapter7 section 7.5.).

HPLC analysis

Cadmium(II) nitrate (1 mM), cysteine and glutathione (2 mM) were made in mQ water and were mixed at 1:2 molar ratios. The pH was adjusted to 7.4 for each mixture by adding 0.01 M NaOH. The mixtures were left at room temperature for 24 h. At the end of the period 50 μL aliquot was injected to HPLC, the retention times applying to major peaks for the mixtures and the corresponding blanks noted. Another set of experiments were carried out in which Cd^{2+}-cysteine (1:2) or Cd^{2+}-glutathione (1:2) mixtures were incubated with 1 mM adenine or 1 mM guanine at pH 7.4 for 24 h and the resulting mixtures were subjected to HPLC analysis as described above.

UV-difference spectral analysis

Solutions of cadmium (II) acetate (1 mM), ascorbate (1 mM each) were made in 0.10 M $NaNO_3$ and mixed in varying proportions so that the concentration of each varied from 0 to 1 mM. The reaction mixtures were kept in the dark for 24 h. At the end of the period, each solution was scanned to determine its UV-difference spectra. The absorbance values were recorded. The absorbance values at λ_{max} or λ_{min} were plotted against added concentration of the reactants to determine the binding ratios of the reactants.

6.2.5 Interaction between Cd^{2+}ion and plasmid DNA

Plasmids are naturally occurring, self-replicating extrachromosomal, circular duplex DNA ranging in size from two kilobases to a few hundred kilobases. These are found in some bacterial cells and carry genes encoding the information needed for the growth and division of the bacteria. Plasmids exist in three forms

Form I: An intact covalently closed circular supercoiled DNA
Form II: A single stranded nicked, relaxed circular DNA
Form III: A double-stranded nicked linear DNA

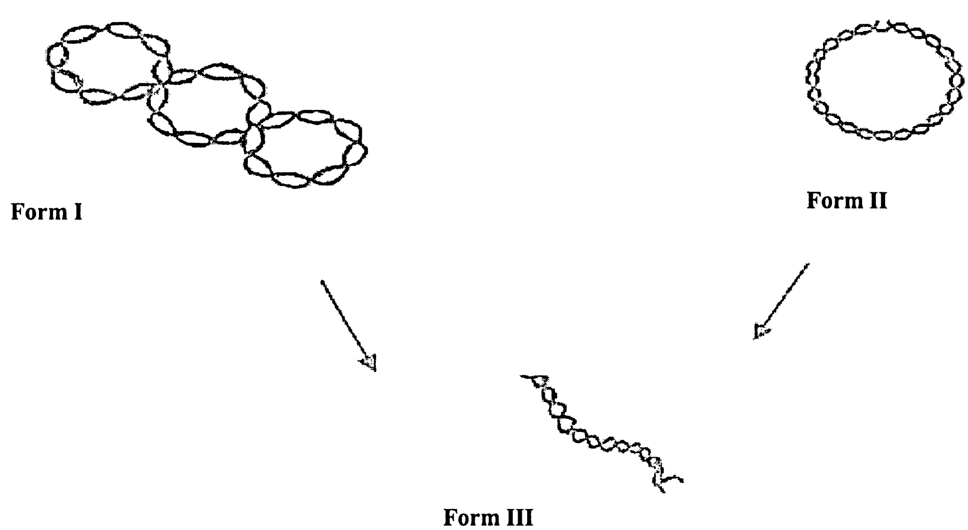


FIGURE 6.1: Three forms of plasmid DNA: Form I: supercoiled, Form II: singly nicked, Form III: doubly nicked

Form I and Form II plasmid DNA can be cleaved at a variety of unique sites by different restriction enzymes which are endonucleases that recognise specific DNA sequences of four to eight pairs and hydrolyse phosphodiester bond in each strand of

DNA in or near the sequences. If this happens the linear Form III DNA will be produced.

The three forms of plasmid DNA can be readily separated by agarose gel electrophoresis under non-denaturating conditions. In an applied electric field, the DNA samples, being negatively charged due to the phosphate backbone, will migrate through the gel from the negative to the positive electrodes.

The rate of migration is dependent upon the size and the compactness of the DNA molecules. Form I being supercoiled and compacted, will migrate at the fastest rate. The flexible and relaxed Form II DNA is the lowest in migration rate whereas the migration rate of linear Form III DNA is in between those two forms.

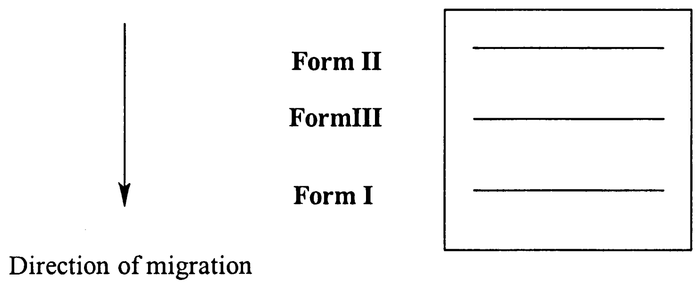


FIGURE 6.2: Migration of three forms of plasmid DNA in gel

The plasmid-binding assay is a rapid *in vitro* technique employed for the study of interaction of metal ions with DNA. The assay involves the use of plasmid DNA and restriction enzymes. Bam H1 and Ssp1 are known to hydrolyse a phosphodiester bond between adjacent guanines and adenines respectively in each strand of the DNA.

When small molecules like cadmium bind to nucleotides immediately adjacent to the recognition sequence or the cleave sites, the cleaving of DNA may be inhibited.

As guanines and adenines are potential binding sites for metal ions, Bam H1 or Ssp1 digestion of the Form I and Form II plasmid DNA into the linear Form III will be inhibited if the metal ions are able to bind to DNA at these sites. Conversely, the non-inhibition of those enzymes in question implies that the metal ions of interest can not bind to DNA.

Cd^{2+}-plasmid binding elecrophoretic assay

For this series of experiments, either cadmium(II) nitrate or cadmium(II) acetate was used as the source of Cd^{2+} ions. Cadmium(II) nitrate (0.30 mg/mL) or cadmium(II) acetate (0.26 mg/mL) solution was prepared in mQ water and was sterilised by passing through 0.22 μm Millipore filter. Solution of pBR322 plasmid DNA (0.50 mg/mL) in the buffer consisting of 1 mM Tris-HCl at pH 7.5, 1 mM NaCl and 1 mM EDTA (as supplied) was used. Appropriate volume of cadmium(II) nitrate or cadmium(II) acetate solution was added to 1 μL of plasmid DNA and the total volume was made up to 20 μL by adding sterilised mQ water so that the concentration of Cd^{2+} ranged from 0 to 100 mM while that of DNA remained unchanged at 0.025 mg/mL (= 60.5 μmol L^{-1} in terms of phosphates). The mixtures were then incubated at 37° C for 5.5 h at the end of which the reaction was quenched by rapid cooling to 0°C.

Submarine gel electrophoresis

Agarose gel (1.5% w/v) in TBE buffer (45 mM Tris, 45 mM Boric acid, and 1 mM EDTA, pH 8.0) containing 0.5 μg/mL ethidium bromide was prepared. 16 μL of each of the incubated Cd^{2+}-plasmid mixtures containing tracking dye (0.25 % bromophenol blue and 40% sucrose in water) was loaded on to the gel and the electrophoresis was carried out under TBE buffer system at 20 V for 20 h and then at 50 V for 1 h. Some of the experiments were also carried out using 1× TAE buffer (2 M Tris base, 2 M acetic acid, 50 mM EDTA at pH 8.0 constitute 50× TAE buffer concentrate) at 120 V for 3 h. At the end of electrophoresis the gel was visualised under UV light using the BIO-RAD Trans illuminator IEC 1010. The illuminated gel was photographed by a Polaroid Camera (an orange filter and Polaroid type of film was used.). The experiments were repeated by adjusting the pH of cadmium(II) nitrate or cadmium(II) acetate and DNA mixtures to 7.4 with 0.01 M NaOH.

BamH1 Restriction enzyme digestion

Another set of Cd^{2+}-plasmid DNA reaction mixtures (prepared and incubated as described earlier) was subjected to restriction enzyme digestion with BamH1 (2 μL, activity 11 U/μL) (which is known to hydrolyse a phosphodiester bond between adjacent guanine sites in each strand of the DNA). To each of the reaction mixtures in microfuge tube was added 3 μL of 10× diluted restriction enzyme and 5 μL of mQ water so that the final volume was 28 μL. The mixture was then left in a shaking water bath at 37° C for 1 h, at the end of which the reaction was terminated by rapid cooling. Finally DNA was electrophoresed as described earlier.

Ssp1 Restriction enzyme digestion

Ssp1 restriction enzyme is known to hydrolyse a phosphodiester bond between adjacent adenine sites in each DNA strand. A set of experiments was also carried out with Ssp1 restriction enzyme digestion to determine whether or not Cd^{2+} binds to adenine sites of DNA strand.

In this case cadmium(II) acetate and plasmid (pBR322) DNA mixtures were prepared, incubated as described earlier and were subjected to restriction enzyme digestion with Ssp1. To each of the reaction mixtures was added 6 μL of 10× restriction enzyme digestion buffer SH and then added 0.60 unit (0.60 μL) of enzyme. A quantity of mQ water was added to adjust the final volume to 28 μL. The mixtures were then incubated at 37°C for 1 h at the end of which the reaction was terminated by rapid cooling. Finally the enzyme digested Cd^{2+}-plasmid mixtures were electrophoresed by the method described earlier.

Cd^{2+}-plasmid binding in presence of antioxidants

Cd^{2+} was also allowed to react with plasmid DNA in presence of antioxidants cysteine, glutathione and ascorbic acid, followed by electrophoretic assay of DNA as described earlier. Solutions of cadmium(II) nitrate (0.30 g/mL) and cysteine (0.35 g/mL) or glutathione (0.60 g/mL) were mixed at 1:2 volume ratio, those of cadmium(II) acetate (0.26 gm/mL) and ascorbic acid (0.17 g/mL) were mixed at 1:1 volume ratio. The mixtures (pH 4.0) were left standing overnight for reaction. For another set of mixtures pH was adjusted to 7.4 by adding 0.01 M NaOH and left standing overnight at room temperature. Appropriate volumes of the mixtures of cadmium(II) nitrate and cysteine or glutathione were added to plasmid DNA at pH 4.0 and at pH 7.4 so that the concentration of Cd^{2+} was varied from 0 to 100 mM and 0 to 6.25 mM (3.12 mM in the case of its mixture with glutathione) respectively. Plasmid DNA was allowed to interact with cysteine only both at pH 1.50 and pH 4.0 for cysteine solutions. Plasmid DNA was also allowed to interact with glutathione only at pH 2.50. In these experiments, the concentration of cysteine or glutathione was varied from 0 to 100 mM.

In the case of cadmium(II) acetate-ascorbate mixture at pH 7.4 the concentration of Cd^{2+} was varied from 0 to 100 mM. Two series of experiments were also carried out, in one of which the concentration of ascorbate only was varied and in the other that of cadmium(II) acetate only was varied.

For the mixture of Cd^{2+} and cysteine, plasmid binding was also carried out under nitrogen, keeping all other experimental parameters unchanged. The results of the experiments were compared with those found in the case of Cd^{2+} or the antioxidant interacting with DNA alone.

6.2.6 Interaction between Cd^{2+} and genomic DNA

Salmon sperm DNA (ssDNA) and calf thymus DNA were chosen as genomic DNA to investigate the damage or change in the structure of DNA due to interaction of heavy metal cadmium. It was stated earlier that plasmid is a mixture of three forms of DNA of which supercoiled and relaxed forms are prominent (10:1 ratio) which show two distinct bands upon electrophoresis. Salmon sperm and calf thymus DNAs are of single form and show a single band on electrophoresis. Salmon sperm DNA is a low molecular weight DNA having 0.60-0.80 kilobase whereas calf thymus DNA having molecular weights > 13 kilobases. In order to compare the effect of interaction of Cd^{2+} between plasmid DNA and genomic DNA a number of electrophoretic assays were carried out.

Cd^{2+}-ssDNA/Cd^{2+}-calf thymus DNA binding electrophoretic assay

For this series of experiments cadmium(II) acetate was used as the source of Cd^{2+} ions. Cadmium(II) acetate (0.26 gm/mL) was prepared in mQ water and the pH of the solution was adjusted to 7.4. The solution was then sterilised by passing through 0.22 μm Millipore filter. ssDNA (1 mg/mL) in mQ water and calf thymus DNA (0.10 mg/mL) in TE (Tris-HCL 1 M, EDTA 1 M) buffer (as supplied) were used for this series of experiment. Appropriate volume of cadmium(II) acetate solution was added to 1 μL of ssDNA (1 mg/mL) or calf thymus DNA (0.10 mg/mL) and the total volume was made up to 20 μL by adding mQ water so that the concentration of Cd^{2+} ranged from 0 to 100 mM while that of DNA remained unchanged. The mixtures were then incubated at 37° C for 5.50 h and the reaction was quenched by rapid cooling to 0°C.

Submarine gel electrophoresis

Agarose gel (1.0% w/v for ssDNA electrophoresis) or (0.7 % w/v for calf thymus DNA elecrtrophoresis) in 1× TAE buffer containing 0.5 μg/mL ethidium bromide was prepared and was solidified at room temperature. At the end of incubation the mixtures were mixed with appropriate volume of DNA loading dye (0.25% bromophenol blue in 40% sucrose). 16 μL of each of the incubated Cd^{2+}-ss DNA or Cd^{2+}-calf thymus DNA mixtures was loaded on to the gel. The electrophoresis was carried out under 1× TAE buffer system at 80 V for ssDNA and 70 V for calf thymus

DNA respectively. The period for electrophoresis was 2.5 h in both cases. At the end of electrophoresis, the gel was visualised under UV light using the BIO-RAD Trans illuminator. The illuminated gel was photographed by a Polaroid Camera. The results were noted.

Cd^{2+}-ssDNA or Cd^{2+}-calf thymus DNA binding in the presence of antioxidant ascorbate

Cd^{2+} was also allowed to interact with ssDNA in the presence of the antioxidant ascorbate followed by electrophoretic assay as described earlier.
Solutions of cadmium(II) acetate (0.26 g/mL) and ascorbate (0.17 g/mL) were mixed at 1:1 volume ratio and were adjusted to pH 7.4. Appropriate volumes of the mixture were added to 1 μL (1 mg/mL) of ssDNA or 1 μL (0.10 mg/mL) of calf thymus DNA so that the concentration of Cd^{2+} was varied from 0 to 100 mM and that of DNA unchanged. The mixtures were incubated and quenched at 0°C. At the end of period the mixtures were electrophoresed as described earlier. The results of the experiments were noted.

6.2.7 Interaction between Ag^+ and plasmid or genomic DNA

Since like Cd^{2+}, Ag^+ is known to bind covalently with nucleobases, experiments were carried out on the interaction between DNA and Ag^+. These were done to determine whether there was a pronounced damage to DNA due to any cooperative interaction between Ag^+ and antioxidants, similar to that observed for Cd^{2+} (See chapter 7, section 7.7, page 174). Plasmid DNA and genomic DNA (salmon sperm and calf thymus DNA) were used to study the interaction of silver ion. It will be seen in the discussion (See chapter 8, section 8.7, page 249) that similar experiments were also done on the interaction between Zn^{2+} and plasmid DNA.

Ag^+-plasmid DNA binding in absence of ascorbate

For this series of experiments, silver(I) acetate was used as the source of Ag^+ ions. Silver acetate (0.17 mg/mL) solution was prepared in mQ water and was sterilised by passing through 0.22 μm Millipore filter. The pH of the solution was adjusted to 7.4 by adding slowly 0.005 M NaOH solution. Solution of pBR322 plasmid DNA (0.025 mg/mL) in the buffer consisting of 1 mM Tris-HCl at pH 7.5, 1 mM NaCl and 1 mM EDTA (as supplied) was used. Appropriate volume of silver(I) acetate solution was added to 1 μL of plasmid DNA and the total volume was made up to 20 μL by adding mQ water so that the concentration of Ag^+ ranged from 0 to 50 mM while that of DNA remained unchanged at 0.025 mg/mL (= 60.5 μmol L^{-1} in terms of phosphates 60.5 μM (in terms of phosphate). The experiments could not be extended beyond 50 mM concentration for Ag^+ since it precipitated as AgOH (or Ag_2O). The mixtures were then incubated at 37° C for 6 h and the reaction was quenched by rapid cooling to 0°C. At the end of incubation appropriate volume of loading dye (0.25 % bromo phenol blue in 40% sucrose solution) was added to the mixtures and the mixtures were electrophoresed as described in Cd^{2+}-plasmid binding assay.

Ag^+-plasmid DNA binding in presence of ascorbate

Ag^+ was also allowed to react with plasmid DNA in the presence of ascorbate, followed by electrophoretic assay of DNA as described earlier. Solutions of silver(I) acetate (0.17 g/mL) and ascorbic acid (0.17 g/mL) were mixed at 1:1 molar ratio. The mixtures with pH adjusted to 7.4 by adding 0.005 M NaOH were left standing overnight at room temperature in the dark. Appropriate volumes of the mixtures were added to plasmid DNA so that the concentration of Ag^+ was varied from 0 to 50 mM. The results of the experiments were compared with those found in the interaction of DNA with Ag^+ or ascorbate.

Ag^+ -genomic DNA binding in absence or in presence of ascorbate

Salmon sperm DNA (ssDNA) and calf thymus DNA were allowed to interact with silver(1) acetate. In order to compare the effect of interaction of Ag^+ between plasmid DNA and genomic DNA, two sets of electrophoretic assays were carried out. Solutions of silver(I) acetate (0.17 g/ml) and ascorbate (0.17 g/ml) were mixed at 1:1 volume ratio and were adjusted to pH 7.4. Appropriate volumes of the mixture were added to 1 μL of ssDNA or calf thymus DNA so that the concentration of Ag^+ was varied from 0 to 50 mM. The mixtures were incubated for 5.50 h and were quenched at 0°C. At the end of incubation the mixtures were electrophoresed as described earlier. In another set of experiments, appropriate volumes of solutions of ascorbate (0.17 g/ml) only were added to 1 μL of ssDNA or calf thymus DNA so that the concentration of ascorbate was varied from 0 to 100 mM. Still in another set of experiments, appropriate volumes of solutions of silver(I) acetate (0.17 g/ml) only were added to 1 μL of ssDNA or calf thymus DNA so that the concentration of Ag^+ was varied from 0 to 50 mM. The mixtures were incubated for 5.50 h and quenched at 0°C, at the end of which the mixtures were electrophoresed as described earlier. The time period for electrophoresis in the case of ascorbate-ssDNA binding was 3.25 h instead of the usual 2.50 h.

6.2.8 PCR analysis of metallated plasmid DNA

In this series of experiments, attempts were made to amplify Cd^{2+}-treated plasmid DNA by polymerase chain reaction. The experiments were carried out to further investigate the changes in DNA due to its interaction with Cd^{2+}. Specifically the Form I band of plasmid DNA was amplified after its interaction with cadmium(II) acetate and its gel mobility compared with the corresponding band in untreated plasmid DNA. Our initial aim was to amplify DNA fragments produced from the interaction of plasmid DNA with high concentrations of cadmium(II) acetate followed by the determination of base sequences of the amplified fragments. Comparing the base sequences of the fragments with that of the untreated plasmid DNA, would have allowed us to determine the positions at which the DNA chain was nicked due its binding with Cd^{2+} ions which in turn would have given us knowledge about the positions at which Cd^{2+} got bound to DNA. This will remain as work to be done in the future since further optimisation of PCR parameters (eg choice of primers) would be required to amplify the fragments.

Plasmid pUC8 DNA having 2655 base pairs was chosen for interaction with cadmium(II) acetate instead of pBR322 DNA that was used in other experiments. The reason why pBR322 plasmid was not used for PCR amplification was that it was not possible to design specific primer sequences (both forward and reverse) for pBR322. On the other hand, for plasmids of the pUC series (or series derived from it), the M13 (Jean-Paul, 1994) forward and reverse primers known as universal primers could be used for priming the DNA.

Base sequence pattern of M13 is as follows:
M13 forward primer – 5' GTAAAACGACGGCCAGT 3'
M13 reverse primer – 5' AACAGCTATGACCATG 3'
The steps followed in polymerase chain reaction are given below where ds and ss are abbreviations standing for double stranded and single stranded respectively.

PCR Steps

Denaturation of DNA (ds DNA$\rightarrow$ ss DNA)

$\downarrow$

Annealation (Primer linking with ss DNA)

$\downarrow$

Amplification (Elongation of ss DNA by polymerase)

$\downarrow$

Repeatation of the steps for more amplification)

$\downarrow$

Amplified DNA is now subjected to Agarose gel Electrophoresis

$\downarrow$

Comparison of the amplified band after metal treatment with that of untreated one

Solution of pUC8 plasmid DNA (1 mg/mL) in the buffer consisting of 1 mM Tris-HCl at pH 7.5, 1 mM NaCl and 1 mM EDTA (as supplied) was used for interaction with Cd^{2+}. Appropriate volume of cadmium(II) acetate solution (0.26 mg/mL) (prepared by following the procedure described earlier) was added to 1 μL of plasmid pUC8 DNA and the total volume was made up to 20 μL by adding TE (1 M Tris-HCl, 1M Na_2EDTA) buffer so that the concentration of Cd^{2+} varied from 0 to 200 mM while that of DNA remained unchanged. The mixtures were then incubated at 37° C for 5.50 h, at the end of which the reaction was quenched by rapid cooling to 0°C. The mixtures were then thawed and appropriate volume of gel loading dye (0.25 % bromo phenol blue in 40% sucrose) was added to each of the mixtures.

Each of the incubated Cd^{2+}-plasmid mixtures (16 μL) was loaded on to the gel (1% w/v agarose gel) and electrophoresis was carried out under 1× TAE buffer system at 120 V for 3 h. At the end of the electrophoresis, the gel was immersed in a tank containing 0.50 μg/mL ethidium bromide solution for 15 min. At the end of staining,

the gel was washed by soaking in distilled water for another 15 min so that the trace of ethidium bromide was removed. The gel was illuminated by UV light and the position of the Form I band (the form chosen for PCR amplification) for each of the mixtures was marked and was subjected to gel extraction.
QIAquick gel extraction kit protocol was followed after a slight modification (suggested by QIAGEN Australia) for extracting Form I bands.

Gel extraction

Form I band from the mixtures were excised from the agarose gel with a sterile sharp scalpel. Extra gel from the sides of the gel slices was removed. The gel slices were weighed and then 900 μL of buffer QG was added to each of the slices taken in sterile microfuge tubes.
The tubes with contents were incubated at 50°C for sufficient time (about 10 min) until the gel slices completely dissolved.

The dissolved content from each tube was then transferred to a QIAquick spin column mounted on a 2-mL collection tube and centrifuged for 1 min at 10000 rpm. The flow-through in the collection tube was discarded and the spin column was placed back on the collection tube again.

750 μL of buffer PE (diluted by adding 100% ethanol) was added to the column, which was left standing for a few min. It was then centrifuged for a further 1 min. The flow-through was discarded followed by additional centrifugation of the remaining contents of the column for a further 1 min at 13000 rpm.

The spin column was then placed inside a sterile 1.5 mL microfuge tube. DNA in the spin column was eluted by adding 50 μL of buffer EB (10 mM Tris. Cl, pH 8.5) to the centre of the membrane of the spin column and was centrifuged for 1 min at 13000 rpm. The eluted volume was found to be 45 μL, the concentration of DNA was calculated to be about 22 ng/μL (as compared to the standard electrophoretogram of pUC8 DNA at different concentrations).

The purified template pUC8 plasmid was subjected to PCR amplification
PCR cocktailing (i.e., mixing of plasmid DNA with different PCR reagents) was carried out on trial and error basis by adopting Taq PCR Core Kit protocol (obtained from QIAGEN Australia) so as to get the optimised protocol for cocktailing. Similarly the parameters for thermocycling operation were also optimised.
The optimised PCR cocktailing and the thermo cycling parameters were as follows:

TABLE: 6.5: Optimised PCR cocktailing parameters

Master mix	Volume (μL)
10×PCR Buffer	10.0
5×Q solution	20.0
dNTP mix (200 μm each dNTP)	2.0 (10 mM each dNTP)
Primer A (forward 5 μM)	2.0
Primer B (reverse 5 μM)	2.0
Taq DNA polymerase (2.5 U)	0.5 (5 U/ μL)
Water (sterile mQ)	61.5
Template (pUC8 44 ng)	2.0
Total	100

Procedure

Cocktailing

Prior to cocktailing aliquoted Taq PCR master mix reagents and the diluted primers were thawed, mixed well by tilting several times and kept in an ice bucket. The reaction mixtures were set up in a laminar flow cabinet. Disposable aerosol resistant tips were used to minimise cross-contamination. During cocktailing utmost care was taken to maintain sterile conditions.

Appropriate volumes of master mix reagents as per table 6.17 were dispensed carefully to the previously sterile PCR tubes and were mixed by pipetting up and down for a number of times. At the end of mixing, the contents in the tubes were kept on ice.

Template DNA (44 ng) was added to the individual tubes containing master mix and was mixed thoroughly by vortexing. At the end of mixing, each tube was overlayed with 100 μL of mineral oil.

The thermocycler was programmed according to table 6.18 and the PCR tubes were placed in the cycler. At the end of reaction the contents of the tubes were subjected to agarose gel electrophoresis.

TABLE 6.6: Optimised thermal sequencing program

Stage	Step	Temperature (°C)	Time	Number of cycle
1 (Denaturation)	1	94	1 min	1
2 (Annealation)	1	92	30 sec	5
	2	55	1.5 min	
	3	72	1.5 min	
3 (Elongation)	1	92	30 sec	30
	2	55	1.5 min	
	3	72	50 sec	
4 (Final elongation)	1	72	5 min	1

Agarose gel electrophoresis

Agarose gel (4%) in 1× TAE buffer was prepared and solidified at room temperature. Appropriate volume of DNA loading dye (30% glycerol in distilled water, 0.25% bromophenol blue and 0.25% xylene cyanol FF. stored at 4°C, especially used for PCR analysis in the gel) was added to each of the PCR product in the tube. 16 μL from each tube was loaded on to the gel and the electrophoresis was carried out under 1× TAE buffer system at 120 V for 3 hours. At the end of electrophoresis, the gel was stained with ethidium bromide and then destained as described. After destaining the gel was photographed by using a Polaroid Camera.

CHAPTER 7

RESULTS

7.1 Interaction between Cd^{2+} and nucleobases or nucleotides

7.1.1 UV-difference spectral analysis

This series of experiments was carried out to investigate the formation of different adducts in solution between Cd^{2+} and nucleobases or nucleotides. 1 mM solutions of cadmium(II) nitrate and nucleobases or nucleotides were mixed in continuously varying proportion and incubated for 24 h at room temperature, at the end of which the UV-difference spectra of the incubated mixtures were recorded. For each combination of reactants $\lambda_{max}/\lambda_{min}$ i.e., the wavelength at which the absorbance was a maximum or a minimum, was determined using the solution that contained equimolar mixtures of the reactants. The absorbance values (at $\lambda_{max}/\lambda_{min}$) of different combinations of reactants were plotted against the concentrations of reactants. The binding ratios corresponding to any maxima or minima in the graph were noted. The experiments were initially carried out by dissolving the reactants in mQ water and repeated by dissolving them in 0.10 M $NaNO_3$.

Reactants dissolved in mQ water

Table 7.1 gives the absorbance values applying to continuously varying mixtures of solutions of cadmium(II) nitrate and adenine against adenine blanks, taken at 265 nm where the absorption maximum was observed. A plot of absorbance versus concentration at 265 nm (Figure 7.1) showed that, as expected (Aoki, *et al.*, 1984) Cd^{2+} formed closely 1:1 adduct with adenine.

TABLE 7.1

Interaction between $Cd(NO_3)_2$ (1 mM) and adenine (1 mM) made in mQ water: Incubated for 24 h at room temperature

Absorbances taken against adenine blanks

Solution	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S
mLs of $Cd(NO_3)_2$	4.0	3.75	3.50	3.25	3.0	2.75	2.50	2.25	2.00	1.75	1.50	1.25	1.13	1.00	0.88	0.75	0.50	0.25	0.00
mLs of adenine	0.0	0.25	0.50	0.75	1.0	1.25	1.50	1.75	2.00	2.25	2.50	2.75	2.88	3.00	3.13	3.25	3.50	3.75	4.00
[Cd^{2+}] mM	1.0	0.94	0.88	0.81	0.75	0.69	0.63	0.56	0.50	0.44	0.38	0.31	0.28	0.25	0.22	0.19	0.13	0.06	0.00
[adenine] mM	0.0	0.06	0.12	0.19	0.25	0.31	0.37	0.44	0.50	0.56	0.62	0.69	0.72	0.75	0.78	0.81	0.87	0.94	0.00
Absorbance at 265 nm	0.0	-0.02	-0.04	-0.02	-0.10	-0.07	0.02	0.88	0.16	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0

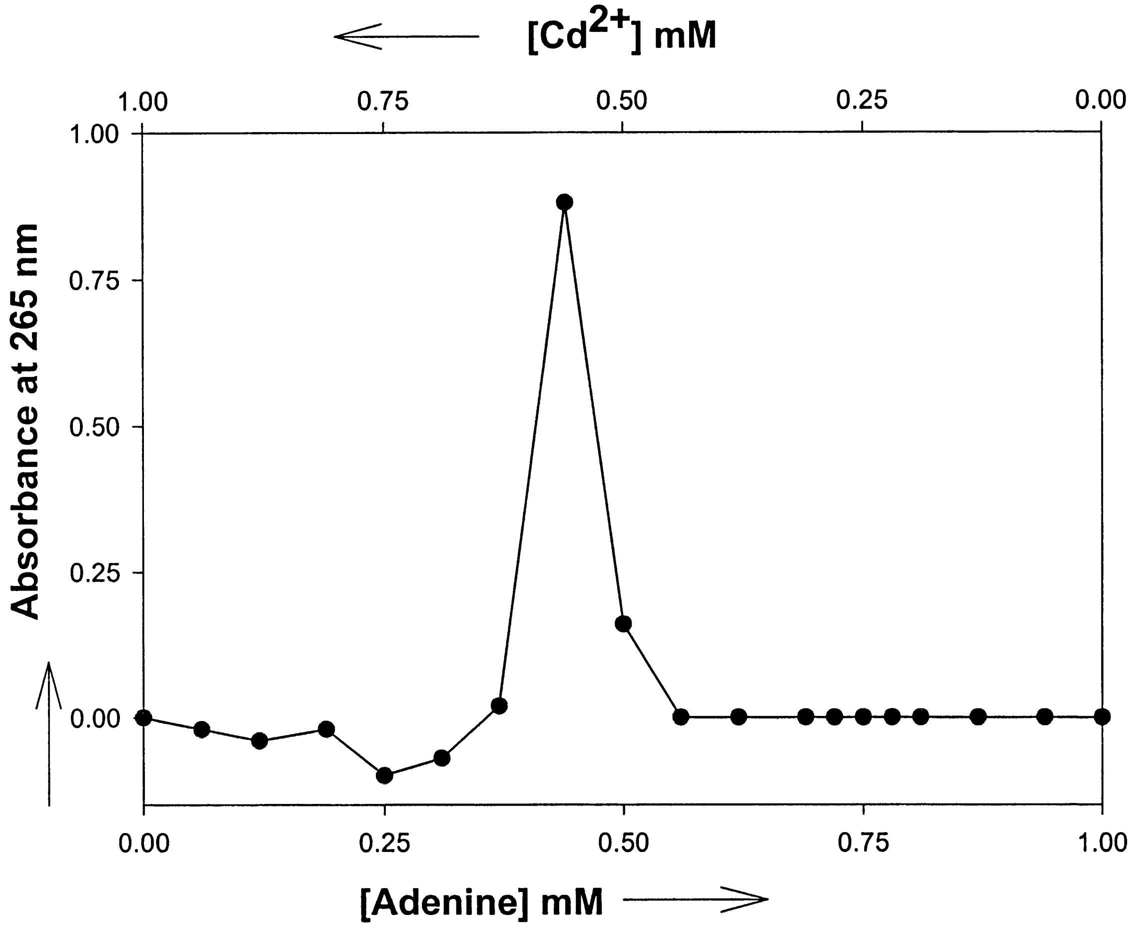


FIGURE 7.1: Interaction between Cd^{2+} and adenine: Plot of absorbance versus concentration at 265 nm, indicating almost corresponding to 1:1 adduct between Cd^{2+} and adenine

In the case of guanine interacting with cadmium(II) nitrate, the situation appeared to be more complex (Table 7.2). It was found that for the 1:1 mixture, the absorbance values in the difference spectrum remained very close to zero as the wavelength was changed. However, it was found that for some combinations of the reactants, the absorbance values at 265 nm were significantly greater than zero. When the absorbance values at this wavelength were plotted against concentrations (Figure 7.2), a broad maximum was observed corresponding to a cadmium:guanine binding ratio ranging from 11:1 to 4:1- values which are difficult to explain. However, in addition, a minimum was also observed corresponding to the cadmium:guanine binding ratio of almost 1:3.

TABLE 7.2

Interaction between $Cd(NO_3)_2$ (1 mM) and guanine (1 mM) made in mQ water (to dissolve guanine, appropriate volume of 0.01 M NaOH was added to reach a pH of about 9.7): Incubated for 24 h at room temperature

Absorbances taken against guanine blanks

Solution	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S
mLs of $Cd(NO_3)_2$	4.0	3.75	3.50	3.25	3.0	2.75	2.50	2.25	2.00	1.75	1.50	1.25	1.13	1.00	0.88	0.75	0.50	0.25	0.00
mLs of guanine	0.0	0.25	0.50	0.75	1.0	1.25	1.50	1.75	2.00	2.25	2.50	2.75	2.88	3.00	3.13	3.25	3.50	3.75	4.00
$[Cd^{2+}]$ mM	1.0	0.94	0.88	0.81	0.75	0.69	0.63	0.56	0.50	0.44	0.38	0.31	0.28	0.25	0.22	0.19	0.13	0.06	0.00
[guanine] mM	0.0	0.06	0.12	0.19	0.25	0.31	0.37	0.44	0.50	0.56	0.62	0.69	0.72	0.75	0.78	0.81	0.87	0.94	0.00
Absorbance at 265 nm	0.0	0.73	0.73	0.72	0.46	0.31	0.01	0.0	0.0	0.0	0.0	0.0	-0.14	-0.09	0.0	0.0	0.0	0.0	0.0

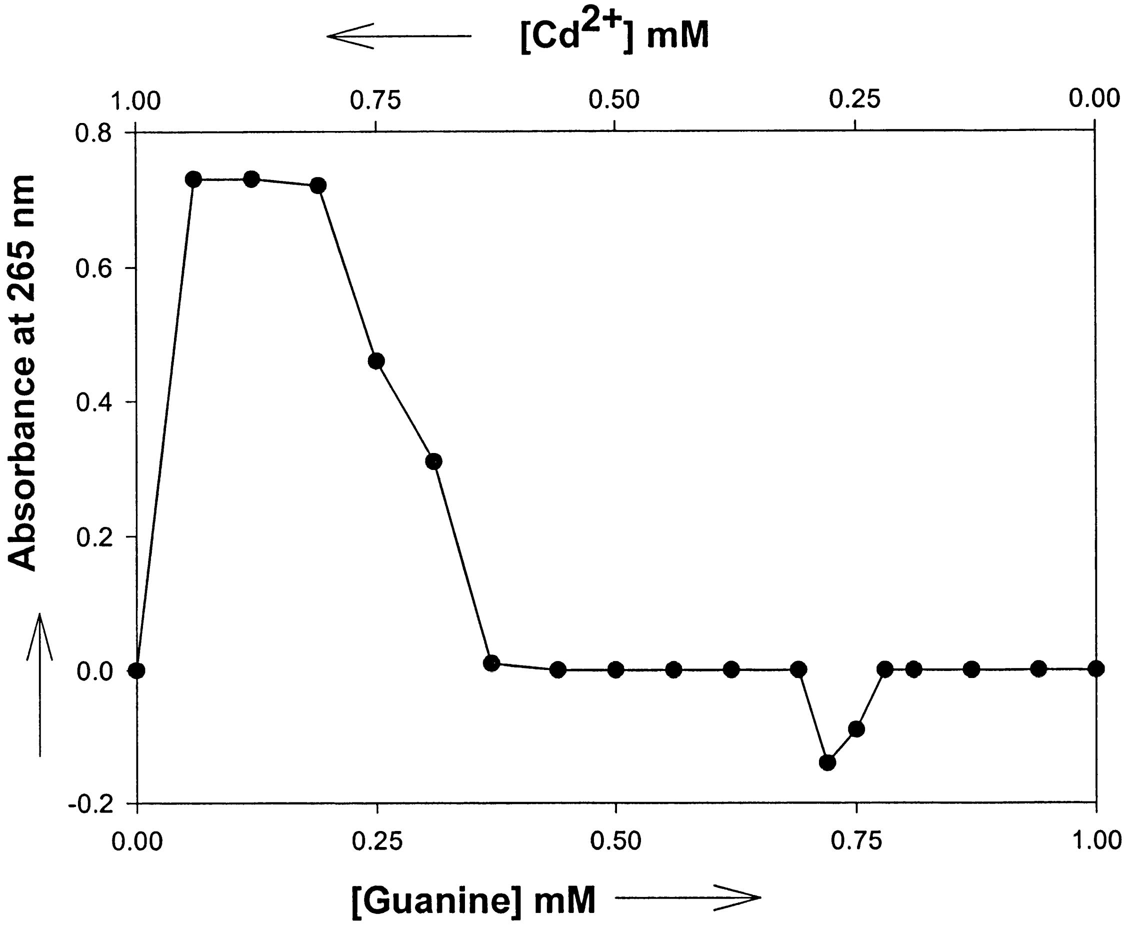


FIGURE 7.2: Interaction between Cd^{2+} and guanine: Plot of absorbance values at 265 nm, indicating almost corresponding to 1:3 adduct between Cd^{2+} and guanine

Tables 7.3 and 7.4 give the absorbance values applying to the incubated mixtures of solutions of cadmium(II) nitrate and thymine or cytosine at 265 nm. When the absorbance values were plotted against concentrations no clearly defined maxima or minima were found (Figures not shown).

TABLE 7.3

Interaction between $Cd(NO_3)_2$ (1 mM) and thymine (1 mM) made in mQ water: Incubated for 24 h at room temperature

Absorbances taken against thymine blanks

Solution	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S
mLs of $Cd(NO_3)_2$	4.0	3.75	3.50	3.25	3.0	2.75	2.50	2.25	2.00	1.75	1.50	1.25	1.13	1.00	0.88	0.75	0.50	0.25	0.00
mLs of thymine	0.0	0.25	0.50	0.75	1.0	1.25	1.50	1.75	2.00	2.25	2.50	2.75	2.88	3.00	3.13	3.25	3.50	3.75	4.00
[Cd^{2+}] mM	1.0	0.94	0.88	0.81	0.75	0.69	0.63	0.56	0.50	0.44	0.38	0.31	0.28	0.25	0.22	0.19	0.13	0.06	0.00
[thymine] mM	0.0	0.06	0.12	0.19	0.25	0.31	0.37	0.44	0.50	0.56	0.62	0.69	0.72	0.75	0.78	0.81	0.87	0.94	0.00
Absorbance at 265 nm	0.0	0.0	0.0	0.0	0.00	0.0	0.0	0.001	0.002	0.001	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0

TABLE 7.4

Interaction between $Cd(NO_3)_2$ (1 mM) and cytosine (1 mM) made in mQ water: Incubated for 24 h at room temperature

Absorbances taken against cytosine blank

Solution	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S
mLs of $Cd(NO_3)_2$	4.0	3.75	3.50	3.25	3.0	2.75	2.50	2.25	2.00	1.75	1.50	1.25	1.13	1.00	0.88	0.75	0.50	0.25	0.00
mLs of cytosine	0.0	0.25	0.50	0.75	1.0	1.25	1.50	1.75	2.00	2.25	2.50	2.75	2.88	3.00	3.13	3.25	3.50	3.75	4.00
[Cd^{2+}] mM	1.0	0.94	0.88	0.81	0.75	0.69	0.63	0.56	0.50	0.44	0.38	0.31	0.28	0.25	0.22	0.19	0.13	0.06	0.00
[cytosine] mM	0.0	0.06	0.12	0.19	0.25	0.31	0.37	0.44	0.50	0.56	0.62	0.69	0.72	0.75	0.78	0.81	0.87	0.94	0.00
Absorbance at 265 nm	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.001	0.002	0.001	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0

Table 7.5 gives absorbance versus concentration values applying to continuously varying mixtures of cadmium(II) nitrate and AMP at 278 nm. When the absorbance values were plotted against concentrations a minimum was observed corresponding to the formation of 1:3 adduct (Figure 7. 3) between Cd^{2+} and AMP.

TABLE 7.5

Interaction between $Cd(NO_3)_2$ (1 mM) and AMP (1 mM) made in mQ water: Incubated for 24 h at room temperature

Absorbances taken against AMP blanks

Solution	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S
mLs of $Cd(NO_3)_2$	4.0	3.75	3.50	3.25	3.0	2.75	2.50	2.25	2.00	1.75	1.50	1.25	1.13	1.00	0.88	0.75	0.50	0.25	0.00
mLs of AMP	0.0	0.25	0.50	0.75	1.0	1.25	1.50	1.75	2.00	2.25	2.50	2.75	2.88	3.00	3.13	3.25	3.50	3.75	4.00
$[Cd^{2+}]$ mM	1.0	0.94	0.88	0.81	0.75	0.69	0.63	0.56	0.50	0.44	0.38	0.31	0.28	0.25	0.22	0.19	0.13	0.06	0.00
[AMP] mM	0.0	0.06	0.12	0.19	0.25	0.31	0.37	0.44	0.50	0.56	0.62	0.69	0.72	0.75	0.78	0.81	0.87	0.94	0.00
Absorbance at 278 nm	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	-0.05	-0.04	-0.03	-0.03	-0.04	0.0

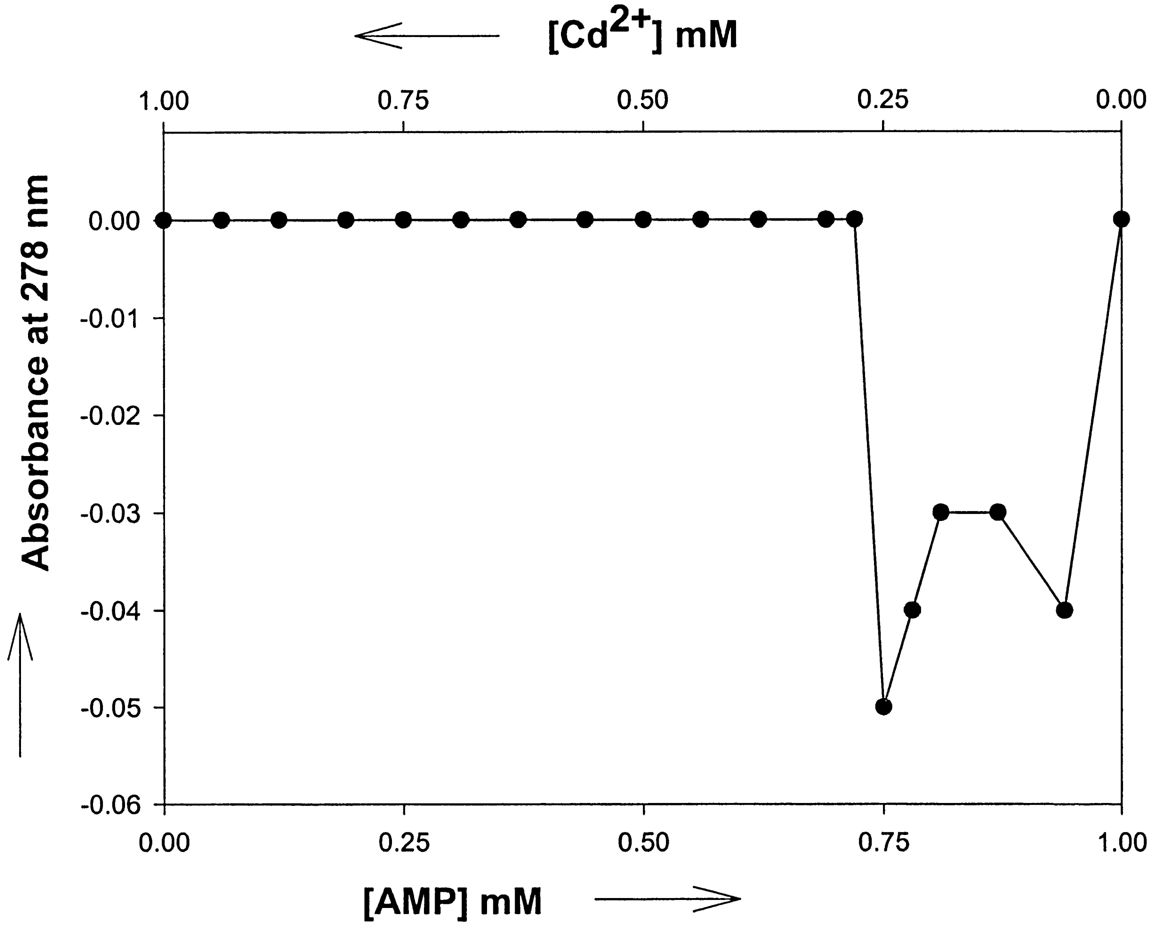


FIGURE 7.3: Interaction between Cd^{2+} and AMP: Plot of absorbance values at 278 nm, indicating the formation of 1:3 adduct between Cd^{2+} and AMP

Table 7.6 shows absorbance values applying to continuously varying mixtures of cadmium(II) nitrate and 2'-dGMP at 285 nm. The plot of absorbance against concentration applying to the continuously varying mixtures of cadmium(II) nitrate and 2'-dGMP at 285 nm indicates the formation of 1:1 and 1:3 adducts between Cd^{2+} and 2'-dGMP (Figure 7. 4).

TABLE 7.6

Interaction between $Cd(NO_3)_2$ (1 mM) and 2'-dGMP (1 mM) made in mQ water: Incubated for 24 h at room temperature

Absorbances taken against 2'-dGMP blanks

Solution	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S
mLs of $Cd(NO_3)_2$	4.0	3.75	3.50	3.25	3.0	2.75	2.50	2.25	2.00	1.75	1.50	1.25	1.13	1.00	0.88	0.75	0.50	0.25	0.00
mLs of 2'-dGMP	0.0	0.25	0.50	0.75	1.0	1.25	1.50	1.75	2.00	2.25	2.50	2.75	2.88	3.00	3.13	3.25	3.50	3.75	4.00
[Cd^{2+}] mM	1.0	0.94	0.88	0.81	0.75	0.69	0.63	0.56	0.50	0.44	0.38	0.31	0.28	0.25	0.22	0.19	0.13	0.06	0.00
[2'-dGMP] mM	0.0	0.06	0.12	0.19	0.25	0.31	0.37	0.44	0.50	0.56	0.62	0.69	0.72	0.75	0.78	0.81	0.87	0.94	0.00
Absorbance at 285 nm	0.0	0.05	0.05	0.25	0.28	0.33	0.15	0.45	0.48	0.30	0.25	0.25	0.75	-0.08	0.90	0.45	0.55	0.0	0.0

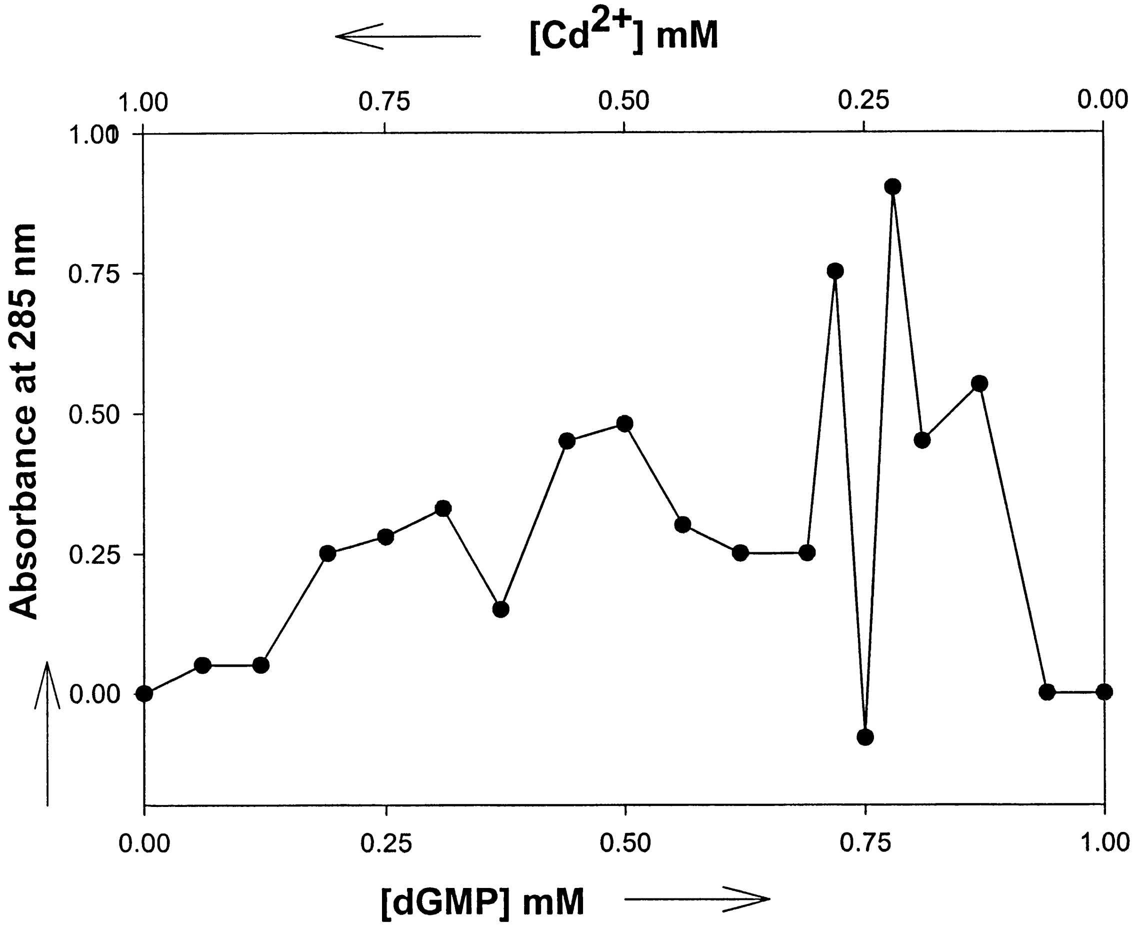


FIGURE 7.4: Interaction between Cd^{2+} and 2'-dGMP: Plot of absorbance values at 285 nm, indicating the formation of 1:1 and 1:3 adducts between Cd^{2+} and 2'-dGMP

Reactants dissolved in 0.10 M $NaNO_3$

As described earlier, the above experiments were repeated by dissolving the reactants in 0.10 M $NaNO_3$ (instead of mQ water). This was done to eliminate any confounding effect resulting from a change in ionic strength of the solutions.

Table 7.7 gives absorbance values applying to continuously varying mixtures of cadmium(II) nitrate and adenine against adenine blanks, taken at 259 nm (the wavelength at which absorbance was found to be a minimum).

The plot of absorbance against concentration applying to continuously varying mixtures of cadmium(II) nitrate and adenine showed that as expected (Sorokin, *et al.*, 1997) cadmium formed mainly 1:1 adduct with adenine. However, there were two other minima corresponding to the formation of 1:2 and 1:3 adducts (Figure 7. 5).

TABLE 7.7

Interaction between $Cd(NO_3)_2$ (1 mM) and adenine (1 mM) made in 0.10 M $NaNO_3$: Incubated for 24 h at room temperature

Absorbances taken against adenine blanks

Solution	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S	T
mLs of $Cd(NO_3)_2$	4.0	3.75	3.50	3.25	3.0	2.75	2.50	2.25	2.00	1.75	1.50	1.38	1.25	1.13	1.00	0.88	0.75	0.50	0.25	0.0
mLs of adenine	0.00	0.25	0.50	0.75	1.0	1.25	1.50	1.75	2.00	2.25	2.50	2.62	2.75	2.87	3.00	3.13	3.25	3.50	3.75	4.0
[Cd^{2+}] mM	1.0	0.94	0.88	0.81	0.75	0.69	0.63	0.56	0.50	0.44	0.38	0.35	0.31	0.28	0.25	0.22	0.19	0.13	0.06	0.0
[adenine] mM	0.0	0.06	0.12	0.19	0.25	0.31	0.37	0.44	0.50	0.56	0.62	0.65	0.69	0.72	0.75	0.78	0.81	0.87	0.94	1.0
Absorbance at 259 nm	0.0	0.0	-0.01	-0.05	-0.07	-0.10	-0.13	-0.15	-0.18	-0.02	-0.04	-0.08	-0.02	-0.02	-0.13	-0.10	-0.05	-0.01	0.0	0.0

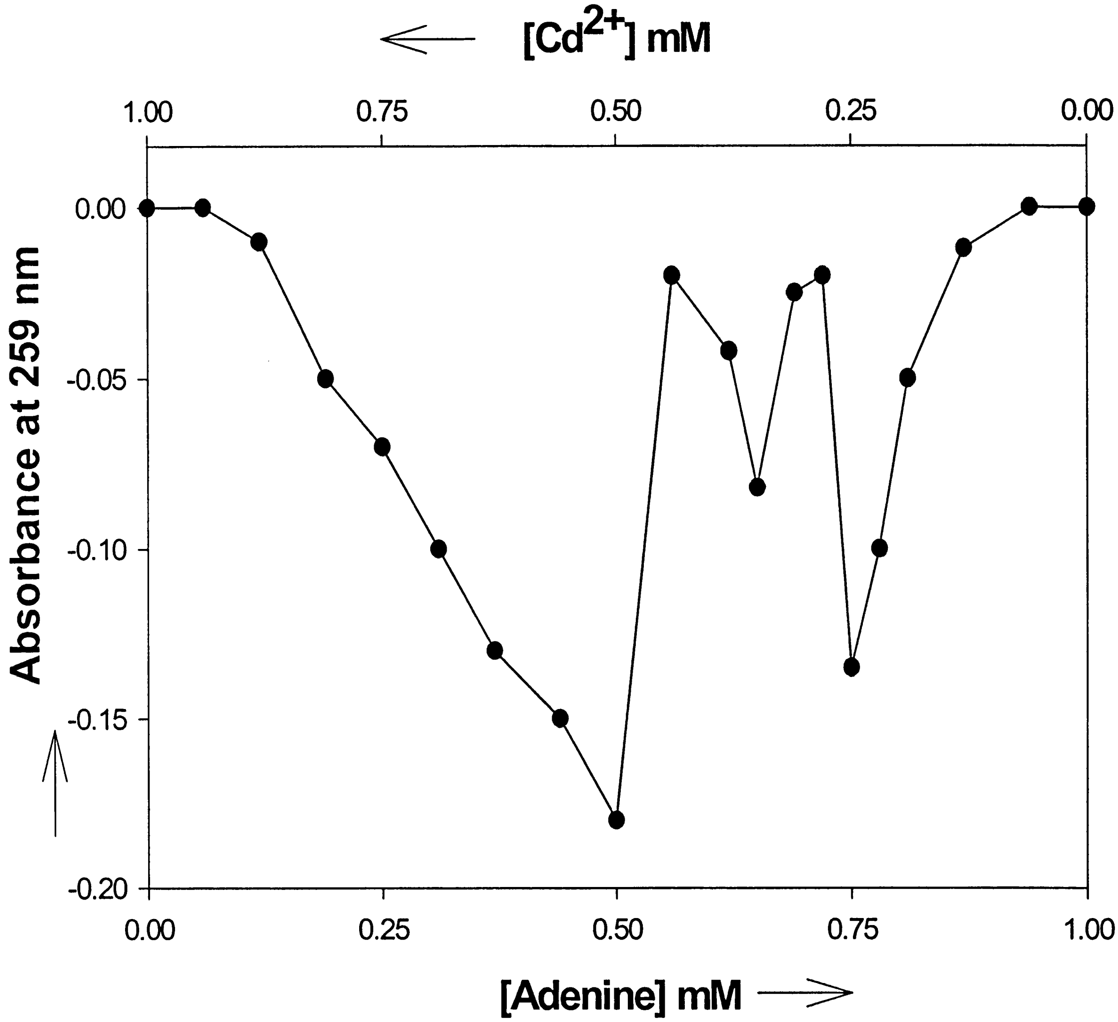


FIGURE 7.5: Interaction between Cd^{2+} and adenine: Plot of absorbance values at 259 nm, indicating the formation of 1:1, 1:2 and 1:3 adducts between Cd^{2+} and adenine

Table 7.8 gives the absorbance values applying to continuously varying mixtures of cadmium(II) nitrate and guanine against guanine blanks at 264 nm (the wavelength at which absorbance was found to be maximum).

When the absorbance values were plotted against concentrations of reactants, a sharply defined maximum was observed corresponding to the formation of 1:1 adduct between Cd^{2+} and guanine (Figure 7. 6), which is in agreement with 1:1 binding ratio between metal ions and the nucleobases, nucleosides and nucleotides found in the structures of crystals (Aoki, 1976, Prakash, *et al.*, 1998).

TABLE 7.8

Interaction between $Cd(NO_3)_2$ (1 mM) and guanine (1 mM) made in 0.10 M $NaNO_3$: Incubated for 24 h at room temperature Absorbances taken against guanine blanks

Solution	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S	T
mLs of $Cd(NO_3)_2$	4.0	3.75	3.50	3.25	3.0	2.75	2.50	2.25	2.00	1.75	1.50	1.38	1.25	1.13	1.0	0.88	0.75	0.50	0.25	0.0
mLs of guanine	0.0	0.25	0.50	0.75	1.0	1.25	1.50	1.75	2.00	2.25	2.50	2.62	2.75	2.87	3.00	3.13	3.25	3.50	3.75	4.0
$[Cd^{2+}]$ mM	1.0	0.94	0.88	0.81	0.75	0.69	0.63	0.56	0.50	0.44	0.38	0.35	0.31	0.28	0.25	0.22	0.19	0.13	0.06	0.0
[guanine] mM	0.0	0.06	0.12	0.19	0.25	0.31	0.37	0.44	0.50	0.56	0.62	0.65	0.69	0.72	0.75	0.78	0.81	0.87	0.94	1.0
Absorbance at 264 nm	0.0	0.03	0.06	0.13	0.20	0.27	0.33	0.40	0.48	0.36	0.22	0.14	0.11	0.08	0.04	0.01	0.01	0.0	0.0	0.0

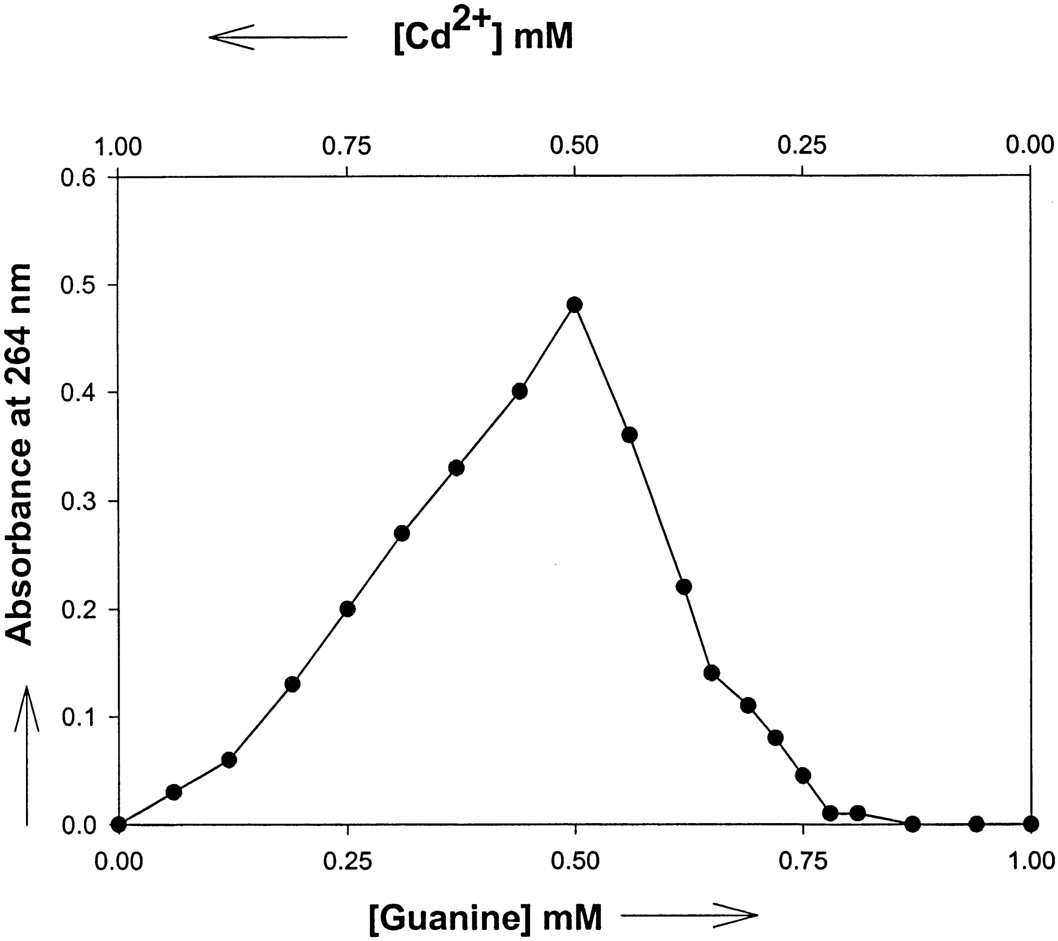


FIGURE 7.6: Interaction between Cd^{2+} and guanine: Plot of absorbance values at 264 nm, indicating the formation of 1:1 adduct between Cd^{2+} and guanine

Table 7.9 shows the absorbance values applying to continuously varying mixtures of cadmium(II) nitrate and thymine against thymine blanks, taken at 280 nm after 48 h incubation at room temperature.

When the absorbance values were plotted against concentrations, two minima were observed corresponding to the formation of 1:2 and 1:3 adducts between Cd^{2+} and thymine (Figure 7.7). In addition, two maxima were also observed corresponding to the formation of 2:1 and 7:1 adducts.

TABLE 7.9

Interaction between $Cd(NO_3)_2$ (1 mM) and thymine (1 mM) made in 0.10 M $NaNO_3$: Incubated for 48 h at room temperature

Absorbances taken against thymine blanks

Solution	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S	T
mLs of $Cd(NO_3)_2$	4.0	3.75	3.5	3.25	3.0	2.75	2.5	2.25	2.0	1.75	1.5	1.38	1.25	1.13	1	0.88	0.75	0.5	0.25	0.0
mLs of thymine	0.0	0.25	0.5	0.75	1.0	1.25	1.5	1.75	2.0	2.25	2.5	2.62	2.75	2.87	3	3.13	3.25	3.5	3.75	4.0
[Cd^{2+}] mM	1.0	0.94	0.88	0.81	0.75	0.69	0.63	0.56	0.5	0.44	0.38	0.35	0.31	0.28	0.25	0.22	0.19	0.13	0.06	0.0
[thymine] mM	0.0	0.06	0.12	0.19	0.25	0.31	0.37	0.44	0.5	0.56	0.62	0.65	0.69	0.72	0.75	0.78	0.81	0.87	0.94	1.0
Absorbance at 280nm	0.02	0.003	0.138	0.006	0.032	0.057	0.04	0.013	-0.02	-0.001	-0.02	-0.008	-0.02	-0.04	-0.02	-0.011	-0.01	-0.01	0.0	0.0

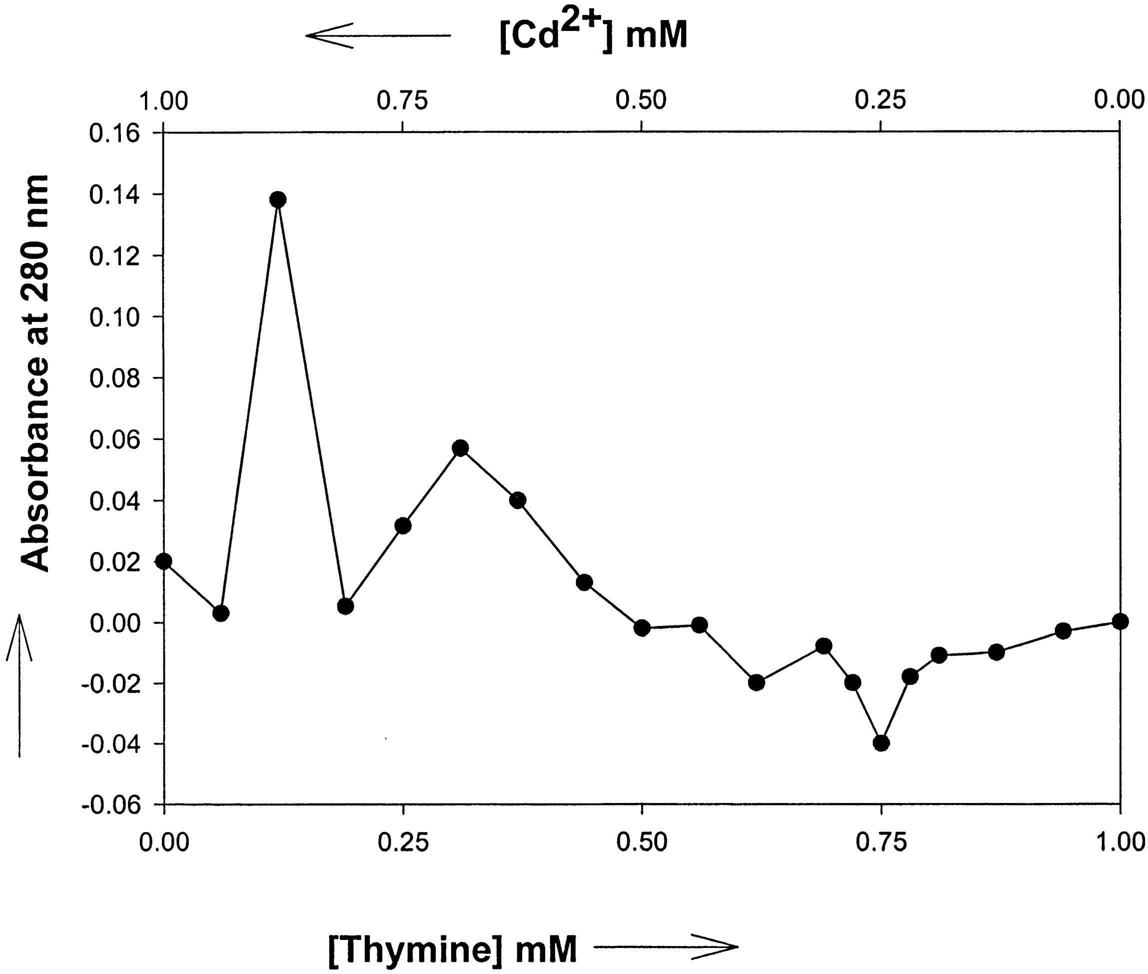


FIGURE 7.7: Interaction between Cd^{2+} and thymine: Plot of absorbance values at 280 nm, indicating the formation of 1:2, 1:3 and 2:1 adducts between Cd^{2+} and thymine

Table 7.10 shows the absorbance values applying to continuously varying mixtures of cadmium(II) nitrate and cytosine against cytosine blanks, taken at 283 nm after 48 h of incubation at room temperature.

When the absorbance values were plotted against concentration both a minimum and a maximum were observed. The minimum corresponded to the formation of 1:1 adduct and the maximum to the formation of 1:3 adduct between Cd^{2+} and cytosine (Figure 7. 8).

TABLE 7.10

Interaction between $Cd(NO_3)_2$ (1 mM) and cytosine (1 mM) made in 0.10 M $NaNO_3$: Incubated for 48 h at room temperature

Absorbances taken against cytosine blanks

Solution	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S	T
mLs of $Cd(NO_3)_2$	4	3.75	3.5	3.25	3	2.75	2.5	2.25	2	1.75	1.5	1.38	1.25	1.13	1	0.88	0.75	0.5	0.25	0.0
mLs of cytosine	0	0.25	0.5	0.75	1	1.25	1.5	1.75	2	2.25	2.5	2.62	2.75	2.87	3	3.13	3.25	3.5	3.75	4.0
[Cd^{2+}] mM	1	0.94	0.88	0.81	0.75	0.69	0.63	0.56	0.5	0.44	0.38	0.35	0.31	0.28	0.25	0.22	0.19	0.13	0.06	0.0
[cytosine] mM	0	0.06	0.12	0.19	0.25	0.31	0.37	0.44	0.5	0.56	0.62	0.65	0.69	0.72	0.75	0.78	0.81	0.87	0.94	1.0
Absorbance at 283 nm	0.028	0.021	0.023	0.024	0.026	0.006	0.0	-0.06	0.003	0.02	0.03	0.01	0.01	0.25	0.03	0.003	0.0003	0.0	0.0	0.0

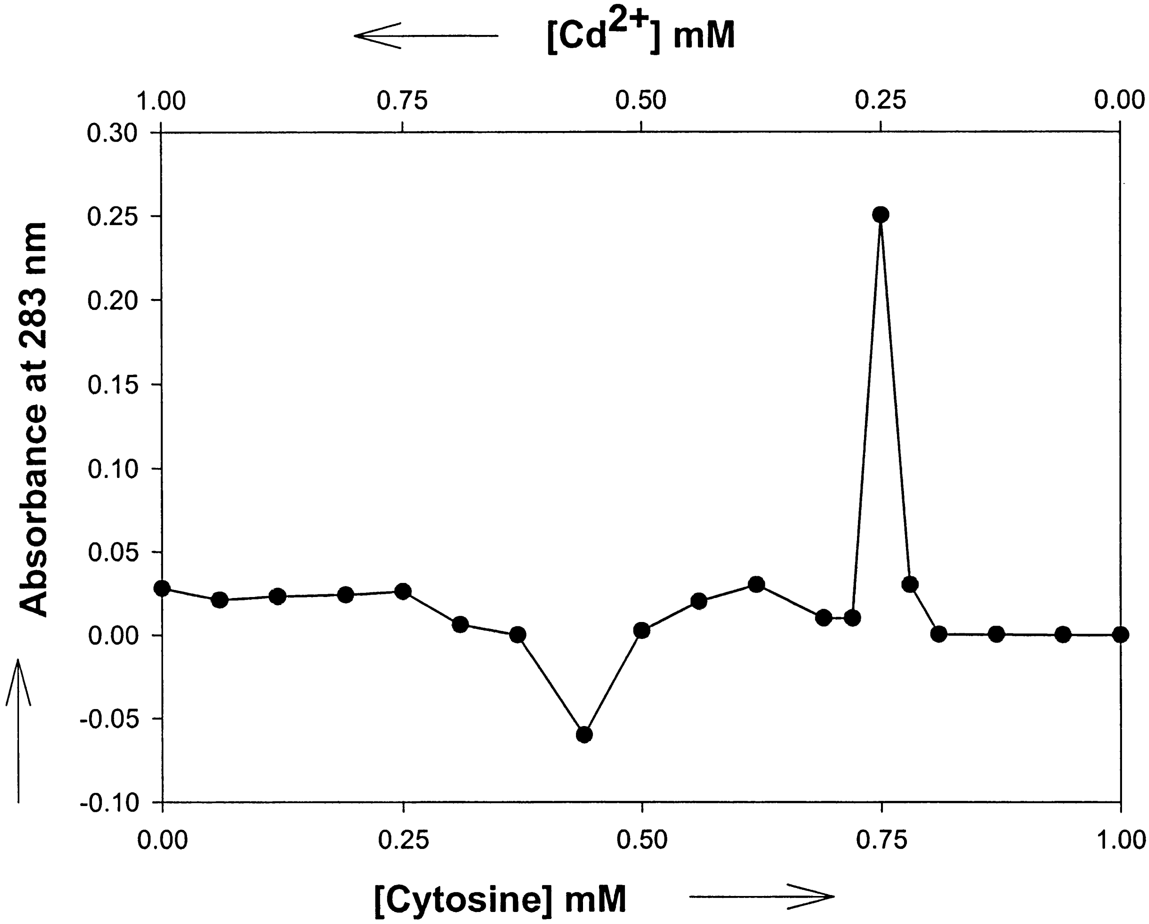


FIGURE 7.8: Interaction between Cd^{2+} and cytosine: Plot of absorbance values at 283 nm, indicating the formation of 1:1 and 1:3 adducts between Cd^{2+} and cytosine

Table 7.11 gives absorbance values applying to continuously varying mixtures of cadmium(II) nitrate and AMP at 274 nm. The plot of absorbance against concentration showed a minimum corresponding to the formation of 1:3 adduct (Figure 7.9) between Cd^{2+} and AMP.

TABLE 7.11

Interaction between $Cd(NO_3)_2$ (1 mM) and AMP (1 mM) made in 0.10 M $NaNO_3$: Incubated for 24 h at room temperature

Absorbances taken against AMP blanks

Solution	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S	T
mLs of $Cd(NO_3)_2$	4.0	3.75	3.50	3.25	3.0	2.75	2.50	2.25	2.00	1.75	1.50	1.25	1.13	1.00	0.88	0.75	0.50	0.25	0.0	0.0
mLs of AMP	0.0	0.25	0.50	0.75	1.0	1.25	1.50	1.75	2.00	2.25	2.50	2.75	2.87	3.00	3.12	3.25	3.50	3.75	4.0	4.0
[Cd^{2+}] mM	1.0	0.94	0.88	0.81	0.75	0.69	0.63	0.56	0.50	0.44	0.38	0.31	0.28	0.25	0.22	0.19	0.13	0.06	0.0	0.0
[AMP] mM	0.0	0.06	0.12	0.19	0.25	0.31	0.37	0.44	0.50	0.56	0.62	0.69	0.72	0.75	0.78	0.81	0.87	0.94	0.0	1.0
Absorbance at 274 nm	0.0	0.0	0.0	-0.012	-0.014	-0.017	-0.019	0.0	0.0	0.0	-0.07	-0.08	-0.10	-0.09	-0.26	-0.04	-0.01	-0.01	0.0	0.0

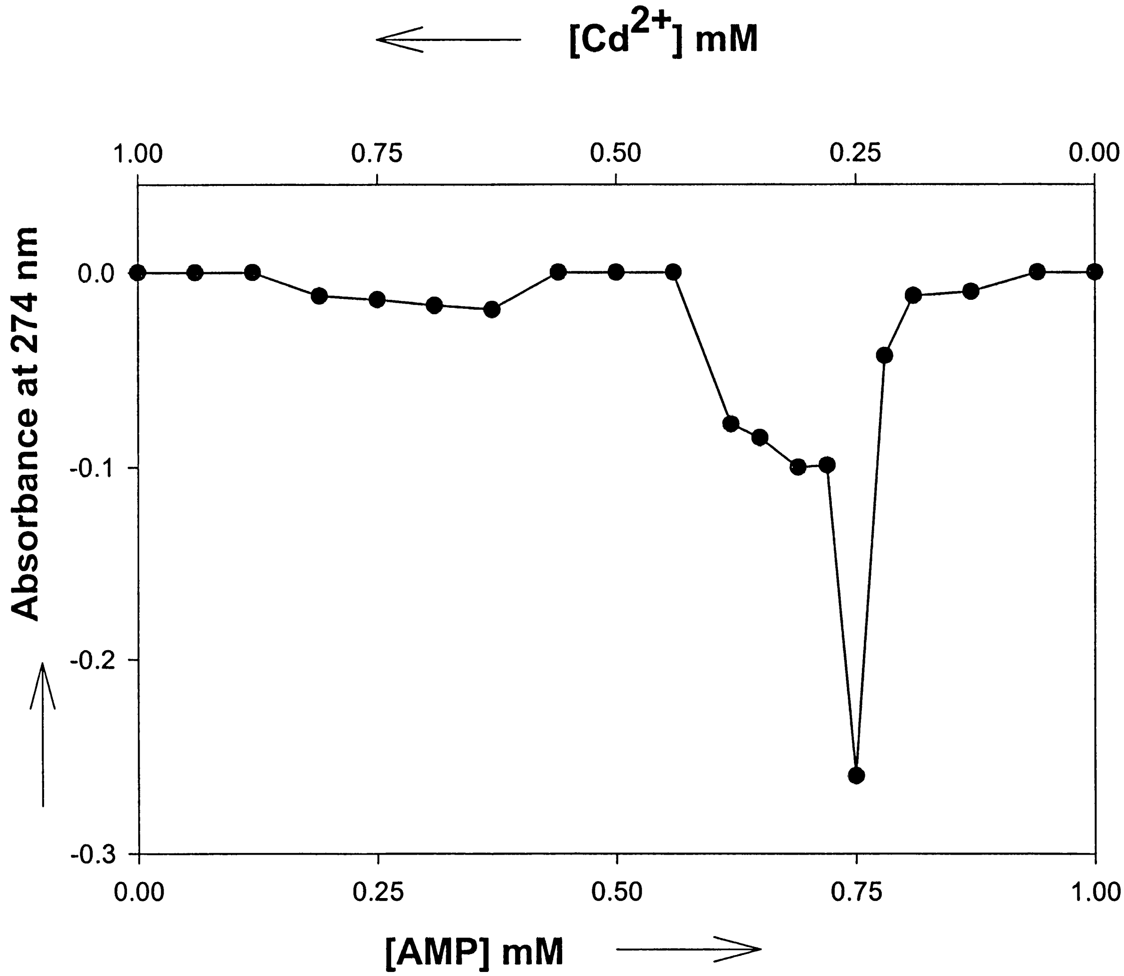


FIGURE 7.9: Interaction between Cd^{2+} and AMP: Plot of absorbance values at 274 nm, indicating the formation of 1:3 adduct between Cd^{2+} and AMP

Table 7.12 gives absorbance values applying to continuously varying mixtures of cadmium(II) nitrate and 2'-dGMP at 287 nm. The maximum observed in the absorbance versus concentration plot indicated the formation of 1:1 adduct between Cd^{2+} and 2'-dGMP (Figure 7.10).

TABLE 7.12

Interaction between $Cd(NO_3)_2$ (1 mM) and 2'-dGMP (1 mM) made in 0.10 M $NaNO_3$: Incubated for 24 h at room temperature

Absorbances taken against 2'-dGMP blanks

Solution	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S	T
mLs of $Cd(NO_3)_2$	4.0	3.75	3.50	3.25	3.0	2.75	2.50	2.25	2.00	1.75	1.50	1.38	1.25	1.13	1.0	0.88	0.75	0.50	0.25	0.0
mLs of 2'-dGMP	0.00	0.25	0.50	0.75	1.0	1.25	1.50	1.75	2.00	2.25	2.50	2.62	2.75	2.87	3.00	3.13	3.25	3.50	3.75	4.0
[Cd^{2+}] mM	1.0	0.94	0.88	0.81	0.75	0.69	0.63	0.56	0.50	0.44	0.38	0.35	0.31	0.28	0.25	0.22	0.19	0.13	0.06	0.0
[2'-dGMP] mM	0.0	0.06	0.12	0.19	0.25	0.31	0.37	0.44	0.50	0.56	0.62	0.65	0.69	0.72	0.75	0.78	0.81	0.87	0.94	1.0
Absorbance at 287 nm	0.0	0.02	0.03	0.02	0.01	0.0	0.25	0.36	0.48	0.40	0.32	0.10	0.07	0.05	0.03	0.02	0.01	0.01	0.0	0.0

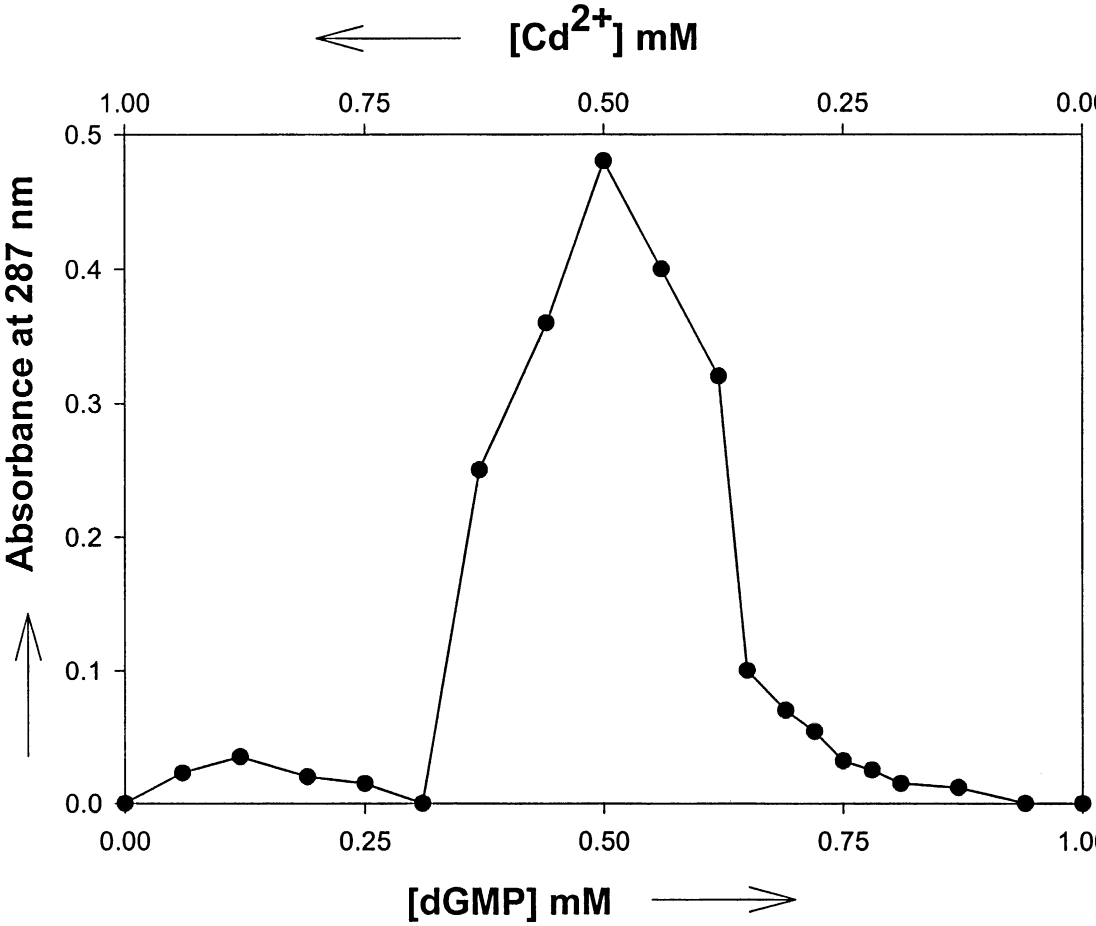


FIGURE 7.10: Interaction between Cd^{2+} and 2'-dGMP: Plot of absorbance versus concentration at 287 nm indicating the formation of 1:1 adduct between Cd^{2+} and 2'-dGMP

7.1.2 HPLC analysis

The retention time applying to the major peak in the chromatogram of incubated mixture of Cd^{2+} and adenine was observed to be different from that of adenine (6.65/6.67 versus 7.45 min) (Table 7.13). However, when the peak fraction was analysed for cadmium content by AAS, no significant amount of cadmium was found to be present. It was also found that the retention time of the major peak did not change when the molar ratio of the reactants was changed. The analyses of minor peak fractions indicated the formation of 1:1, 1:2 adducts between cadmium and adenine (results not shown in the table). There was also a tiny peak corresponding to the formation of 1:4 adducts. When the period of incubation was varied from 20 min to 24 h no change in the retention time of the major peak was found, suggesting that cadmium reacted with adenine quickly.

In the case of Cd^{2+} interacting with guanine, the retention time of the major peak in the chromatogram of the incubated mixture was found to be practically the same as that of guanine (3.43 min versus 3.48 min) (Table 7.13). It was also found that the peak fraction did not have any significant cadmium.

In the case of Cd^{2+} interacting with AMP, the fraction corresponding to the major peak was not found to have any significant cadmium although there was a change in retention time as compared to that of AMP (4.73 versus 4.92 min) (Table 7.13).

In the case of Cd^{2+} interacting with 2'-dGMP there was no significant change in retention time (4.19 versus 4.25 min) (Table 7.13) as compared to that of 2'-dGMP and also the peak fraction did not have any significant amount of cadmium.

TABLE 7.13: Interaction between 1 mM $Cd(NO_3)_2$ or $Cd(OOCCH_3)_2$ and 1 mM nucleobase or nucleotide (N) mixed at 1:1 molar ratio

Reactant A	Reactant B	λ_{max} (nm)	HPLC Retention time (min) for the major peak	UV- absorbance of the major peak fraction at λ_{max}	Cadmium Content (ppb)	Binding ratio $[Cd^{2+}]$:[N]
Adenine (0.50 mM)	None	260	7.45	0.08	N/A[1]	N/A
Adenine (1 mM)	$Cd(NO_3)_2$ (1 mM)	260	6.65	N/A	20	1:36
Adenine (1 mM)	$Cd(OOCCH_3)_2$ (1 mM)	260	6.67		18	1:37
Guanine (0.50 mM)	None	260	3.48	0.18	N/A	N/A
Guanine (1 mM)	$Cd(NO_3)_2$ (1 mM)	260	3.43	N/A	4.60	1:178
AMP (0.5 mM)	None	260	4.92	0.07	N/A	
AMP (1 mM)	$Cd(NO_3)_2$ (1 mM)	260	4.73	N/A	3.75	1:166
2'-dGMP (0.50 mM)	None	260	4.25	0.01	N/A	
2'-dGMP (1 mM)	$Cd(NO_3)_2$ (1 mM)	260	4.13	N/A	60	1: 19

[1]: N/A means not applicable

7.2 Interaction between Cd^{2+} and salmon sperm DNA (ssDNA)

7.2.1 UV-difference Spectral analysis

Table 7.14 shows the UV difference spectral results at 260 nm applying to the mixtures of cadmium(II) nitrate and ssDNA taken against ssDNA blank where the concentration of cadmium(II) nitrate was varied from 0.005 to 20 mM and that of ssDNA was kept constant at 0.07 mM (in terms of phosphate). The absorbance values were found to be negative for the [Cd^{2+}] range: 0.005 to 0.30 mM and positive for 0.50 up to 20 mM. Within the two ranges, as the concentration of Cd^{2+} was increased the magnitude of the absorbance was found to increase as well. The change in absorbance (decrease or increase) is believed to be associated with the increase in the amount of Cd^{2+} ions that are bound to ssDNA.

TABLE 7.14: Interaction between $Cd(NO_3)_2$ (0.005 mM to 20 mM) and salmon sperm DNA (ssDNA) (0.07 mM): Incubated for 24 h at 37°C: Absorbance values taken at 260 nm against DNA blanks

Solution	A	B	C	D	E	F	G	H	I	J	K	L	M	N
[$Cd(NO_3)_2$] in mM	0.0	0.005	0.01	0.02	0.05	0.10	0.20	0.30	0.50	1.0	1.50	5.0	10.0	20.0
[ssDNA] in mM	0.07	0.07	0.07	0.07	0.07	0.07	0.07	0.07	0.07	0.07	0.07	0.07	0.07	0.07
Absorbance at 260 nm	0.0	-0.1	-0.15	-0.20	-0.25	-0.32	-0.48	-0.48	0.20	0.34	0.37	0.52	0.58	0.02

7.2.2 HPLC analysis

From the UV-difference spectral results, it was inferred that Cd^{2+} interacted covalently with ssDNA. To further investigate the interaction between Cd^{2+} and ssDNA, the HPLC studies were carried out.

Table 7.15 gives the retention times applying to the major peaks in the chromatogram of the incubated mixtures of Cd^{2+} and ssDNA (for which the concentration of Cd^{2+} was varied from 0 to 50 mM and that of ssDNA kept unchanged at 0.07 mM). Three major peaks were observed in the chromatogram of ssDNA alone and also in the chromatograms of mixtures for which the concentration of Cd^{2+} ranged from 0 to 10 mM. In addition, three minor peaks were also observed when the concentration of Cd^{2+} was 12.5 mM. No peak was observed when the concentration of Cd^{2+} ranged from 25 to 50 mM. The retention times for the three major peaks of ssDNA were 3.43, 7.55 and 9.22 min. In the case of mixtures of Cd^{2+} and ssDNA, it was found that the retention time of the first peak remained essentially unchanged up to 10 mM. There was a slight decrease in the retention time for peak 2 (from 7.55 to 7.13 min) and a marked decrease in retention time for peak 3 (from 9.22 to 8.07 min).

The retention times for the three minor peaks were found to be 3.43, 7.23 and 8.78 min. The results suggest that with the increase in the concentration of Cd^{2+} more and more of the metal ions get bound to DNA, ultimately causing extensive damage to ssDNA. This will be considered in a greater detail in the discussion (See chapter 8 in section 8.2.2, page 226).

TABLE 7.15: Interaction between $Cd(NO_3)_2$ and salmon sperm DNA (ssDNA) at 1:1volume ratio

Reactant A	Reactant B	λ_{max} (nm)	Retention time (min) for the major peak 1	Retention time (min) for the major peak 2	Retention time (min) for the major peak 3
ssDNA (0.07 mM)	None	260	3.43	7.55	9.22
ssDNA (0.07 mM)	$Cd(NO_3)_2$ (1 mM)	260	3.40	7.13	8.07
ssDNA (0.07 mM)	$Cd(NO_3)_2$ (5 mM)	260	3.37	7.13	8.07
ssDNA (0.07 mM)	$Cd(NO_3)_2$ (10 mM)	260	3.37	7.13	8.07
ssDNA (0.07 mM)	$Cd(NO_3)_2$ (12.50 mM)	260	3.43 (minor peak)	7.23 (minor peak)	8.78 (minor peak)
ssDNA (0.07 mM)	$Cd(NO_3)_2$ (25 mM)	260	No peak	No peak	No peak
ssDNA (0.07 mM)	$Cd(NO_3)_2$ (50 mM)	260	No peak	No peak	No peak

7.3 HPLC analysis of 8-oxoguanine and 8-oxoadenine

These experiments were carried out to determine whether the observed change in retention time for adenine (or guanine) due to interaction with Cd^{2+} (or Ni^{2+}) was due to the formation of 8-oxoadenine (or 8-oxoguanine). Table 7.16 shows the retention times applying to the major peaks in the chromatogram of 8-oxoguanine and 8-oxoadenine (prepared from guanine and adenine respectively by reaction with H_2O_2 in presence of ascorbic acid). The retention time for 8-oxoguanine was found to be different from that of guanine (1.50 versus 3.83 min). Similarly, the retention time of 8-oxoadenine was found to be different from that of adenine and (2.80 versus 7.35 min). More importantly, the retention time of 8-oxoadenine was found to be different from that of the major peak in the incubated mixture of Cd^{2+} and adenine (Table 7.13) clearly showing that 8-oxoadenine was not produced from the interaction of adenine with Cd^{2+}. This will be considered in a greater detail in the discussion (See chapter 8 in section 8.1.2, page 219).

TABLE 7.16: Retention time applied to the major peaks of 8-oxoguanine and 8-oxo adenine against those of guanine and adenine

Reactant	λ_{max} (nm) at which peak was detected	Retention time (min) for major peak
Guanine (0.06 mM)	260	3.83
8-oxoguanine (0.06 mM)	260	1.50
Adenine (0.06 mM)	260	7.35
8-oxoadenine (0.06 mM)	260	2.80

7.4 Interaction between Cd^{2+} and antioxidants

Cd^{2+} being a soft metal ion is expected to bind covalently with sulfur donor ligands cysteine and glutathione which also act as antioxidants. As Cd^{2+} enters the biological system, it is likely to encounter these antioxidant ligands before it can interact with DNA. Thus, it was considered appropriate to investigate the interaction of cadmium-cysteine or cadmium-glutathione complex with DNA constituents.

7.4.1 Interaction between Cd^{2+} and cysteine or glutathione: *HPLC analysis*

Table 7.17 shows the retention times applying to the major peaks for 1:2 mixtures of cadmium(II) nitrate and cysteine or glutathione. It is found that whereas only one major peak is observed for cysteine (retention time: 1.37 min) or glutathione (retention time: 1.47), two peaks are found in the incubated mixtures of Cd^{2+} and cysteine (retention times: 1.40 and 3.50 min) or Cd^{2+} and glutathione (retention times:1.49 and 3.53 min). The extra peak found in the chromatogram of each of the incubated mixtures is believed to be due to the formation a complex between Cd^{2+} and the ligand. It is found that in each case, the size of the extra peak is much greater than that due to the free ligand, indicating that most of the ligand has complexed with cadmium. It should also be noted that on a relative scale the size of the peak due to free glutathione is smaller than that due to free cysteine showing that glutathione has complexed more fully with cadmium than cysteine did.

TABLE 7.17: Retention times applied to the major peaks after interaction between Cd^{2+} and cysteine or glutathione against that of cysteine or glutathione

Reactant A	Reactant B	λ_{max} (nm)	Retention time (min) for the major peak 1	Retention time (min) for the major peak 2
Cysteine (2 mM)	None	260	1.37	None
Cysteine (2 mM)	$Cd(NO_3)_2$ (1 mM)	260	1.40	3.50
Glutathione (2 mM)	None	260	1.47	None
Glutathione (2 mM)	$Cd(NO_3)_2$ (1 mM)	260	1.49	3.53

7.4.2 Interaction between Cd^{2+}-cysteine complex and adenine: *HPLC analysis*

Table 7.18 shows the retention times applying to the major peaks for incubated mixtures of Cd^{2+}-cysteine complex and adenine. It is found that the chromatogram contains three major peaks having retention times of 3.50, 3.99 and 4.50 min. The peak at 3.50 min is due to Cd(II)-cysteine complex (Table 7.17) and that at 3.99 min is believed due to a cadmium-cysteine-adenine complex. The peak at 4.50 min is believed to be due to modified adenine produced from its interaction with Cd^{2+} (See chapter 8 in section 8.1.2 page 220).

7.4.3 Interaction between Cd^{2+}-cysteine complex and guanine: *HPLC analysis*

Table 7.18 shows the retention times applying to the major peaks for incubated mixtures of Cd^{2+}-cysteine complex and guanine. It is found that the chromatogram contains three major peaks having retention times of 3.50, 3.57 and 3.90 min. The peak at 3.50 min is due to guanine whereas that at 3.57 min is believed to be due to Cd^{2+}-cysteine complex. The peak at 3.90 min (which is the largest) is believed to be due to cadmium-cysteine-guanine complex.

TABLE 7.18: Retention time applied to the major peaks of the mixtures of Cd^{2+}-cysteine complex and adenine or guanine against that of adenine or guanine blank

Reactant A	Reactant B	λ_{max} (nm)	Retention time (min) for the major peak 1	Retention time (min) for the major peak 2	Retention time (min) for the major peak 3
Adenine (1 mM)	None	260	7. 40	None	None
Cd^{2+}-cysteine (1:2)	Adenine (1 mM)	260	3.50	3.99	4.50
Guanine (1 mM)	None	260	3.48	None	None
Cd^{2+}-cysteine (1:2)	Guanine (1.0 mM)	260	3.50	3.57	3.90

7.4.4 Interaction between Cd^{2+}-glutathione complex and adenine: *HPLC analysis*

Table 7.19 shows the retention times applying to the major peaks for incubated mixtures of Cd^{2+}-glutathione complex and adenine. It is found that the chromatogram contains three major peaks having retention times of 3.57, 3.63 and 4.57 min. The peak at 3.57 min is believed to be due to Cd^{2+}-glutathione complex whereas that at 3.63 min (which is the largest in size) is believed to be due to cadmium-glutathione-adenine complex. The peak at 4.57 min (which is smallest in size) is due to modified adenine produced from the interaction of free adenine with cadmium (See chapter 8 in section 8.1.2 page 220).

7.4.5 Interaction between Cd^{2+}-glutathione complex and guanine: *HPLC analysis*

Table 7.19 shows the retention times applying to the major peaks for incubated mixtures of Cd^{2+}-glutathione complex and guanine. It is found that the chromatogram contains three major peaks having retention times of 3.47, 3.56 and 3.62 min. The peak at 3.47 min is believed to be due to free guanine, that at 3.56 min is due to Cd^{2+}-glutathione complex. The peak at 3.62 min (which is the largest in size) is believed to be due to cadmium-glutathione-guanine complex.

TABLE 7.19: Retention time applied to the major peaks of the mixtures of Cd^{2+}-glutathione complex and adenine or guanine against that of adenine or guanine

Reactant A	Reactant B	λ_{max} (nm)	Retention time (min) for the major peak 1	Retention time (min) for the major peak 2	Retention time (min) for the major peak 3
Adenine (1 mM)	None	260	7. 35	None	None
Cd^{2+}-glutathone (1:2)	Adenine (1 mM)	260	3.57	3.63	4.57
Guanine (1 mM)	None	260	3.48	None	None
Cd^{2+}-glutathione (1:2)	Guanine (1 mM)	260	3.47	3.56	3.62

7.5 Interaction between Cd^{2+} and ascorbate: *UV-difference spectral analysis*

Table 7.20 shows the absorbance values applying to continuously varying mixtures of cadmium(II) acetate and ascorbate against ascorbate blanks, taken at 266 nm. When the absorbances were plotted against concentrations a minimum was observed corresponding to the formation of 1:1 adduct between Cd^{2+} and ascorbate (Figure 7.11).

TABLE 7.20

Interaction between $Cd(CH_3COO)_2$ (1 mM) and ascorbate (1 mM) made in 0.10 M $NaNO_3$: Incubated for 24 h in the dark at room temperature

Absorbances taken against ascorbate blanks

Solution	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S	T
mLs of $Cd(CH_3COO)_2$	4.0	3.75	3.50	3.25	3.0	2.75	2.50	2.25	2.00	1.75	1.50	1.38	1.25	1.13	1.00	0.88	0.75	0.50	0.25	0.0
mLs of ascorbate	0.00	0.25	0.50	0.75	1.0	1.25	1.50	1.75	2.00	2.25	2.50	2.62	2.75	2.87	3.00	3.13	3.25	3.50	3.75	4.0
$[Cd^{2+}]$ mM	1.0	0.94	0.88	0.81	0.75	0.69	0.63	0.56	0.50	0.44	0.38	0.35	0.31	0.28	0.25	0.22	0.19	0.13	0.06	0.0
[ascorbate] mM	0.00	0.06	0.12	0.19	0.25	0.31	0.37	0.44	0.50	0.56	0.62	0.65	0.69	0.72	0.75	0.78	0.81	0.87	0.94	1.0
Absorbance at 266 nm	0.0	0.0	-0.04	-0.06	-0.07	-0.07	-0.10	-0.11	-0.13	-0.10	-0.07	-0.10	-0.11	-0.11	-0.10	-0.08	-0.01	-0.01	0.00	0.0

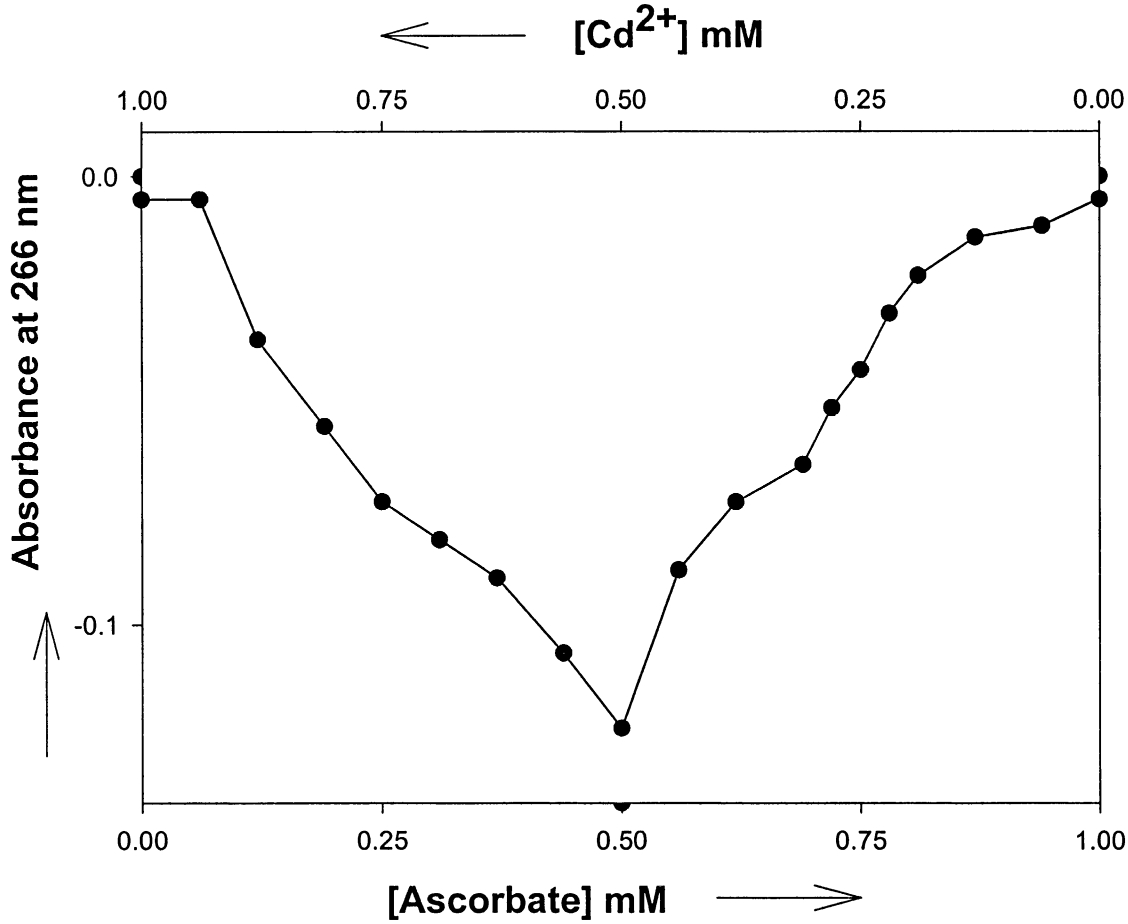


FIGURE 7.11: Interaction between Cd^{2+} and ascorbate: Plot of absorbance versus concentration at 266 nm indicating the formation of 1:1 adduct between Cd^{2+} and ascorbate

7.6 Interaction between Cd^{2+} and DNA

7.6.1 Cd^{2+}-plasmid binding assays

Plasmid (pBR322) DNA used in the experiment, was found to be a mixture of Form I and Form II bands (mainly Form I). It was found that when the concentration of cadmium(II) nitrate was ≥ 100 mM, total cleavage of DNA occurred so that no band was visible (Figure 7.12(a) *lane 3*). In fact, even when the concentration of cadmium(II) nitrate was 50 mM, the bands were found to be extremely faint (Figure 7.12 (a) *lane 4*)

[Cd(II) nitrate] decreases from left to right

Key: Lane 1: Untreated pBR322 plasmid DNA, Lane 2: none, Lanes 3-11: DNA + Cd(II) nitrate: Lane 3: [Cd^{2+}] 100 mM, Lane 4: [Cd^{2+}] 50 mM, Lane 5: [Cd^{2+}] 25 mM, Lane 6: [Cd^{2+}] 12.5 mM, Lane 7: [Cd^{2+}] 6.25 mM, Lane 8: [Cd^{2+}] 1 mM, Lane 9: [Cd^{2+}] 100 μM, Lane 10: [Cd^{2+}] 50 μM, Lane 11: [Cd^{2+}] 25 μM

FIGURE 7.12 (a): Interaction between Cd(II) nitrate and plasmid DNA in TBE buffer at the pH of the solution

It was also found that the presence of a smaller amount of cadmium(II) nitrate (concentration 25 mM) caused a significant increase in the intensity of the Form II band as compared to that observed in the untreated DNA (Figure 7.12 (a) *lane 5*). No noticeable change in the migration rates of the bands was observed. Since Cd^{2+} is not redox active, it is believed that the partial or the total cleavage of DNA in presence of cadmium(II) nitrate is the result of covalent binding of Cd^{2+} with the nucleobases in DNA (most likely adenine and guanine). Because cadmium(II) nitrate solution in water is acidic (pH about 5.8), it is possible that H^+ ions could have contributed to the damage to DNA, especially in the presence of molecular oxygen (More about in chapter 8 in section 8.4.1 page 228).

To determine whether this was indeed the case, varying amounts of 0.01 M HCl or 0.01 M NaOH was added to 1 μL plasmid DNA so that pH of the mixtures varied from 3 to 9. The mixtures were then incubated for 5.50 h at 37°C, following which electrophoresis was carried out. It should be noted that all the incubation experiments in DNA binding works were done under sterilised conditions. It was found that, judging from the absence of any DNA band, an extensive damage to DNA occurred at pH 3. Between pH 5 to 9 two bands (Form I and Form II) were observed without any significant change in relative intensities of the bands (Figure 7.12 (b)).

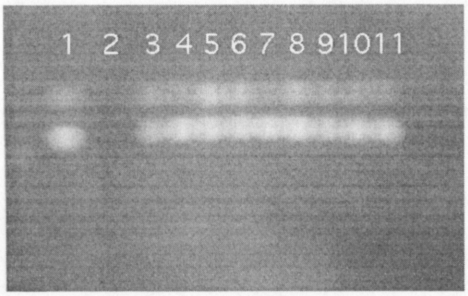
Key: Lane 1: Untreated pBR322 plasmid DNA in water, Lanes 2-3: none, Lane 4: DNA at pH 3, Lane 5: DNA at pH 5, Lane 6 DNA at pH 6, Lane 7: DNA at pH 7, Lane 8: DNA at pH 8, Lane 9: DNA at pH 9.

FIGURE 7.12 (b): Electrophoresis of plasmid DNA in TBE buffer at different pH

Thus, it can be concluded that the observed damage to the DNA at pH 5.8 in the presence of cadmium(II) nitrate is associated with its interaction with Cd^{2+} ions.

To determine whether the acidic pH played a contributory role, the pH of the cadmium(II) nitrate solution was adjusted to 7.4 by adding 0.01 M NaOH before its addition to the DNA. Two bands were observed at all concentrations of cadmium(II) nitrate from 0 to 100 mM, and the relative intensities of the bands remained almost unchanged, although the mobility of the bands were found to increase slightly as $[Cd^{2+}]$ decreased (Figure 7.12 (c)). It can therefore be concluded that the damage to the DNA due to its binding with Cd^{2+} ions is accentuated by the presence of H^+ ions.

[Cd(II) nitrate] decreases from left to right



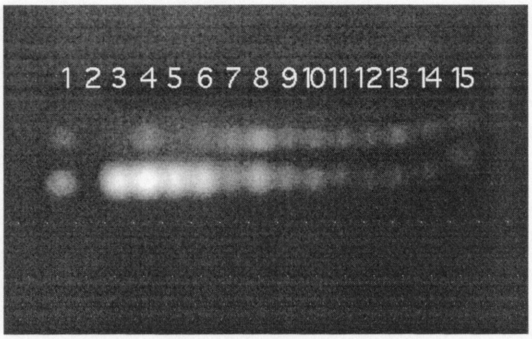
Key: Lane 1: Untreated pBR322 plasmid DNA, Lane 2: none, Lanes 3-11: DNA + Cd(II) nitrate: Lane 3: [Cd^{2+}] 100 mM, Lane 4: [Cd^{2+}] 50 mM, Lane 5: [Cd^{2+}] 25 mM, Lane 6 [Cd^{2+}] 12.5 mM, Lane 7: [Cd^{2+}] 6.25 mM, Lane 8: [Cd^{2+}] 1 mM, Lane 9: [Cd^{2+}] 100 μM, Lane 10: [Cd^{2+}] 50 μM, Lane 11: [Cd^{2+}] 25 μM

FIGURE 7.12 (c): Interaction between Cd(II) nitrate and plasmid DNA in TBE buffer at pH 7.4

As the pH was increased to obtain a neutral condition for cadmium(II) nitrate solution some quantity of cadmium was removed from solution (at high [Cd^{2+}]) due to the precipitation of cadmium(II) hydroxide (characterised by elemental analysis) so that the actual concentration of Cd^{2+} in solution was lowered.

Since, cadmium(II) acetate in solution has a pH close to 7.0; the plasmid binding experiments were also carried out using cadmium(II) acetate instead of cadmium(II) nitrate as the source of Cd^{2+} ions. It was found that when cadmium(II) acetate was used, the Form I band was more prominent than Form II band at low concentrations (from 0 up to 100 μM). At higher concentrations of cadmium(II) acetate (1 mM to 6.25 mM), the intensity of the Form I band was found to decrease, that of Form II band was found to increase slightly. At still higher concentrations of cadmium(II) acetate (12.5 mM to 100 mM), no noticeable change in the intensity of either band could be detected. However, the mobility of both the bands were found to decrease, as the concentration of cadmium(II) acetate was increased (Figure 7.12 (d)). The result was similar to that observed in the case of cadmium(II) nitrate except that the intensity of the bands was greater in the case of cadmium(II) acetate.

[Cd(II) acetate] increases from left to right



Key: Lane 1: Untreated pBR322 plasmid DNA, Lane 2: none, Lanes 3-15: DNA + Cd(II) acetate: Lane 3: [Cd^{2+}] 12.5 μM, Lane 4: [Cd^{2+}] 25 μM, , Lane 5: [Cd^{2+}] 50 μM, Lane 6: [Cd^{2+}] 100 μM, Lane 7: [Cd^{2+}] 1 mM, Lane 8: [Cd^{2+}] 2 mM, Lane 9: [Cd^{2+}] 4 mM, Lane 10: [Cd^{2+}] 6.25 mM, Lane 11: [Cd^{2+}] 12.5 mM, Lane 12: [Cd^{2+}] 25 mM, Lane 13: [Cd^{2+}] 37.5 mM, Lane 14: [Cd^{2+}] 50 mM, Lane 15: [Cd^{2+}] 100 mM

FIGURE 7.12 (d): Interaction between Cd(II) acetate and plasmid DNA in TAE buffer at pH 7.4

These results clearly show that Cd^{2+} ions bind covalently with DNA.

7.6.2 Enzyme digestion

Restriction enzyme digestion is an important biochemical technique providing information about the nature of binding of small molecules or ions to DNA. When small molecules bind to nucleotides immediately adjacent to the recognition sequence or the cleave sites, the cutting of DNA by restriction enzyme may be inhibited. For example, cis-$Pt(NH_3)_2(H_2O)_2^{2+}$ is found to bind predominantly to the adjacent GG sites in the single strand of a DNA molecule (Fichtinger-Schempman, *et al.*, 1982, Fichtinger-Schempman, *et al.*, 1985) which prevents the hydrolysis of the phosphodiester bond between the two nucleotide units. Since hydrated Cd^{2+} is expected to bind to purine nucleobases guanine and adenine in a DNA strand, it is possible that such binding may prevent the restriction-enzyme digestion of the DNA. Thus, the binding of hydrated Cd^{2+} to the GG sites, may prevent (or retard) the BamH1 digestion at this site.

When plasmid DNA was digested with BamH1 (which cuts at the GG sites), only the Form III band was observed irrespective of whether it was preceded by the interaction of DNA with Cd^{2+} or not (Figure 7.13 (a)). However, the mobility of the band was found to decrease with the increase in the concentration of Cd^{2+} in a manner similar to that observed in the absence of enzyme digestion (Figures 7.12 (c) and 7.12 (d)).

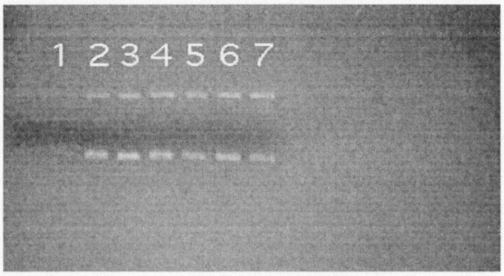
[Cd(II) acetate] increases from left to right

Key: Lane 1: Untreated pBR322 plasmid DNA, Lane 2: none, Lanes 3-14: Plasmid DNA + Cd(II) acetate: Lane 3: [Cd^{2+}] 12.5 μM, Lane 4: [Cd^{2+}] 25 μM, Lane 5: [Cd^{2+}] 50 μM, Lane 6: [Cd^{2+}] 100 μM, Lane 7: [Cd^{2+}] 1 mM, Lane 8: [Cd^{2+}] 2 mM, Lane 9: [Cd^{2+}] 4 mM, Lane 10: [Cd^{2+}] 6.25 mM, Lane 11: [Cd^{2+}] 12.5 mM, Lane 12: [Cd^{2+}] 25 mM, Lane 13: [Cd^{2+}] 50 mM, Lane 14: [Cd^{2+}] 100 mM

FIGURE 7. 13 (a): Interaction between Cd(II) acetate and plasmid DNA in TAE buffer at pH 7.4 followed by Bam H1 enzyme digestion

When the enzyme Ssp1 (which cuts at the AT sites) was used in stead of BamH1, two bands were observed when the enzyme digestion was preceded by interaction of DNA with Cd^{2+} as against one band for the untreated DNA (Figure 7.1 3 (b)). The results suggest that Cd^{2+} binds covalently with DNA (possibly at G, A T sites).

[Cd(II) acetate] increases from left to right



Lane 1: Untreated pBR322 plasmid DNA, Lanes 2-7: DNA + Cd(II) acetate: Lane 2: [Cd^{2+}] 100 μM, Lane 3: [Cd^{2+}] 1 mM, Lane 4: [Cd^{2+}] 12.5 mM, Lane 5: [Cd^{2+}] 25 mM, Lane 6: [Cd^{2+}] 50 mM, Lane 7: [Cd^{2+}] 100 mM

FIGURE 7.13 (b): Interaction between Cd(II) acetate and plasmid in TAE buffer at pH 7.4 followed by Ssp1 enzyme digestion

7.7 Cd^{2+}-plasmid binding in presence of antioxidants

Cellular antioxidants include ascorbate, cysteine and glutathione. It is believed that the antioxidants can protect cellular DNA from damage due to ROS and reactive nitrogen species (RNS) as they react more readily with ROS and RNS than DNA does. Cd^{2+} can modulate the effect of antioxidants such as cysteine and glutathione as it can bind covalently with these sulfur-donor ligands. It will be seen later that Cd^{2+} also binds covalently with ascorbate forming 1:1 adduct.

7.7.1 Cd^{2+}-cysteine and plasmid

The plasmid DNA used for interaction with $Cd(cysteine)_2$ was found to be of Form I (Figure 7.14 (a) *lane 1*). When it was allowed to interact with $Cd(cysteine)_2$ (formed in solution due to interaction between cadmium(II) nitrate and L-cysteine), only Form II band (instead of Form I band present in the unreacted DNA) was observed at a concentration of $\leq$ 12.5 mM for the cadmium-cysteine complex. (Figure 7.14 (a) *lane 5*). The results show that supercoiled Form I DNA is changed to singly-nicked relaxed Form II DNA due its interaction with the complex. When the concentration of the complex was greater than or equal to 25 mM, an extensive damage to DNA occurred (Figure 7.14 (a) *lane 4*).

[Cd-cysteine complex] and [Cysteine]

decrease from left to right

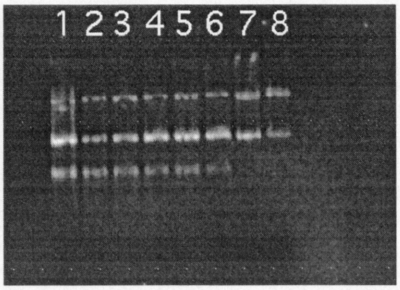
Key: Lane 1: Untreated pBR322 plasmid DNA, Lanes 2-6: Plasmid DNA + Cd-cysteine complex, Lanes 7-12: DNA + cysteine: Lane 2: [Cd-cysteine complex] 100 mM, Lane 3: [Cd-cysteine complex] 50 mM, Lane 4: [Cd-cysteine complex] 25 mM, Lane 5: [Cd-cysteine complex] 12.5 mM, Lane 6: [Cd-cysteine complex] 6.25 mM, Lane 7: [Cysteine] 100 mM Lane 8: [Cysteine] 50 mM, Lane 9: [Cysteine] 25 mM, Lane 10: [Cysteine] 12.5 mM, Lane 11: [Cysteine] 1 mM, Lane 12: [Cysteine] 12.5 μm

FIGURE 7.14 (a): Interaction between $Cd(cysteine)_2$ or cysteine and plasmid DNA in TBE buffer at the pH of the solution in air

It should be recalled that when cadmium(II) nitrate was allowed to interact with plasmid DNA, the extensive damage to DNA occurred at a concentration of Cd^{2+} equal to or greater than 100 mM (Figure 7.12 (a)). It can be seen that the cadmium-cysteine complex is more damaging to DNA than cadmium(II) nitrate.

When plasmid DNA was allowed to interact with cysteine hydrochloride solution (pH 1.50) an extensive damage to DNA occurred even when the concentration of cysteine was as low as 12.5 μM (Figure 7.14 (a) *lane 12*). When the pH of the cysteine solution was increased to 4, three bands corresponding to Forms I, II and III were observed for all concentrations of cysteine up to 6.25 mM (Figure 7.14 (b) beyond that only Form II and Form III bands were observed.

[Cysteine] increases from left to right



Key: Lane 1: Untreated pBR322 plasmid DNA, Lanes 2-8: DNA + cysteine: Lane 2: [Cysteine] 100 μM, Lane 3: [Cysteine] 1 mM, Lane 4: [Cysteine] 6.25 mM, Lane 5: [Cysteine] 12.5 mM, Lane 6: [Cysteine] 25 mM, Lane 7: [Cysteine] 50 mM, Lane 8: [Cysteine] 100 mM

FIGURE 7.14 (b): Interaction between cysteine and plasmid DNA in TBE buffer at pH 4 in air

To remove the effect of acidity, the pH of the mixture of cadmium(II) nitrate and cysteine was adjusted to pH 7.4 and plasmid binding experiments were repeated. However, at pH 7.4, the study could not be extended to $[Cd^{2+}] > 6.25$ mM because of the precipitation of cadmium(II) hydroxide. It was found that under neutral condition an extensive damage to DNA occurred when the concentration of $Cd(cysteine)_2$ complex was equal to 6.25 mM as against 25 mM for the same under acidic conditions (Figures 14 (c) and 14 (a)).

The results suggest that the production of ROS due to interaction of between Cd(cysteine)$_2$ complex and molecular oxygen is pronounced at a neutral pH than under acidic conditions (as was the case for cysteine interacting with molecular oxygen).

[Cd-cysteine complex] and [Cysteine]
increase from left to right

Key; Lane 1: Untreated pBR322 plasmid DNA, Lanes 2-7: DNA + Cd-cysteine complex, Lanes 11-15: DNA + cysteine: Lane 2: [Cd-cysteine complex] 12.5 μm, Lane 3: [Cd-cysteine complex] 25 μm, Lane 4: [Cd-cysteine complex] 50 μm, Lane 5: [Cd-cysteine complex] 100 μm, Lane 6: [Cd-cysteine complex] 1 mM, Lane 7: [Cd-cysteine complex] 6.25 mM, Lane 8-10: none, Lane 11: [Cysteine] 25 μm, Lane 12: [Cysteine] 50 μm, Lane 13: [Cysteine] 100 μm, Lane 14: [Cysteine] 1 mM, Lane 15: [Cysteine] 6.25 mM

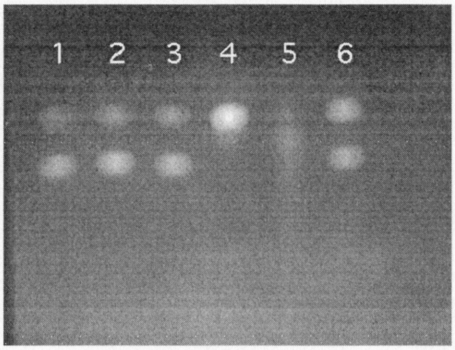
FIGURE 7.14 (c): Interaction between Cd(cysteine)$_2$ or cysteine and plasmid DNA in TBE buffer at pH 7.4 in air

The results show that the solution of cysteine is more damaging to DNA at pH 7.4 than at pH 4.

To observe whether molecular oxygen was involved in causing damage to DNA or not these experiments were repeated under a nitrogen atmosphere. In this case Forms I and

Form II bands were observed that remained unchanged in intensity with the change in concentration of the cadmium-cysteine complex (Figure 7.14 (d)).

[Cd-cysteine complex] increases
from left to right



Key: Lane 1: Untreated pBR322 plasmid DNA, Lanes 2-6: DNA + Cd-cysteine complex: Lane 2: [Cd-cysteine complex] 25 μm, Lane 3: [Cd-cysteine complex] 50 μm, Lane 4: [Cd-cysteine complex] 100 μm, Lane 5: [Cd-cysteine complex] 1 mM, Lane 6: [Cd-cysteine complex] 6.25 mM

FIGURE 7.14 (d): Interaction between Cd(cysteine)$_2$ and plasmid DNA in TBE buffer at pH 7.4 under nitrogen

The tripeptide glutathione (γ-L-glutamyl-L-cysteinylglycine) abbreviated as GSH is widely present in living systems and it plays a key role in metal detoxification. (Rabenstein,1989). It offers a number of binding sites for metal ions including the thiol group, which has a special affinity for heavy metal ions. Since Cd^{2+} can form stable 1:1 and 1:2 complexes with GSH (Diaz-Cruz, *et al.*, 1997), it can reduce the amount of free Cd^{2+} ions available for interaction with DNA. Also, glutathione can act as an antioxidant. It is thus possible that the presence of glutathione can alter the nature of interaction of Cd^{2+} ions with DNA.

7.7.2 Cd^{2+}-glutathione and plasmid

In this series of experiment, we studied the interaction between Cd(glutathione)$_2$ (i.e., a 1:2 mixture of cadmium(II) nitrate and glutathione) and plasmid DNA. The plasmid DNA used was found to be initially a mixture of Forms I and II bands. When it was allowed to interact with Cd(glutathione)$_2$, only Form II band (Figure 7.15 (a)) was observed up to a concentration 100 mM (the highest concentration used in the experiment). The band decreased in intensity as the concentration of the mixture of cadmium(II) nitrate and glutathione was increased. Although the damage to DNA caused by the mixture was found to be similar to that caused by a 1:2 mixture of cadmium(II) nitrate and cysteine, it was found that Cd(glutathione)$_2$ caused a lesser damage than Cd(cysteine)$_2$ (whereas a 25 mM concentration caused an extensive damage in the case of cadmium(II) nitrate and cysteine mixture, even 100 mM concentration did not cause such a damage in the case of cadmium(II) nitrate and glutathione mixture).

[Cd-glutathione complex] and [glutathione]

decrease from left to right

Key: Lane 1: Untreated pBR322 plasmid DNA, Lanes 2-6: DNA + Cd-glutathione complex, Lanes 7-12: DNA + glutathione Lane 2: [Cd-glutathione complex] 100 mM, Lane 3: [Cd-glutathione complex] 50 mM, Lane 4: [Cd-glutathione complex] 25 mM, Lane 5: [Cd-glutathione complex] 12.5 mM, Lane 6: [Cd-glutathione complex] 6.25 mM, Lane 7: [glutathione] 100 mM Lane 8: [glutathione] 50 mM, Lane 9: [glutathione] 25 mM, Lane 10: [glutathione] 6.25 mM, Lane 11: [glutathione] 1 mM, Lane 12: [glutathione] 12.5 μm

FIGURE 7.15 (a): Interaction between $Cd(glutathione)_2$ or glutathione and plasmid DNA in TBE buffer at the pH of the solution in air

When glutathione alone at pH 2.50 (pH of the solution) was allowed to interact with plasmid DNA, total damage to DNA occurred even when the concentration of glutathione was as low as 12.5 μm (Figure 7.15 (a) *lane 12*). However, when the pH of glutathione solution was adjusted to 7.4 by adding 0.01 M NaOH, it was found that no significant damage to DNA occurred (Forms I and II bands observed in all cases) up to 100 mM for glutathione (Figure 7.15 (b), *lanes 11-15*). Similarly, when the pH of the mixture of cadmium(II) nitrate and glutathione was adjusted to 7.4 (by adding 0.01 M NaOH) before its interaction with plasmid DNA, it was found that no damage to DNA occurred up to a concentration of 3.12 mM (Figure 7.15 (b), *lane 6*) for the mixture (beyond which experiment could not be done due to precipitation of cadmium possibly as cadmium hydroxide or as a complex of Cd^{2+} and glutathione).

[Cd-glutathione complex] and [glutathione]
decrease from left to right

Key: Lane 1: Untreated pBR322 plasmid DNA, Lanes 3-6: DNA + Cd-glutathione complex, Lanes 11-15: DNA + glutathione: Lane 2: none, Lane 3: [Cd-glutathione complex] 3.12 mM, Lane 4: [Cd-glutathione complex] 1.56 mM, Lane 5 [Cd-glutathione complex] 0.78 mM, Lane 6: [Cd-glutathione complex] 100 μm, Lane 7-10: none, Lane 11: [glutathione] 100 mM, Lane 12: [glutathione] 50 mM, Lane 13: [glutathione] 25 mM, Lane 14: [glutathione] 12.5 mM, Lane 15: [glutathione] 6.25 mM

FIGURE 7.15 (b): Interaction between $Cd(glutathione)_2$ or glutathione and plasmid DNA in TBE buffer at pH 7.4 in air

Ascorbic acid is a potent water-soluble antioxidant that plays an important role in cellular defense mechanism. It has a protective effect against carcinogenesis (Henson, *et al.*, 1991, Liehr, *et al.*, 1989) by scavenging physiologically relevant ROS and RNS. In addition to scavenging ROS and RNS, ascorbic acid can regenerate other small molecule antioxidants such as glutathione, urate and β-carotene (Carr, A., *et al.*, 1999). There is also recent evidence that suggests that in the presence of heavy metal ions, ascorbate may produce ROS which can then damage DNA (Littlefield, 1995). The authors found that while neither ascorbate nor cadmium(II) chloride alone caused any significant damage to DNA of lymphocyte cells, a much greater damage occurred when they were present together. To determine the exact role of Cd^{2+} in DNA damage due to ascorbate, we carried out experiments on the interaction between ascorbate and plasmid DNA and that between a mixture of cadmium(II) acetate and ascorbate and plasmid DNA.

7.7.3 Cd^{2+}-ascorbate and plasmid

When ascorbate alone at pH 7.4 was allowed to interact with plasmid DNA, two bands were observed for all concentrations of ascorbate from 0 to 100 mM. However, the intensity of the Form II band was found to increase and that of Form I band decrease as the concentration of ascorbate was increased (Figure 7.16 (a)). The changes in intensity of the bands indicate the occurrence of a partial damage to supercoiled DNA to produce more of the singly-nicked Form II plasmid DNA.

[ascorbate] increases from left to right

Key: Lane 1: Untreated pBR322 plasmid DNA, Lane 2: none, Lanes 3-10: Plasmid DNA+ ascorbate: Lane 3: [ascorbate] 50 μm, Lane 4: [ascorbate] 100 μm, Lane 5: [ascorbate] 1 mM, Lane 6: [ascorbate] 6.25 mM, Lane 7: [ascorbate] 12.5 mM, Lane 8: [ascorbate] 25 mM, Lane 9: [ascorbate] 50 mM, Lane 10: [ascorbate] 100 mM

FIGURE 7.16 (a): Interaction between ascorbate and plasmid DNA in TBE buffer at pH 7.4 in air

The results suggest that ascorbate alone at pH 7.4 causes only a minor damage to DNA.

When cadmium(II) acetate and ascorbate were present together (at 1:1 molar ratio), total damage to DNA occurred at 1 mM concentration of the mixture and a significant damage occurred for lower concentrations up to 25 μM (Figure 7.16 (b)) (indicated by a complex pattern of DNA bands).

[Cd(II) acetate-ascorbate mixture]
decreases from left to right

Key: Lane 1: Untreated pBR322 plasmid DNA, Lanes 2-13: Plasmid DNA + Cd(II) acetate-ascorbate mixture: Lane 2: [Cd(II) acetate-ascorbate mixture] 100 mM, Lane 3: [Cd(II) acetate-ascorbate mixture] 50 mM, Lane 4: [Cd(II) acetate-ascorbate mixture] 25 mM, Lane 5: [Cd(II) acetate-ascorbate mixture] 12.5 mM, Lane 6: [Cd(II) acetate-ascorbate mixture] 6.25 mM, Lane 7: [Cd(II) acetate-ascorbate mixture] 1 mM, Lane 8: [Cd(II) acetate-ascorbate mixture] 100 μM, Lane 9: [Cd(II) acetate-ascorbate mixture] 50 μM, Lane 10: [Cd(II) acetate-ascorbate mixture] 25 μM, Lane 11: [Cd(II) acetate-ascorbate mixture] 12.5 μM, Lane 12: [Cd(II) acetate-ascorbate mixture] 6.25 μM, Lane 13: [Cd(II) acetate-ascorbate mixture] 1 μM

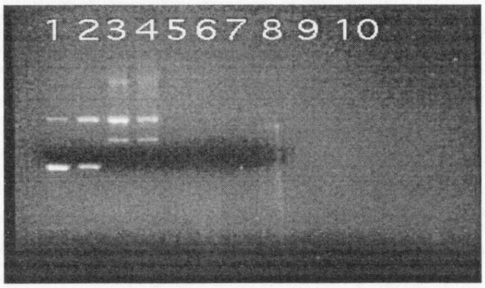
FIGURE 7.16 (b): Interaction between Cd(II) acetate and plasmid DNA in the presence of ascorbate in TBE buffer at pH 7.4 in air

To determine which component in the mixture (cadmium(II) acetate or ascorbate) has contributed more to DNA damage, two series of experiments were done, in one of which the concentration of ascorbate only was varied and in the other that of cadmium(II) acetate only was varied.

In the series of experiments in which the concentration of ascorbate was varied from 0 to 100 mM but that of cadmium(II) acetate was kept constant at 50 mM at pH 7.4 both Form I and II bands of DNA were observed for all concentrations ranging up to 100 µm (similar to that observed in unreacted plasmid) and above that concentration no band was observed (Figure 7.16 (c)). However, it was found that the mobility of the Form I band was much less in the presence of ascorbate and cadmium(II) acetate.

[Cd(II) acetate-ascorbate mixture]

increases from left to right



Key: Lane 1: Untreated pBR322 plasmid DNA, Lanes 2-10: DNA + ascorbate (in varied amount)+Cd(II) acetate (in constant amount 50 mM): Lane 2: [ascorbate] 25 μm Lane 3: [ascorbate] 50 μm, Lane 4: [ascorbate] 100 μm, Lane 5: [ascorbate] 1 mM, Lane 6: [ascorbate] 6.25 mM, Lane 7: [ascorbate] 12.5 mM, Lane 8: [ascorbate] 25 mM, Lane 9: [ascorbate] 50 mM, Lane 10: [ascorbate] 100 mM

FIGURE 7.16 (c): Interaction between varying concentration of ascorbate and plasmid DNA in the presence of fixed concentration of Cd(II) acetate in TAE buffer at pH 7.4 in air

The decreased mobility may be due to one or more of the following: (1) a change in the nature of super-coiling, (2) an increase in molecular mass due to associative interaction, and (3) a decrease in the overall negative charge on the DNA due its binding with Cd^{2+} ions. It was also found that extensive damage to DNA occurred when the concentration of ascorbate was greater than or equal to 1 mM (which was the same as that required for the 1:1 mixture of cadmium(II) acetate and ascorbate). The results show that a higher concentration of cadmium(II) acetate (50 mM) did not alter the 1 mM critical concentration of ascorbate required to cause total damage to DNA.

In the other series of experiments in which the concentration of cadmium(II) acetate was varied from 0.0 to 100 mM but that of ascorbate was kept constant (at 50 mM), at pH 7.4 both Form I and II bands of DNA were observed at all concentrations ranging from 0 to 100 μm above which no DNA band was observed (Figure 7.16 (d)). The mobility of the Form I band was found to be much less than that in unreacted plasmid DNA but similar to that observed in experiments in which the concentration of ascorbate was varied. Total damage to DNA occurred when the concentration of cadmium(II) acetate was greater than or equal to 1 mM which was exactly the same as that observed for ascorbate when the concentration of ascorbate was varied but that of cadmium(II) acetate was kept constant at 50 mM (Figure 7.16 (c)).

[Cd(II) acetate-ascorbate mixture]

increases from left to right

Key: Lane 1: Untreated pBR322 plasmid DNA, Lanes 2-10: DNA + ascorbate (constant amount 50 mM)+ Cd(II) acetate (in varied amount) Lane 2: [Cd(II) acetate] 25 μm, Lane 3: [Cd(II) acetate] 50 μm, Lane 4: [Cd(II) acetate] 100 μm, Lane 5: [Cd(II) acetate] 1 mM, Lane 6: [Cd(II) acetate] 6.25 mM, Lane 7: [Cd(II) acetate] 12.5 mM, Lane 8: [Cd(II) acetate] 25 mM, Lane 9: [Cd(II) acetate] 50 mM, Lane 10: [Cd(II) acetate] 100 mM

FIGURE 7.16 (d): Interaction between varying concentration of Cd(II) acetate and plasmid DNA in the presence of fixed concentration of ascorbate in TAE buffer at pH 7.4 in air

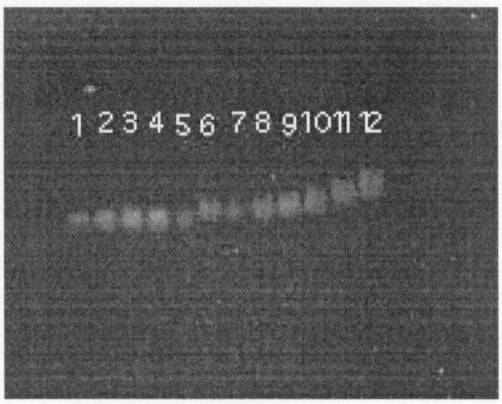
The results show that the damage to DNA is potentiated when cadmium(II) acetate and ascorbate are present together at 1:1 molar ratio. Thus, whereas neither 1 mM ascorbate nor 1 mM cadmium(II) acetate alone could cause any significant damage to DNA at pH 7.4 (Figures 7.16 (a), 7.12 (c) or 7.12 (d)) when present together they caused an extensive damage to DNA (7.16 (b) or 7.16 (c) or 7.16 (d)).

7.8 Interaction between Cd^{2+} and genomic DNA

7.8.1 Interaction between cadmium(II) acetate and salmon sperm DNA (ssDNA)

Submarine gel electrophoresis of ssDNA at pH 7.4 gave a single band. When it was electrophoresed after its interaction with cadmium(II) acetate, no change in intensity and mobility of the band were observed up to 100 μM concentration of Cd^{2+} (Figure 7.17 (a). However, when the concentration of Cd^{2+} was increased from 100 μM to 100 mM the mobility of the band was found to decrease with the increase in concentration. There was also a corresponding decrease in the intensity of the band.

[Cd(II) acetate] increases from left to right



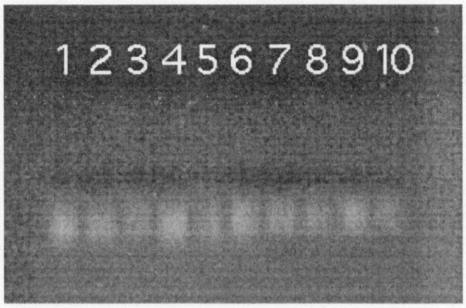
Lane 1: Untreated ssDNA; Lanes 2- 12: DNA + Cd(II) acetate: Lane 2: [Cd^{2+}] 12.5 μM, Lane 3: [Cd^{2+}] 25 μM, Lane 4: [Cd^{2+}] 50 μM, Lane 5: [Cd^{2+}] 100 μM, Lane 6: [Cd^{2+}] 1 mM, Lane 7: [Cd^{2+}] 6.25 mM, Lane 8: [Cd^{2+}] 12.5 mM, Lane 9: [Cd^{2+}] 25 mM, Lane 10: [Cd^{2+}] 50 mM, Lane 11: [Cd^{2+}] 75 mM, Lane 12: [Cd^{2+}] 100 mM

FIGURE 7.17 (a): Interaction between Cd(II) acetate and salmon sperm DNA in TAE buffer at pH 7.4

7.8.2 Interaction between ascorbate and ssDNA

When ascorbate at pH 7.4 was allowed to interact with ssDNA, a single band was observed for all concentration from 0 to 100 mM for ascorbate although there was a slight decrease in mobility of the band at higher concentrations (50 to 100 mM) (Figure 7.17 (b).

[Ascorbate] increases from left to right



Lane 1: Untreated ssDNA; Lanes 2-10: DNA + ascorbate

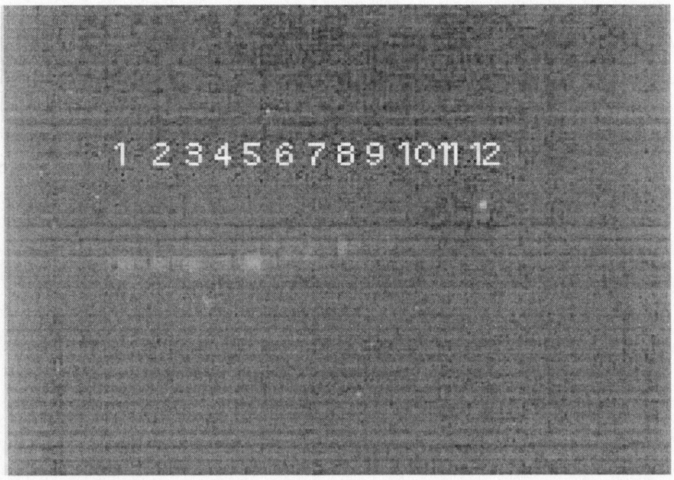
Lane 2: [ascorbate] 25 μM, Lane 3: [ascorbate] 50 μM, Lane 4: [ascorbate] 100 μM, Lane 5: [ascorbate] 1 mM, Lane 6: [ascorbate] 6.25 mM, Lane 7: [ascorbate] 12.5 mM, Lane 8: [ascorbate] 25 mM, Lane 9: [ascorbate] 50 mM, Lane 10: [ascorbate] 100 mM

FIGURE 7.17 (b): Interaction between ascorbate and salmon sperm DNA in TAE buffer at pH 7.4 in air

7.8.3 Interaction between cadmium(II) acetate, ascorbate and ssDNA

When 1:1 mixtures of cadmium(II) acetate and ascorbate were allowed to interact with ssDNA at pH 7.4, no significant change in intensity and mobility of the band were observed up to a concentration of 100 μM. A partial damage to the band was observed for concentrations ranging from 1 mM to 12.5 mM and also the mobility of the band was found to decrease with the increase in concentration (similar to what was observed in the interaction with Cd^{2+} alone). Extensive damage to DNA occurred at concentrations ≥ 25 mM (Figure 7.17 (c) (a value which was significantly greater than 1 mM concentration that was required to cause total damage to plasmid DNA).

[Cd(II) acetate-ascorbate] increases from left to right



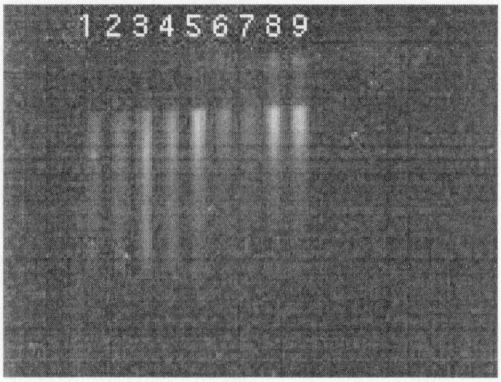
Lane 1: Untreated ssDNA; Lanes 2-12: DNA + Cd(II) acetate-ascorbate mixture: Lane 2: [Cd(II) acetate-ascorbate mixture] 12.5 μM, Lane 3: [Cd(II) acetate-ascorbate mixture] 25 μM, Lane 4: [Cd(II) acetate-ascorbate mixture] 50 μM, Lane 5: [Cd(II) acetate-ascorbate mixture] 100 μM, Lane 6: [Cd(II) acetate-ascorbate mixture] 1 mM, Lane 7: [Cd(II) acetate-ascorbate mixture] 6.25 mM, Lane 8: [Cd(II) acetate-ascorbate mixture] 12.5 mM, Lane 9: [Cd(II) acetate-ascorbate mixture] 25 mM, Lane 10: [Cd(II) acetate-ascorbate mixture] 50 mM, Lane 11: [Cd(II) acetate-ascorbate mixture] 75 mM, Lane 12: [Cd(II) acetate-ascorbate mixture] 100 mM

FIGURE 7.17 (c): Interaction between Cd(II) acetate and salmon sperm DNA in the presence of ascorbate in TAE buffer at pH 7.4 in air

7.8.4 Interaction between cadmium(II) acetate and calf thymus DNA

Submarine gel electrophoresis of calf thymus DNA at pH 7.4 gave a single streaking band. When it was allowed to interact with cadmium(II) acetate at the same pH, it was found that the head of the band decreased in intensity and the tail increased in intensity with the increase in concentration of Cd^{2+}. Also, the mobility of the band was found to decrease as the concentration of Cd^{2+} was increased (7.18 (a)).

[Cd(II) acetate] increases from left to right



Lane 1: Calf thymus DNA; Lanes 2-9: Calf thymus DNA +

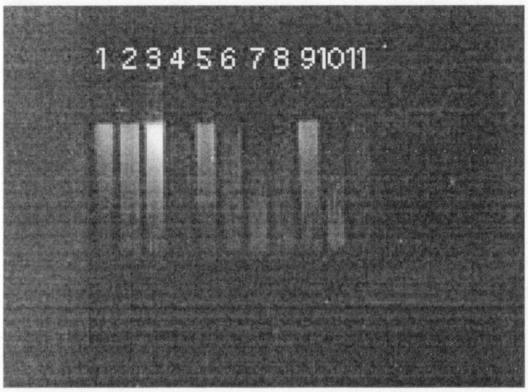
Cd(II) acetate: Lane 1: Untreated calf thymus DNA, Lane 2: [Cd^{2+}] 50 μM, Lane 3: [Cd^{2+}] 100 μM, Lane 4: [Cd^{2+}] 1 mM, Lane 5: [Cd^{2+}] 6.25 mM, Lane 6: [Cd^{2+}] 12.5 mM, Lane 7: [Cd^{2+}] 25 mM, Lane 8: [Cd^{2+}] 50 mM, Lane 9: [Cd^{2+}] 100 mM

FIGURE 7.18 (a): Interaction between Cd(II) acetate and Calf thymus DNA in TAE buffer at pH 7.4 in air

7.8.5 Interaction between ascorbate and calf thymus DNA

When ascorbate at pH 7.4 was allowed to interact with calf thymus DNA a single streaking band was observed for all concentration from 0 to 100 mM which generally decreased in intensity with the increase in concentration of the ascorbate. However, mobility of the band remained unchanged (Figure 7.18(b)).

[Ascorbate] increases from left to right



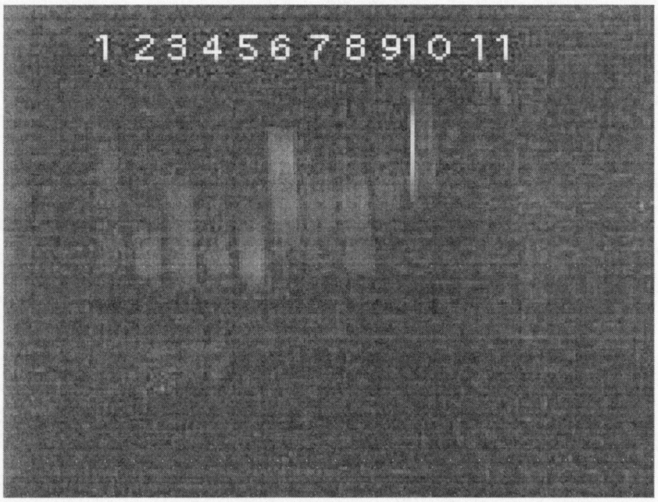
Lane 1: Untreated calf thymus DNA, Lanes 2-11: DNA + ascorbate: Lane 2: [ascorbate] 12.5 μM, Lane 3: [ascorbate] 25 μM, Lane 4: [ascorbate] 50 μM, Lane 5: [ascorbate] 100 μM, Lane 6: [ascorbate] 1 mM, Lane 7: [ascorbate] 6.25 mM, Lane 8: [ascorbate] 12.5 mM, Lane 9: [ascorbate] 25 mM, Lane 10: [ascorbate] 50 mM, Lane 11: [ascorbate] 100 mM

FIGURE 7.18 (b): Interaction between ascorbate and calf thymus DNA in TAE buffer at pH 7.4 in air

7.8.6 Interaction between cadmium(II) acetate, ascorbate and calf thymus DNA

When 1:1 mixtures of cadmium(II) acetate and ascorbate were allowed to interact with calf thymus DNA at pH 7.4, no significant change in intensity of the band was observed up to a concentration of 100 μM (*lanes 1-5*, Figure 7.18 (c)). A noticeable change in the appearance of band occurred for concentrations ranging from 1 mM to 12.5 mM. Extensive damage to DNA occurred at concentration ≥ 25 mM (Figure 7.18 (c)) (a value, which was found to be the same as that, required to cause such a damage to ssDNA).

[Cd(II) acetate- ascorbate mixture] increases from left to right



Lane 1: Calf thymus DNA, Lanes 2-12: DNA+ Cd(II) acetate-ascorbate mixture: Lane 2: [Cd(II) acetate-ascorbate mixture] 12.5 μM, Lane 3: [Cd(II) acetate-ascorbate mixture] 25 μM, Lane 4: [Cd(II) acetate-ascorbate mixture] 50 μM, Lane 5: [Cd(II) acetate-ascorbate mixture] 100 μM, Lane 6: [Cd(II) acetate-ascorbate mixture] 1 mM, Lane 7: [Cd(II) acetate ascorbate mixture] 6.25 mM, Lane 8: [Cd(II) acetate-ascorbate mixture] 12.5 mM, Lane 9: [Cd(II) acetate-ascorbate mixture] 25 mM, Lane 10: [Cd(II) acetate-ascorbate mixture] 50 mM, Lane 11: [Cd(II) acetate-ascorbate mixture] 75 mM, Lane 12: [Cd(II) acetate-ascorbate mixture] 100 mM

FIGURE 7.18 (c): Interaction between Cd(II) acetate and calf thymus DNA in the presence of ascorbate in TAE buffer at pH 7.4 in air

7.9 Interaction between Ag^+ and plasmid or genomicDNA

Having found that in presence of ascorbate and Cd^{2+} together damage due to DNA is greatly potentiated and having established that Cd^{2+} binds covalently with DNA, a natural question to ask was whether ascorbate and other heavy metal ions (that are expected to bind covalently with DNA) together cause a similar pronounced damage to DNA. Thus, experiments were carried out on interaction between DNA and Ag^+ and Zn^{2+} in the absence and presence of ascorbate using protocols similar to those for cadmium. It should be noted that both the metal ions are expected to bind covalently with DNA.

7.9.1 Interaction between silver(I) acetate and plasmid DNA

Submarine gel electrophoresis of pBR322 plasmid at pH 7.4 gave two bands corresponding to Form I and Form II with the Form I band being much more prominent. When it was electrophoresed after its interaction with silver(I) acetate, no significant change in intensity of the bands was observed for all concentrations of Ag^+ ranging from 0 to 50 mM (Figure 7.19 (a)). However, electrophoretic mobility of the bands was found to decrease slightly with the increase in concentration of Ag^+. The experiments could not be extended beyond 50 mM concentration for Ag^+ since above this concentration Ag^+ precipitated as AgOH or Ag_2O at pH 7.4.

[Ag(I) acetate] increases from left to right

Key: Lane 1: Untreated pBR322 plasmid DNA, Lanes 2-9: DNA + Ag(I) acetate Lane 2: [Ag^+] 25 μM, Lane 3: [Ag^+] 50 μM, Lane 4: [Ag^+] 100 μM, Lane 5: [Ag^+] 1 mM, Lane 6: [Ag^+] 6.25 mM, Lane 7: [Ag^+] 12.5 mM, Lane 8: [Ag^+] 25 mM, Lane 9: [Ag^+] 50 mM

FIGURE 7.19 (a): Interaction between Ag(I) acetate and pBR322 plasmid DNA in TAE buffer at pH 7.4 in air

7.9.2 Interaction between silver(I) acetate, ascorbate and plasmid DNA

It was stated earlier that at pH 7.4 ascorbate alone caused only a minor damage to plasmid DNA (Figure 7.16 (a) page 183).

When 1:1 mixtures of silver(I) acetate and ascorbate were allowed to interact with plasmid DNA, total damage to DNA did not occur at any concentration over the range 12.5 μM to 50 mM. It was found that most significant damage occurred not at the highest concentration but at a lower concentration namely 12.5 mM (Figure 7.19 (b) *lane 8*). At this concentration only a faint band with a much higher mobility was observed. At 25 mM, Form II and Form III bands were observed with Form III band being more prominent. At 50 mM concentration, only Form II band was observed. For the concentration range: 100 μM - 6.25 mM, two narrow bands (which were different from the bands observed at lower or higher concentrations and remained practically unchanged in the concentration range) were found. The appearance of the bands between 12.5 μM to 50 μM was almost the same as that for the unreacted DNA although the intensity of Form II band was found to increase as the concentration of the mixture was increased over the range.

[Ag(I) acetate-ascorbate] increases from left to right

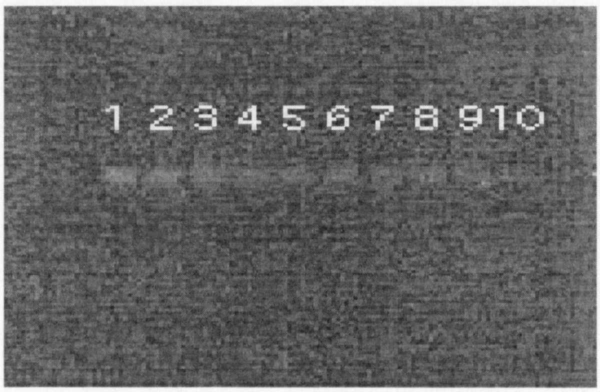
Key: Lane 1: Untreated pBR322 plasmid DNA, Lanes 2-10: DNA + Ag(I) acetate-ascorbate mixture: Lane 2: [Ag(I) acetate-ascorbate mixture] 12.5 μM, Lane 3: [Ag(I) acetate-ascorbate mixture] 25 μM, Lane 4: [Ag(I) acetate-ascorbate mixture] 50 μM, Lane 5: [Ag(I) acetate-ascorbate mixture] 100 μM, Lane 6: [Ag(I) acetate-ascorbate mixture] 1 mM, Lane 7: [Ag(I) acetate-ascorbate mixture] 6.25 mM, Lane 8: [Ag(I) acetate-ascorbate mixture] 12.5 mM, Lane 9: [Ag(I) acetate-ascorbate mixture] 25 mM, Lane 10: [Ag(I) acetate-ascorbate mixture] 50 mM

FIGURE 7.19 (b): Interaction between Ag(I) acetate and plasmid DNA in the presence of ascorbate in TAE buffer at pH 7.4 in air

7.9.3 Interaction between silver(I) acetate and ssDNA

Submarine gel electrophoresis of ssDNA at pH 7.4 gave a single band. When it was electrophoresed after its interaction with silver(I) acetate, no change in mobility of the band was observed from 0 to 50 mM concentration of Ag^+. However, there was a progressive decrease in the intensity of the band as the concentration of Ag^+ increased (Figure 7.20 (a)).

[Ag(I) acetate] increases from left to right



Key: Lane 1: Untreated ssDNA, Lanes 2-10: DNA + Ag(I) acetate: Lane 2: $[Ag^+]$ 12.5 μM, Lane 3: $[Ag^+]$ 25 μM, Lane 4: $[Ag^+]$ 50 μM, Lane 5: $[Ag^+]$ 100 μM, Lane 6: $[Ag^+]$ 1 mM, Lane 7: $[Ag^+]$ 6.25 mM, Lane 8: $[Ag^+]$ 12.5 mM, Lane 9: $[Ag^+]$ 25 mM, Lane 10: $[Ag^+]$ 50 mM

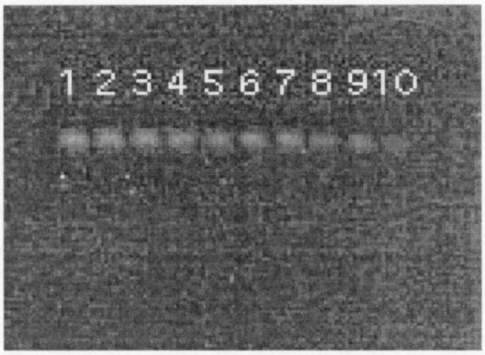
FIGURE 7.20 (a): Interaction between Ag(I) acetate and ssDNA in TAE buffer at pH 7.4 in air

7.9.4 Interaction between silver(I) acetate, ascorbate and ssDNA

When ascorbate at pH 7.4 was allowed to interact with ssDNA, a single band was observed for all concentration from 0 to 100 mM for ascorbate although there was a slight decrease in mobility of the band at higher concentrations (50 to 100 mM) (Figure 7.17 (b) page 190).

When ssDNA was allowed to interact with 1:1 mixture of silver(I) acetate and ascorbate the intensity of the band was found to decrease slightly as the concentration of Ag^+ was increased (Figure 7.20 (b)). However, the mobility of the band was found to increase as the concentration of Ag^+ increased.

[Ag(I) acetate-ascorbate] increases from left to right



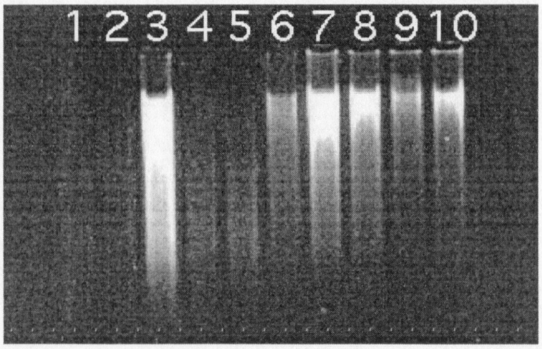
Key: Lane 1: ssDNA, Lanes 2-10: DNA + Ag(I) acetate-ascorbate mixture: Lane 2: [Ag(I) acetate-ascorbate mixture] 12.5 μM, Lane 3: [Ag(I) acetate-ascorbate mixture] 25 μM, Lane 4: [Ag(I) acetate-ascorbate mixture] 50 μM, Lane 5: [Ag(I) acetate-ascorbate mixture] 100 μM, Lane 6: [Ag(I) acetate-ascorbate mixture] 1 mM, Lane 7: [Ag(I) acetate-ascorbate mixture] 6.25 mM, Lane 8: [Ag(I) acetate-ascorbate mixture] 12.5 mM, Lane 9: [Ag(I) acetate-ascorbate mixture] 25 mM, Lane 10: [Ag(I) acetate-ascorbate mixture] 50 mM

FIGURE 7.20 (b): Interaction between Ag(I) acetate and ssDNA in the presence of ascorbate in TAE buffer at pH 7.4 in air

7.9.5 Interaction between silver(I) acetate and calf thymus DNA

Unreacted calf thymus DNA originally appeared as a faint streaking band at pH 7.4 indicating that the molecular mass of the DNA covered a wide range of values. (Figure 7.18 (a)). When it was allowed to interact with silver(I) acetate at pH 7.4, there was first a sharp increase in the intensity of the band followed by a gradual fall-off as the concentration of Ag^+ was increased. Mobility of the band was found to decrease slightly with the increase in concentrations of Ag^+ increased from 0 to 50 mM (Figure 7.21 (a)) similar to that observed in the interaction of the DNA with Cd^{2+} .

[Ag(I) acetate] increases from left to right



Key: Lane 1: Untreated calf thymus DNA, Lanes 2-10:

DNA + Ag(I) acetate: Lane 2: [Ag (I) acetate] 12.5 μM, Lane 3: [Ag (I) acetate] 25 μM, Lane 4: [Ag (I) acetate] 50 μM, Lane 5: [Ag (I) acetate] 100 μM, Lane 6: [Ag (I) acetate] 1 mM, Lane 7: [Ag (I) acetate] 6.25 mM, Lane 8: [Ag (I) acetate] 12.5 mM, Lane 9: [Ag (I) acetate] 25 mM, Lane 10: [Ag (I) acetate] 50 mM

FIGURE 7.21 (a): Interaction between Ag(I) acetate and calf thymus DNA in TAE buffer at pH 7.4 in air

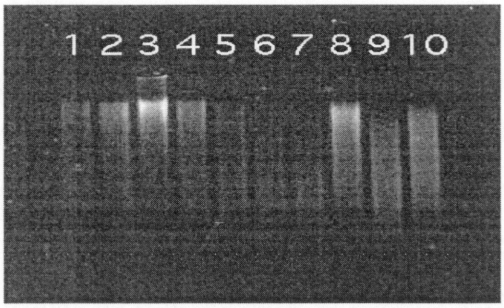
Results suggest that covalent interaction of Ag^+ causes a change in the structure of calf thymus DNA as evidenced by the increase in intensity of the band at high concentrations.

7.9.6 Interaction between silver(I) acetate, ascorbate and calf thymus DNA

When ascorbate at pH 7.4 was allowed to interact with calf thymus DNA a single streaking band was observed for all concentration from 0 to 100 mM which generally decreased in intensity with the increase in concentration of the ascorbate. However, mobility of the band remained unchanged (Figure 7.18 (b) page 194).

When silver(I) acetate was allowed to interact with calf thymus DNA in the presence of ascorbate, the intensity of the band was found to be similar to that of unreacted calf thymus DNA. However, the mobility of the band was found to increase as the concentration of Ag^+ increased (Figure 7.21 (b)).

[Ag(I) acetate-ascorbate] increases from left to right



Lane 1: Untreated calf thymus DNA, Lanes 2-10: DNA + Ag(I) acetate-ascorbate mixture: Lane 2: [Ag(I) acetate-ascorbate mixture] 12.5 μM, Lane 3: [Ag(I) acetate-ascorbate mixture] 25 μM, Lane 4: [Ag(I) acetate-ascorbate mixture] 50 μM, Lane 5: [Ag(I) acetate-ascorbate mixture] 100 μM, Lane 6: [Ag(I) acetate-ascorbate mixture] 1 mM, Lane 7: [Ag(I) acetate-ascorbate mixture] 6.25 mM, Lane 8: [Ag(I) acetate-ascorbate mixture] 12.5 mM, Lane 9: [Ag(I) acetate-ascorbate mixture] 25 mM, Lane 10: [Ag(I) acetate-ascorbate mixture] 50 mM

FIGURE 7.21 (b): Interaction between Ag(I) acetate and calf thymus DNA in the presence of ascorbate in TAE buffer at pH 7.4 in air

Results suggest that Ag^{+} in presence of ascorbate caused a change in the structure of calf thymus DNA that was manifested by increased mobility of the band at higher concentration. This result is not similar to that caused by Ag^{+} alone to calf thymus DNA in which case there was no change in the mobility of the band rather change in intensity of the band at higher concentrations.

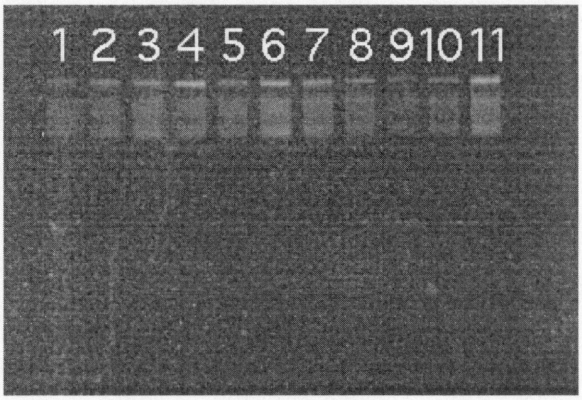
7.10 Interaction between Zn^{2+} and Plasmid DNA

As it has been stated in the introduction, zinc has an inhibitory effect on the carcinogenicity due to cadmium and this may be due to a similarity in their chemical nature, especially related to their interactions in biological systems. Since zinc is involved in the synthesis and function of nucleic acids, it is possible that zinc binds directly to DNA itself. It also binds to heterochromatin in DNA. In order to compare the effect of binding of Zn^{2+} on DNA with that due to Cd^{2+}, a series of electrophoretic experiments were carried out in which varying amounts of zinc(II) acetate or 1:1 mixtures of zinc(II) acetate and ascorbate were allowed to interact with plasmid DNA using similar protocols as were used for its interaction with Ag^{+} and Cd^{2+}. Specifically, the aim of the experiments was to determine whether there was a pronounced damage to pBR322 plasmid DNA due to 1:1 mixture of zinc(II) acetate and ascorbate as was observed with 1:1 mixture of cadmium(II) acetate and ascorbate.

7.10.1 Interaction between Zinc(II) acetate and plasmid DNA in absence or in presence of ascorbate

Electrophoresis of untreated pBR322 plasmid DNA at pH 7.4 gave two bands corresponding to Form 1 and Form II, with the Form I band being much more broad than Form II band. When it was allowed to interact with zinc(II) acetate over the concentration range: 0 to 50 mM for zinc(II) acetate, the diffuse Form I band got separated into Form I and III bands (Figure 7.22 (a)). At low concentrations of zinc(II) acetate, the separation between Form I and Form III bands was found to be blurred whereas at the highest concentration the two bands were more clearly defined. The experiments could not be extended beyond 50 mM concentration for zinc(II) acetate due to precipitation of $Zn(OH)_2$ at pH 7.4. As the concentration of zinc(II) acetate was increased, the mobility of the bands was found to remain essentially unchanged for the concentration range: 0 to 25 μM and then decrease slightly over the concentration range: 25μM to 50 mM. There was no significant change in intensity of the bands as the concentration of zinc(II) acetate was varied.

[Zn(II) acetate] increases from left to right



Lane 1: pBR322 plasmid DNA, Lanes 2-11: DNA + Zn(II) acetate: Lane 2: [Zn^{2+}] 6.25 μM, Lane 3: [Zn^{2+}] 12.5 μM, Lane 4: [Zn^{2+}] 25 μM, Lane 5: [Zn^{2+}] 50 μM, Lane 6: [Zn^{2+}] 100 μM, Lane 7: [Zn^{2+}] 1 mM, Lane 8: [Zn^{2+}] 6.25 mM, Lane 9: [Zn^{2+}] 12.5 mM Lane 10: [Zn^{2+}] 25 mM Lane 11: [Zn^{2+}] 50 mM

FIGURE 7.22 (a): Interaction between Zn(II) acetate and pBR322 plasmid DNA in TAE buffer at pH 7.4 in air

When 1:1 mixtures of zinc(II) acetate and ascorbate were allowed to interact with pBR322 plasmid DNA over the concentration range for the mixture: 0 to 50 mM, two bands were observed corresponding to Form I and Form II. However, as the concentration of the mixture was increased, the broad band corresponding to Form I almost got separated into two bands (Figure 7.22 (b)). The results are similar to those observed in the interaction of the DNA with zinc(II) acetate alone.

[Zn(II) acetate-ascorbate] increases from left to right

Lane 1: pBR322 plasmid DNA, Lanes 2-10: pBR322 plasmid DNA + Zn(II) acetate-ascorbate mixture: Lane 2: [Zn(II) acetate-ascorbate mixture] 12.5 μM, Lane 3: [Zn(II) acetate-ascorbate mixture] 25 μM, Lane 4: [Zn(II) acetate-ascorbate mixture] 50 μM, Lane 5: [Zn(II) acetate-ascorbate mixture] 100 μM, Lane 6: [Zn(II) acetate-ascorbate mixture] 1 mM, Lane 7: [Zn(II) acetate-ascorbate mixture] 6.25 mM, Lane 8: [Zn(II) acetate-ascorbate mixture] 12.5 mM, Lane 9: [Zn(II) acetate-ascorbate mixture] 25 mM Lane 10: [Zn(II) acetate-ascorbate mixture] 50 mM

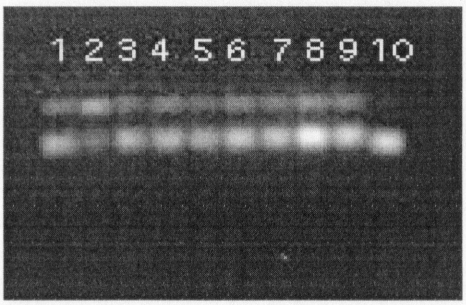
FIGURE 7.22 (b): Interaction between Zn(II) acetate and pBR322 plasmid DNA in presence of ascorbate in TAE buffer at pH 7.4 in air

7.11 PCR analysis of metallated plasmid DNA

It was stated earlier (chapter 6, section 6.2.8) that pUC8 plasmid was allowed to interact with Cd^{2+} at concentrations ranging from 0 to 200 mM, following which the DNA was electrophoresed.

Figure 7.23 (a) shows that pUC8 plasmid DNA consists of a mixture of Form I and Form II. Both of the bands were found to be present when the DNA was allowed to interact with cadmium(II) acetate up to a [Cd^{2+}] of 100 mM whereas at 200 mM only a single band was observed whose mobility was found greater than that of Form I band.

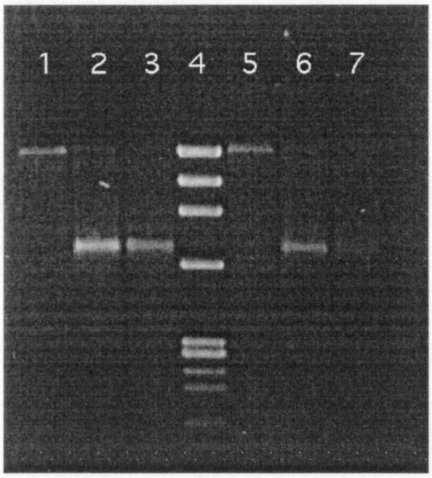
[Cd(II) acetate] increases from left to right



Lane 1: Untreated pUC8 plasmid DNA, Lanes 2-10: pUC8 plasmid DNA + Cd(II) acetate: Lane 2: [Cd^{2+}] 50 μM, Lane 3: [Cd^{2+}] 100 μM Lane 4: [Cd^{2+}] 1 mM Lane 5: [Cd^{2+}] 6.25 mM, Lane 6: [Cd^{2+}] 12.5 mM, Lane 7: [Cd^{2+}] 25 mM Lane 8: [Cd^{2+}] 50 mM, Lane 9: [Cd^{2+}] 100 mM, Lane 10: [Cd^{2+}] 200 mM

FIGURE 7.23 (a): Interaction between Cd(II) acetate and pUC8 plasmid DNA in TAE buffer at pH 7.4 in air

The figure 7.23 (b) shows the amplified Form I band of pUC8 plasmid DNA after its interaction with cadmium(II) acetate at concentrations ranging from 0 to 100 mM. The results show that the mobility of the Form I band after interaction with cadmium(II) acetate at 1 mM concentration is less than that of the corresponding band found in the untreated DNA. When the concentration of cadmium(II) acetate was greater than 1 mM, although the Form I band was still present for all concentrations of Cd^{2+} up to 100 mM, this was not amplified by PCR. At concentrations lower than 1 mM, multiple bands were observed (results not given) which indicated the occurrence of non-specific binding of the primer. It should be noted that under optimised PCR conditions, there would be least possibility for the formation of nonspecific PCR products.



Lane 1: amplified Form I band of cadmium(II) acetate-treated pUC8 plasmid DNA ([Cd^{2+}] 1 mM), Lanes 2-3: amplified Form I band of untreated pUC8 plasmid DNA, Lane 4: DNA ladder for comparing amplified band, Lane 5: replicate of amplified Form I band of cadmium(II) acetate-treated pUC8 plasmid DNA ([Cd^{2+}] 1 mM), Lane 6-7: replicates of amplified Form I band of untreated pUC8 plasmid DNA

FIGURE 7.23 (b): PCR amplification of cadmium-treated and untreated pUC8 plasmid DNA

CHAPTER 8

DISCUSSION

8.1 Interaction between Cd^{2+} and nucleobases or nucleotides

8.1.1 UV-difference spectral analysis

The UV-difference spectral results showed that cadmium(II) nitrate and adenine (both dissolved in mQ water) reacted to form mainly 1:1 adduct (Figure 7.1). It should however be noted that the observed binding ratio was not exactly equal to 1:1. This departure from 1:1 binding ratio is believed to be due to a change in the ionic strength of the solution when the concentrations of the reactants were continuously varied. When the experiments were repeated by dissolving the reactants in 0.10 M $NaNO_3$ instead of mQ water, it was found that Cd^{2+} and adenine reacted to form three adducts corresponding to binding ratios 1:1, 1:2 and 1:3 (Figure 7.5). It should also be noted that in solution in water whereas a maximum was observed for 1:1 binding ratio, the corresponding mixture gave a minimum in solution in 0.10 M $NaNO_3$. In fact, all the peaks observed in 0.10 M $NaNO_3$ were minima rather than maxima, indicating when the ionic strength was kept constant (at about 0.05 M), free adenine absorbed more strongly than cadmium-adenine adducts or modified adenine. It will be considered more about the binding between Cd^{2+} and adenine later in the discussion.

When cadmium(II) nitrate was allowed to react with guanine in solution in water, a broad maximum was observed that corresponded to unrealistic binding ratios (between Cd^{2+} and guanine) ranging from 11:1 to 4:1. These values are believed to be an artifact resulting from a change in the ionic strength of the solutions. The difference spectral plot also gave a minimum corresponding to 1:3 binding ratio between Cd^{2+} and guanine (Figure 7.2). The 1:3 binding between Cd^{2+} and guanine is different from 1:1 binding between Cd^{2+} and guanine that is commonly found in the structures of crystals (Aoki, 1976, Prakash, 1998). However, when cadmium(II) nitrate was allowed to interact with guanine in solution in 0.10 M $NaNO_3$ (rather than mQ water), a clearly defined maximum was observed corresponding to the formation of 1:1 adducts (Figure 7.6). It will be considered more about the binding between Cd^{2+} and guanine later in the discussion.

It was observed that in solution in mQ water Cd^{2+} did not react thymine or cytosine even after forty eight hours of incubation. It was also found that in solution in 0.10 M $NaNO_3$ also, Cd^{2+} did not react thymine or cytosine when the period of incubation was twenty four hours. However, when the period of incubation was extended to forty eight hours, it was found that Cd^{2+} reacted with thymine to form 2:1, 1:2 and 1:3 adducts and with cytosine to form 1:1 and 1:3 adducts. The additional maximum corresponding to 7:1 binding ratio between Cd^{2+} and thymine is unrealistic and difficult to explain. The formation of 2:1 adduct can occur if thymine acts as a bidentate ligands being bonded to two Cd^{2+} ions.

It can be deduced from the above results that cadmium ion binds preferentially with adenine and guanine most likely at their N7 centres, as was concluded by (Sorokin, *et al.*, 1997, Aoki, 1976, Prakash, 1998). The N7 coordination was also found in the 1:1 complex between Cd^{2+} and AMP (Sigel, *et al.*, 1994). However, molecular mechanics and semi-empirical calculations carried out for $Cd(adenine)(H_2O)_5^{2+}$ (considered later in the discussion) using HyperChem 5 (Huq, *et al.*, 2000) show that (from energy consideration) both N1 and N7 coordination are almost equally likely for cadmium. It is also found that when cadmium is considered to be bonded to N1 centre of adenine, an intramolecular H bond between an amino hydrogen and a coordinated water molecule is formed. No such H bond is observed when cadmium is considered to be bonded to the N7 centre of adenine. It should be noted when cadmium is allowed to interact with DNA, coordination with N1 centre of adenine is not likely as it is not available, being involved in H bond with thymine.

In the 1:1 adduct between cadmium and adenine, it is assumed that Cd^{2+}, in addition to being bonded to one adenine unit through at its N7 position, is also bonded to five water molecules so that the complex ion has the stoichiometry $Cd(adenine)(H_2O)_5^{2+}$

(Figure 8.1). Molecular modeling analysis showed that monodentate coordination to N7 position was more favourable energetically than bidentate coordination involving N7 and N1 or N3 positions.

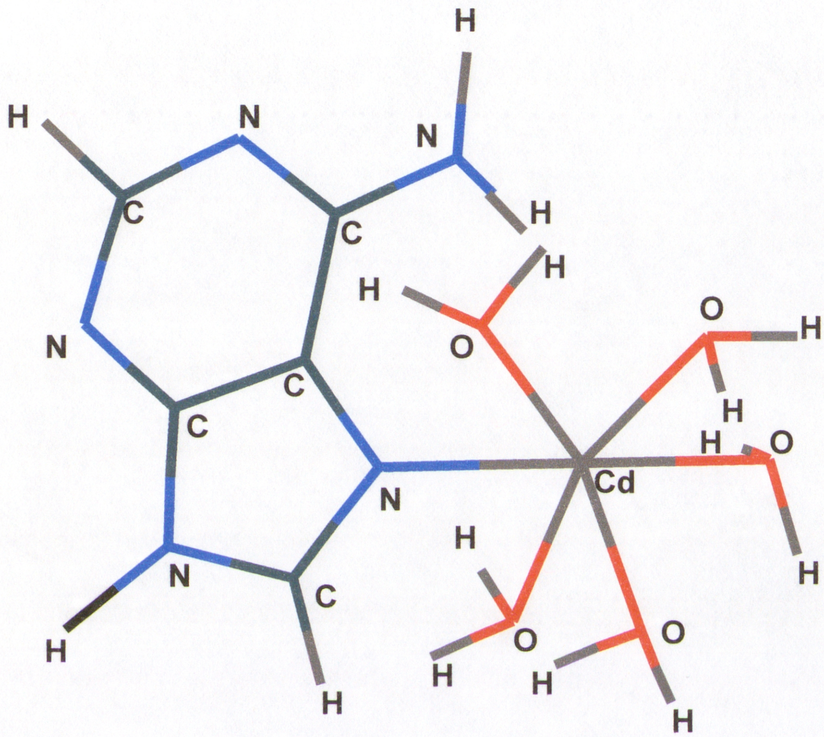


FIGURE 8.1: Proposed structure of Cd(adenine)$(H_2O)_5^{2+}$ in which Cd^{2+} is believed to be bonded to N7 centre of adenine and five water molecules

However, in the crystals of a binuclear complex between cadmium and adenine (grown from acidic solutions), each Cd^{2+} ion was found to be coordinated to the N3 centre of one adenine unit and the N9 centre of another adenine unit (Hsuan, *et al.*, 1981). The N1 and N7 centres were not involved in coordination; rather they were found to be protonated.

Martin (Martin, 1996) points out that N1 centre in purines is more basic than N7 position so that N1 coordination should be more likely than N7 coordination. As to the reason why there are more crystal structures showing N7 than N1 coordination, he suggests that it is probably due to the fact that crystals are grown from acidic solutions where N1 position becomes protonated. However, it was noted earlier that in the crystal of a binuclear complex grown from acidic solutions, cadmium was found to be bonded to N3 and N9 positions whereas N1 and N7 positions were protonated. In the 1:2 adduct between cadmium and adenine, it is assumed that Cd^{2+}, in addition to being bonded to two adenine ligands through their N7 centres, is also bonded to four water molecules so that the complex ion has the stoichiometry $Cd(adenine)_2(H_2O)_4^{2+}$ (Figure 8.2). In this adduct there is an intramolecular H bond between a coordinated water molecule and an amino nitrogen of an adenine molecule.

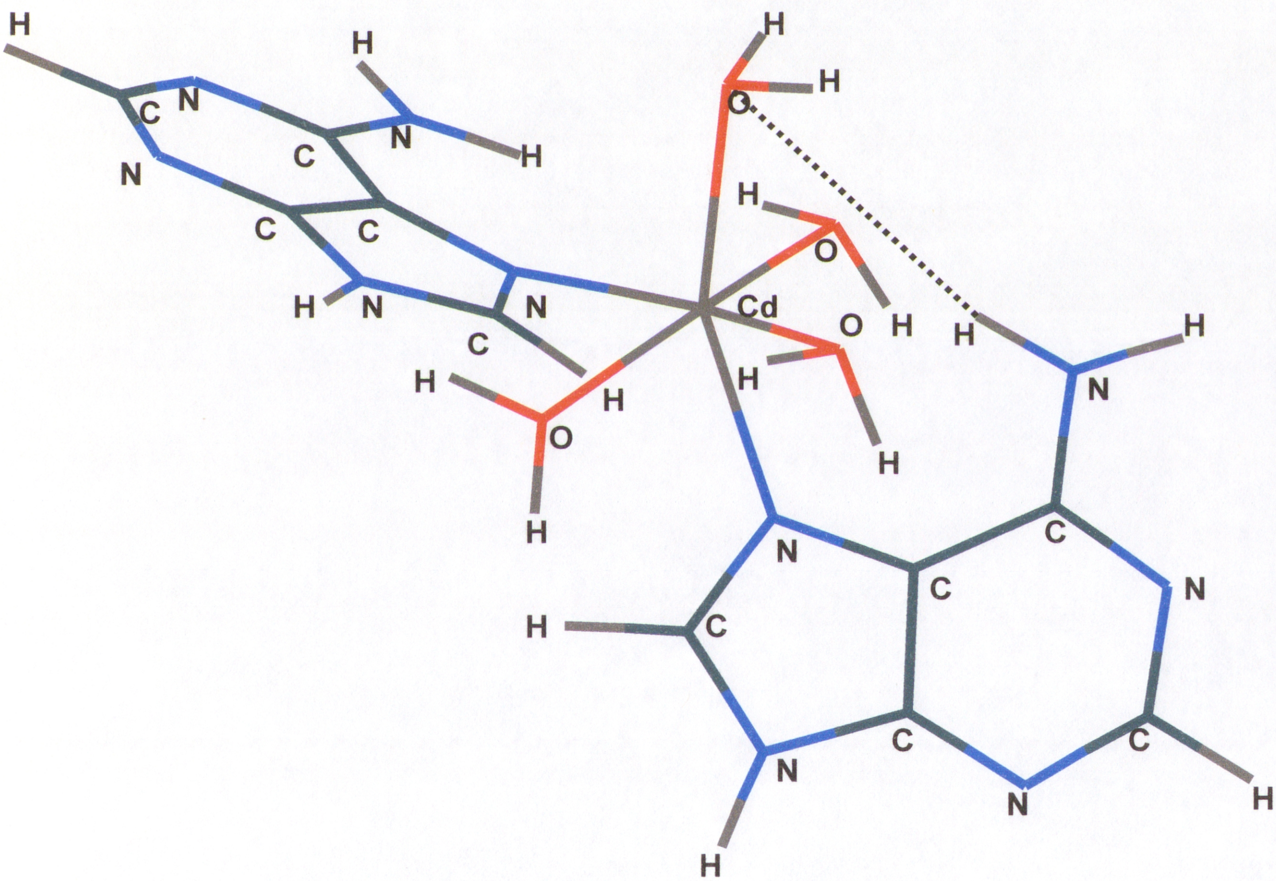


FIGURE 8.2: Proposed structure of $Cd(adenine)_2(H_2O)_4^{2+}$ in which Cd^{2+} is believed to be bonded to N7 centre of two adenine ligands and four water molecules

In the 1:3 adduct between cadmium and adenine, it is assumed that Cd^{2+}, in addition to being bonded to three adenine ligands through their N7 centres, is also bonded to three water molecules so that the complex ion has the stoichiometry $Cd(adenine)_3(H_2O)_3^{2+}$. In this adduct, there are seven intramolecular H bonds resulting into the formation of a number of macrochelates (Figure 8.3).

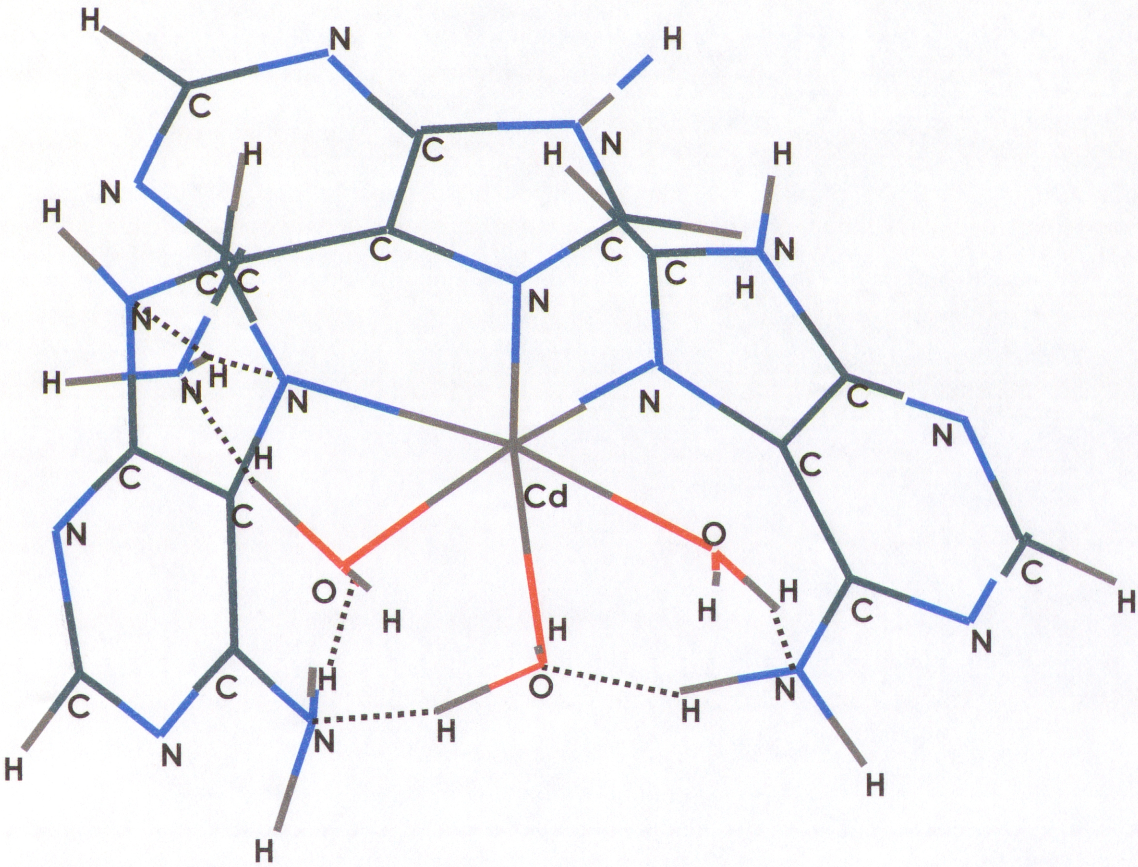


FIGURE 8.3: Proposed structure of $Cd(adenine)_3(H_2O)_3^{2+}$ in which Cd^{2+} is believed to be bonded to N7 centre of three adenine ligands and three water molecules

In the 1:1 adduct between cadmium and guanine, it is assumed that Cd^{2+}, in addition to being bonded to one guanine ligand through at its N7 centre is also bonded to five water molecules so that the complex ion has the stoichiometry $Cd(guanine)(H_2O)_5{}^{2+}$ (Figure 8.4). In this adduct, there are three intramolecular H bonds between coordinated water molecules and guanine.

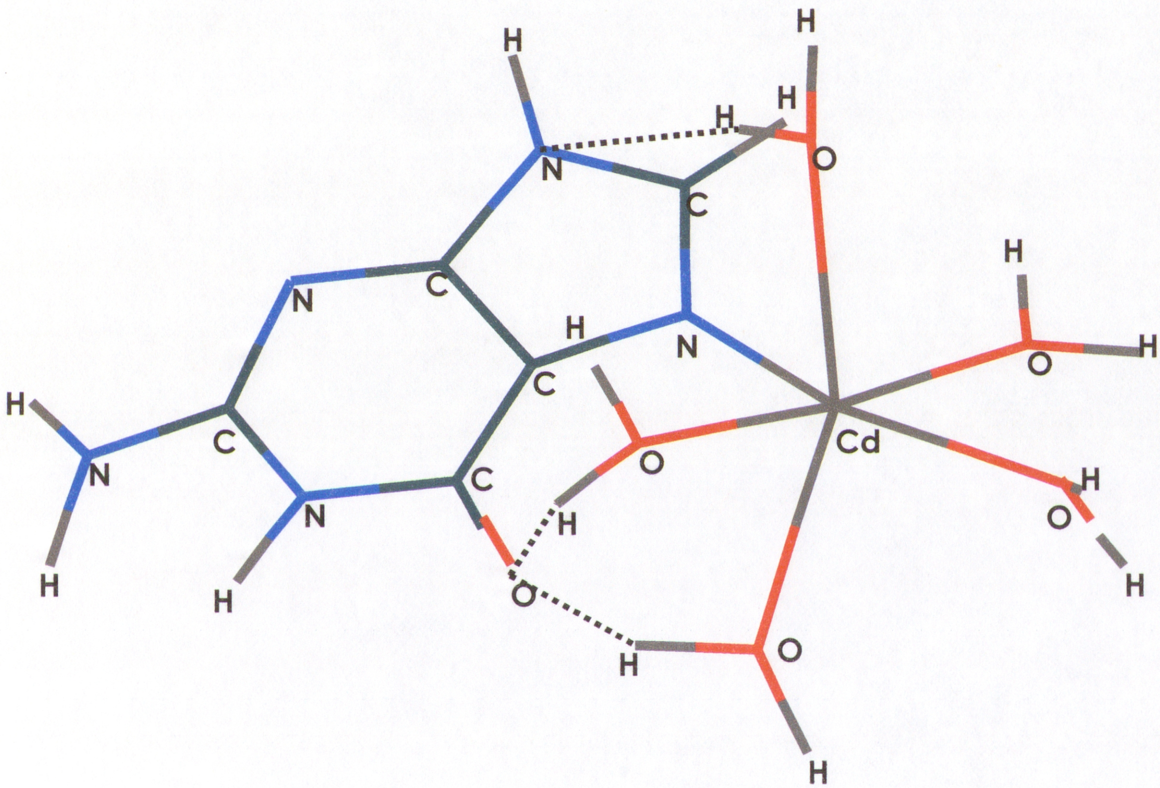


FIGURE 8.4: Proposed structure of $Cd(guanine)(H_2O)_5{}^{2+}$ in which Cd^{2+} is believed to be bonded to N7 centre of guanine and five water molecules

In the case of Cd^{2+} interacting with AMP the minimum in the absorbance versus concentration plot indicated the formation of a 1:3 adduct. This result is unexpected since the formation of $Cd(AMP)_3$ (in which cadmium is coordinated to three AMP ligands), is considered to be highly unlikely since there would be a strong repulsive interaction between $Cd(AMP)_2(H_2O)_4^{2-}$ and AMP^{2-}. The formation of 1:3 adduct can however be the result of stacking interaction between coordinated and uncoordinated nucleotides. It has been suggested that head-to-tail stacking with five-membered and six-membered rings alternating in the stack can occur in purines, especially adenine derivatives (Son, *et al.*, 1973).

The maximum observed in the absorbance versus concentration plot applying to the continuously varying mixtures of cadmium(II) nitrate and 2'-dGMP at 287 nm, suggested the formation of 1:1 adduct between Cd^{2+} and 2'-dGMP. In this adduct, it is believed that Cd^{2+} is bonded to N7 centre of guanine moiety and five water molecules so that the complex ion has the stoichiometry $Cd(2'\text{-}dGMP)(H_2O)_5^+$. In this adduct there are seven intramolecular H bonds forming a number of macrochelates (Figure 8.5).

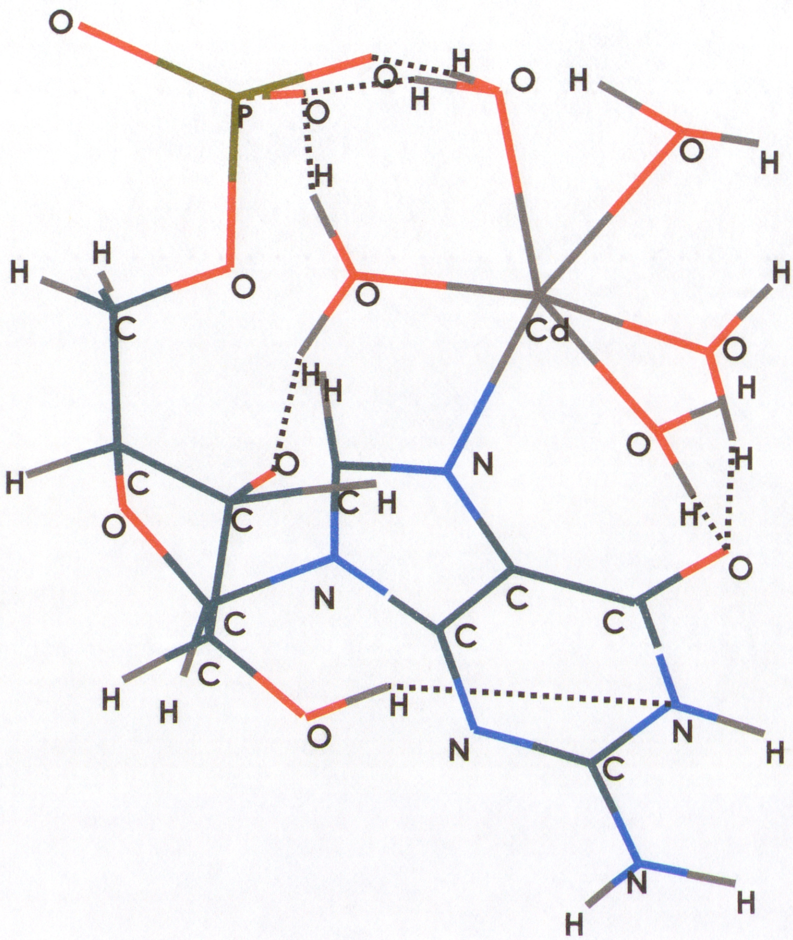


FIGURE 8.5: Proposed structure of $Cd(2'\text{-}dGMP)(H_2O)_5^{2+}$ in which Cd^{2+} is believed to be bonded to N7 centre of guanine moiety and five water molecules

Comment on H-bonding

It should be noted in a number of adducts, intramolecular H bonds formed are often associated with groups which are not available in DNA due to interstrand H bonding between nucleobases. Either such H bonds may not form with DNA or if they are formed, the consequence would be a marked change in the structure of DNA.

8.1.2 HPLC analysis

The retention time applying to the major peak in the chromatogram of incubated mixture of Cd^{2+} and adenine was found to be different from that of adenine (6.65 versus 7.45 min). However, when the peak fraction was analysed for cadmium content by AAS, no significant amount of cadmium was found to be present. It was also found that the retention time for the major peak did not change when the molar ratio of the reactants was varied. The reason why no cadmium was found in the peak-fraction is believed to be due to the dissociation of the labile adducts in the HPLC mobile phase. If that was so, the presence of acetate ions in the mobile phase, which can complex with Cd^{2+}, most possibly have contributed to the process by competing for Cd^{2+}. Then the question that remains to be answered is that of a change in retention time for adenine. It will be seen later that changes in retention time were also observed for adenine nucleotides but not for guanine and guanine nucleotides. The results are different from those observed in the interaction between Ni^{2+} and nucleobases and nucleotides (Huq, *et al.*, 2000) in the following two ways: (1) in interaction with Ni^{2+} changes in retention time were observed for guanine and guanine nucleotides but not for adenine and adenine nucleotides and (2) unlike that in the case of Cd^{2+} where a change in retention time occurred rather quickly (about 20 min after mixing), it occurred only after a prolonged period of incubation (24 h).

To find out whether, as a result of interaction with Cd^{2+}, 8-hydroxyadenine was produced from adenine, it was prepared as described (Bialkowski, *et al.*, 1998), injected into the HPLC and its retention time was noted. It was found that the retention time for 8-hydroxyadenine was different from that of the major peak fraction in the incubated mixture (2.80 min as against 6.65 min). So, what did happen to adenine that was reflected as a change in retention time?

Change in adenine causing a change in its retention time

Although it may be difficult to quantify the exact change in adenine, we may speculate about what might have happened. One possibility is that of a shift in the position of the amino-imino equilibrium due to interaction with Cd^{2+}. It is well known that amino-imino tautomerism can occur in adenine (Figure 8.6), substituted adenines, guanine and substituted guanines (Sabio, *et al.*, 1990, Pullman, *et al.*, 1969) and that one tautomer may be more stable than the other under a given set of conditions. Thus, it is possible that while for unreacted adenine one tautomer predominates, when bound to Cd^{2+} it is the other tautomer that may predominate. The difficulty with the idea is that of reconciling with the absence of cadmium in the peak-fraction (since cadmium is believed to be required to stabilise the tautomer), unless of course a shift in the tautomeric equilibrium is followed by a more permanent change.

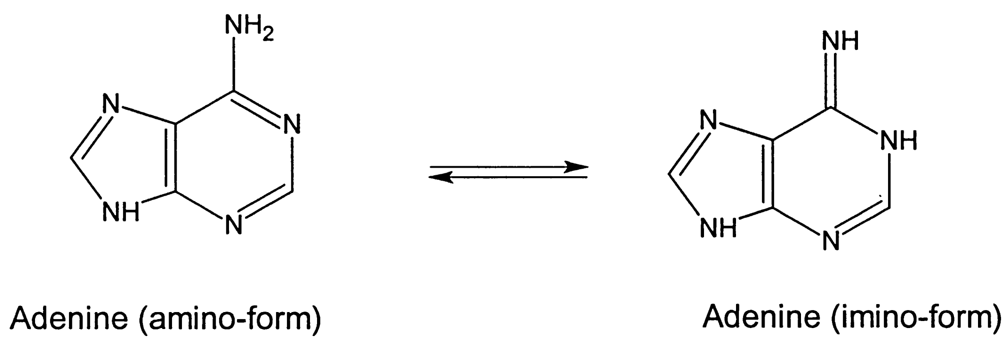


FIGURE 8.6: Tautomeric equilibrium in adenine

It should be noted when a metal ion binds covalently with a nucleobase, it can cause a perturbation of electron density in the heterocyclic ring such that it may be prone to further changes eg deprotonation and breaking of interbase H bonds that are found in DNA. It is also appropriate to note that when a metal ion binds to a nucleobase, nucleotide or DNA, it is likely to migrate from a kinetically preferred site to a thermodynamically preferred one or from a kinetically preferred adduct to a thermodynamically stable form. The situation with Cd^{2+} is clearly different from that with Ni^{2+} where it was found that 8-hydroxyguanine was produced from guanine. If indeed a shift in the tautomeric equilibrium followed by a more permanent change was that what actually occurred (on interaction with Cd^{2+}), it would provide an explanation as to why cadmium is mutagenic since the shift in the tautomeric equilibrium can result into mispairing of nucleobases. As Muller *et al.* (Muller *et al.*, 2000) points out a number of factors could lead to mispairing of nucleobases in DNA resulting from its interaction with metal ions. These include: (1) Blocking of H bonding sites, (2) Cross-linking of nucleobases, (3) Effect of acid-base properties of H bonding sites, (4) Effect on tautomeric equilibrium, (5) Metal-induced hydrolysis of exocyclic base groups (6) Hydrolysis of glycosidic bonds, (7) Oxidative changes of nucleobases and (8) Saturation of C-C double bonds in pyrimidine bases. To find out whether a permanent change was induced in adenine (and adenine nucleotides) due its interaction with Cd^{2+}, the following experiments were carried out. Equimolar solutions of cadmium(II) nitrate and adenine were mixed and incubated as described earlier. Cadmium was then precipitated as cadmium(II) carbonate by adding 0.1 M solution of sodium carbonate. The mixture was then filtered (using 0.22 μm millipore filter) to collect the filtrate whose pH was then adjusted to 7 by adding 0.01 M NaOH. It was then injected into HPLC as described earlier, and the retention times of any peaks observed were noted. The pH of 1 mM solution of adenine was also adjusted to 7 by adding 0.01 M NaOH. It was then injected into HPLC and its retention time noted. It was found that the retention time of the major peak in the incubated adenine after removal of Cd^{2+} was different from that of adenine itself (4.47 min versus 6.55 min). The results clearly show that the interaction with Cd^{2+} introduces a permanent change in adenine.

The following molecular modeling calculations were carried out to further investigate the problem. First the both the amino- and imino-forms of adenine in solution in water were optimized and their heats of formation calculated. It was found that the heats of formation of hydrated amino- and imino-forms of adenine were respectively –

59078.26 and –63047.58 k cal mol^{-1} showing that in solution in water the imino-form is more stable than the amino-form. Next, the imino-form of adenine was hydrated. Two possible additions were considered: (1) with OH added to the side-chain N and H to C6, (2) with OH added to C6 and H to the side-chain N. When the hydrated structures were optimized and the heats of formation of the optimized molecules were calculated, it was found that the adduct with OH added to the side-chain N is much more stable (heats of formation were respectively –73592.22 and –3475.93 kcal mol^{-1}).

It is therefore proposed that binding with Cd^{2+} causes a shift in the amino-imino tautomeric equilibrium so that imino-form predominates which is then hydroxylated to form the following adduct (Figure 8.7).

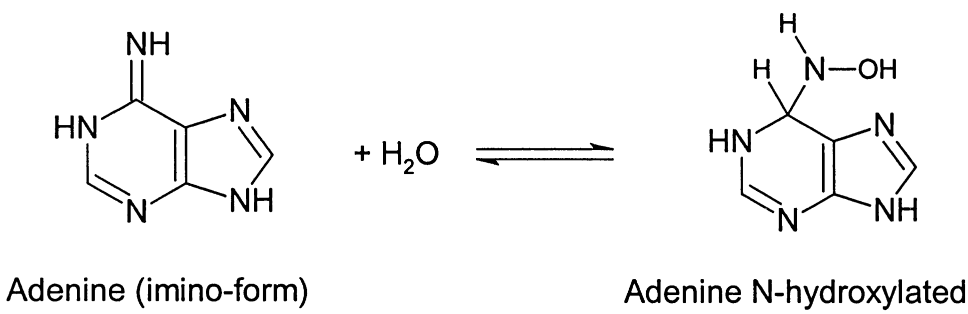


FIGURE 8.7: Addition of water to imino-form of adenine

IR Spectral confirmation of changes in adenine

To determine whether the suggested modification was indeed introduced in adenine due to its interaction with Cd^{2+}, adenine was allowed to interact with cadmium(II) nitrate (as described earlier) following which the metal ion was removed by its precipitation as cadmium(II) carbonate. The reflectance IR spectrum of the modified adenine was recorded using a Bio-Rad FTS-40 FTIR spectrometer with KBr as the dispersion medium. The IR spectrum was found to contain a broad band centered at 3464 cm^{-1}, and two narrow bands at 1397 cm^{-1} and 994 cm^{-1}. These are believed to be due to OH stretch and OH bending and N-O stretch (of N-O-H) respectively. In a related paper it was reported that when pBR322 plasmid DNA was allowed to interact with cadmium(II) acetate,digestion by Ssp1 enzyme was prevented (Hossain and Huq, 2001a). One possible reason for this could be structural modification introduced in adenine so that the enzyme was not able to recognise the AT site.

Finally, it needs to be explained why there was not a change in the retention time in the interaction between Ni^{2+} and adenine (and adenine nucleotides). Assuming that both Cd^{2+} and Ni^{2+} interacted covalently with adenine, the observed difference for the two metal ions may be due to a difference in the actual binding sites or due to a difference in the strengths of binding. It was noted earlier that metal ions could coordinate to both N1 and N7 sites. If indeed the preferred binding sites for Cd^{2+} and Ni^{2+} in adenine were different, they would have different effects on the ring electron density. For example, the binding of a metal ion at the N1 position would have a profound effect on the electron density at the C2 and C6 carbons whereas binding to N7 position is expected to have a profound effect on the electron density at the C8 but not on C2 or C6. Thus, we may consider that whereas nickel binds predominantly to N7, cadmium binds to N1. But this raises another problem since the binding of Cd^{2+} to N1 position will not allow the formation of imino-tautomer considered earlier. Of course, another binding site is the N3 position. It may be that for both the metal ions, the preferred binding site is actually N7 but the two metal ions differ in their strength of binding (Cd^{2+} is expected to bind more strongly than Ni^{2+} to adenine).

The analyses of minor peak fractions observed in the chromatogram indicated the formation of 1:1, 1:2 adducts between cadmium and adenine (results not shown in the table). There was also a tiny peak corresponding to the formation of 1:4 adduct. It should be recalled the UV difference spectral results also suggested the formation of 1:1 and 1:2 adducts between Cd^{2+} and adenine. However, whereas the difference spectral results also indicated the formation of 1:3 adduct, a minor peak in the chromatogram corresponded to the formation of 1:4 adduct. Since the sizes of these peaks were very small compared to the major peak observed in the chromatogram, the best inference that can be drawn from the HPLC results is that cadmium-adenine adducts are labile and therefore one should consider with caution the significance of the observed binding ratios.

In the case of Cd^{2+} interacting with guanine, the retention time of the major peak in the chromatogram of the incubated mixture was found to be practically the same as that of guanine (3.43 min versus 3.48 min). It was also found that the peak fraction did not have any significant cadmium. The results suggest that: (1) cadmium-guanine adducts are labile and (2) cadmium on binding with guanine does not induce any chemical modification in it, unlike that in the case of its interaction with adenine (considered earlier). As stated earlier, these results are different from those reported for the interaction between Ni^{2+} and nucleobases and nucleotides (Huq, *et al.*, 2000) where it was found that Ni^{2+} caused a chemical modification in guanine but not in adenine.

In the case of Cd^{2+} interacting with AMP, the fraction corresponding to the major peak was not found to have any significant cadmium although there was a change in retention time suggests that the binding of Cd^{2+} with adenine moiety induces a permanent change in it similar to that in the binding of cadmium with adenine (see earlier discussion) and the absence of cadmium in the peak fraction points to the lability of the adduct.

In the case of Cd^{2+} interacting with 2'-dGMP there was no significant change in retention time (4.19 min versus 4.25 min) as compared to that of 2'-dGMP and also the peak fraction did not have any significant amount of cadmium. The result suggests that either Cd^{2+} did not bind with 2'-dGMP (which is unlikely the case since binding was inferred from UV-difference spectral results) or if it did, the binding caused no structural modification in 2'-dGMP.

8.2 Interaction between Cd^{2+} and salmon sperm DNA (ssDNA)

The aims of the experiments were to characterise the adducts between Cd^{2+} and ssDNA in terms of UV absorbance and HPLC retention time so as to investigate whether Cd^{2+} binds with ssDNA.

8.2.1 UV-difference spectral analysis

When varying amounts of cadmium(II) nitrate were allowed to interact with ssDNA at a fixed concentration (0.07 mM in terms of phosphate), it was found that the absorbance values of the incubated mixtures (taken at 260 nm against ssDNA blanks) were negative for the [Cd^{2+}] range: 0.005 to 0.30 mM and positive for the range: 0.50 to 20 mM. The change in absorbance (first decrease and then increase) with the increase in [Cd^{2+}] is believed to be associated with increased binding of Cd^{2+} ions with nucleobases in ssDNA. Sorokin *et al.* (Sorokin, *et al.*, 1997) observed that when divalent transition metal ions were allowed to interact with calf thymus DNA, there was a decrease in absorbance around 250 nm and an increase in absorbance around 270 nm when the concentration of the metal ions was increased from 5 x 10^{-3} mM to 20 mM. Based on the results from continuous variation studies, it was concluded earlier (Sorokin, *et al.*, 1997) that Cd^{2+} binds preferentially to adenine and guanine (rather than cytosine and thymine) and if this preferred mode of binding applies to DNA as well, it is logical to assume that at low [Cd^{2+}], the metal ion binds to N7 centres of adenine and guanine. Thus, the decrease in absorbance of DNA at the lower concentration must be associated with the stabilisation of the double helix due to the covalent binding of Cd^{2+} ion to N7 centres of adenine and guanine (the positions that are not involved in inter strand H bonding. That at the higher concentrations, absorbance values increase, indicate that the DNA is denatured (at least partially) in a manner similar to that observed in its melting. The reason why the DNA denatures at the higher concentrations of the metal ion, is likely to be associated with its binding to other positions in adenine and guanine namely N1 and N3 centres, after saturation of the N7 positions. At the higher concentrations, the metal ion may also bind to nitrogen centres in cytosine and thymine. Irrespective of whether the additional binding involves other centres of adenine and guanine or nitrogen centres of cytosine and thymine, this will disrupt interbase H bonding and consequently will denature DNA.

8.2.2 HPLC analysis

From the UV-difference spectral results, it has been inferred that Cd^{2+} interacts covalently with ssDNA. HPLC studies have been carried out in order to investigate the interaction between Cd^{2+} and ssDNA.

Three major peaks are observed in the chromatogram of ssDNA (0.07 mM with respect to phosphate) and also in the chromatograms of mixtures for which the concentration of Cd^{2+} ranges from 0 to 10 mM. In addition, three minor peaks are observed when the concentration of Cd^{2+} is increased to 12.5 mM. No peak is observed when the concentration of Cd^{2+} lies in the range: 25 to 50 mM.

When the retention time of the first peak observed for the incubated mixtures is compared with that observed for the unreacted ssDNA, it is found that the value remains essentially unchanged (about 3.40 min) as the concentration of Cd^{2+} is increased from to 12.5 mM, although the size of the peak decreases significantly above 10 mM. When a similar comparison is made for the second and third peaks, it is found that the retention time of the second peak decreases from 7.55 to 7.13 min and that of the third peak decreases from 9.22 min to 8.07 min, as the concentration of Cd^{2+} is increased from 0 to 10 mM. When the concentration of Cd^{2+} is 12.5 mM, both the peaks decrease in size although there is a slight increase in retention time for both. The existence of three major peaks suggests that there are essentially three fractions of ssDNA based on molecular mass. If this is so, the fraction eluting first would have the lowest molecular mass whereas the fraction eluting last would have the highest molecular mass. The changes in retention time are most likely due to covalent binding between Cd^{2+} and ssDNA. The decrease in size of the peaks at high [Cd^{2+}] indicates the occurrence of a partial damage to ssDNA brought about by covalent binding of Cd^{2+} with the DNA. It should be recalled that when the interaction between plasmid

DNA and Cd^{2+} was studied by submarine gel electrophoresis, it was found that as the concentration of Cd^{2+} was increased, the intensity of both Form I and Form II bands decreased (especially at high concentrations). The decrease in intensity of the bands was attributed (at least in part) to some DNA damage as a result of covalent binding between plasmid DNA and Cd^{2+} (See chapter 7 Figure 7.12 (d) page 170).

Summary

The results of both gel electrophoresis and HPLC indicate that a high concentration of Cd^{2+} can cause damage to plasmid and genomic DNA.

8.3 Interaction between Cd^{2+} and antioxidants

The aims of the experiments were to characterize the adducts between Cd^{2+} and antioxidants in terms of either HPLC retention time and/or the binding ratio between the metal ion and the ligand, so as to serve as a reference to investigate the interaction between cadmium-cysteine and cadmium-glutathione complexes with adenine and guanine or that between Cd^{2+} and DNA in presence of ascorbate.

8.3.1 Interaction between Cd^{2+} and cysteine or glutathione: *HPLC analysis*

The results of HPLC analysis showed that Cd^{2+} combined with both cysteine and glutathione (more strongly with glutathione than cysteine). Relatively large size of the peak due to the complex ion as compared to that due to the free ligand suggests that most of the ligand and Cd^{2+} ions in solution have combined together forming the expected 1:2 complex. The results also show that unlike cadmium-nucleobase or cadmium-nucleotide complexes (which decomposed completely in the HPLC mobile phase because of a greater lability), the complexes of Cd^{2+} with cysteine and glutathione remained mostly intact in the HPLC mobile phase, indicating that Cd^{2+} binds more strongly with cysteine and glutathione than with nucleobases or nucleotides.

8.3.2 Interaction between Cd^{2+}-cysteine complex/Cd^{2+}-glutathione complex and adenine or guanine: *HPLC analysis*

It was established from the results of the preceding experiments that cadmium-cysteine and cadmium-glutathione complexes remained mostly intact in the mobile phase. The results of the present series of experiment show that cadmium-cysteine and cadmium-glutathione complexes combined with adenine and guanine forming cadmium-cysteine-adenine, cadmium-cysteine-guanine, cadmium-glutathione-adenine, cadmium-glutathione-guanine adducts. However, these reactions did not go to completion as indicated by the presence of free cadmium-cysteine or cadmium-glutathione complexes in the HPLC mobile phase. Why cadmium-cysteine-nucleobase or cadmium-glutathione-nucleobase adducts did not decompose completely in the HPLC mobile phase (but cadmium-nucleobase and cadmium-nucleotide adducts did) can be explained in terms of a caging effect produced by cysteine and glutathione around cadmium. This means that the crowding produced around cadmium due to bound cysteine or glutathione, although may retard the approach of a further ligand to come in close proximity to cadmium ion, it also means that once another ligand such as adenine or guanine gets bound to the metal ion (possibly after a prolonged incubation), it may not be easily replaced by a solvent molecule or another ligand.

8.4 Interaction between Cd^{2+} and DNA

8.4.1 Cd^{2+}-plasmid binding assays

These results in the binding assays of Cd^{2+} with plasmid DNA clearly show that Cd^{2+} ions bind covalently with DNA. The reasons why a high concentration of cadmium(II) nitrate solution at the pH of the solution causes extensive damage to the DNA, are probably due to the combined effect of covalent binding of Cd^{2+} with DNA and the acid-catalysed hydrolysis of DNA. According to Bloemink *et al.* (Bloemink, *et al.*, 1996), the acid catalysed hydrolysis of the *N*-glycosidic bond in DNA involves the rupture of the bond (following the protonation of purine bases) producing a free base and a resonance stabilised oxocarbenium ion (Figure 8.8).

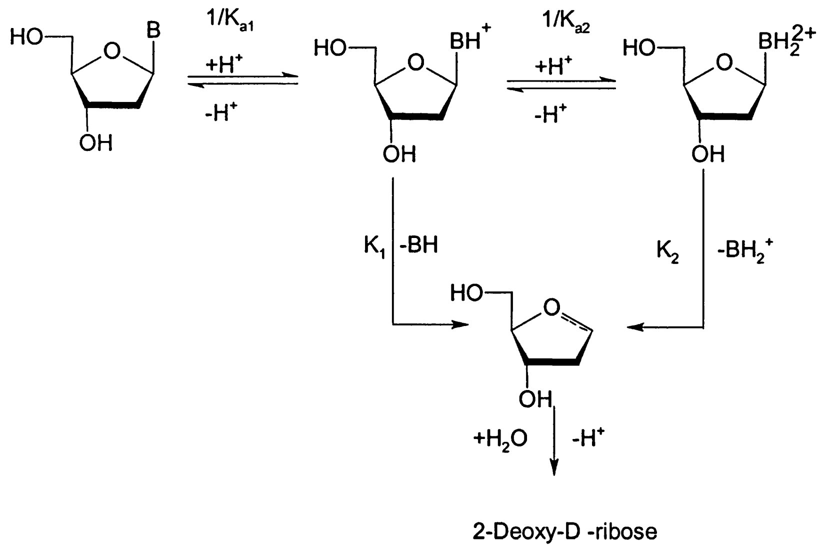


FIGURE 8.8: Reaction scheme depicting the acid-catalysed hydrolysis of *N*-glycosidic bond

The rate of bond rupture is dependent upon the number of protons attached to purine bases. Since the pK_{a2} value in purine nucleosides is 4-5 units lower than pK_{a1}, the diprotonated base moiety is likely to leave the nucleoside structure 10^4-10^5 times more rapidly than the monoprotonated species (Kuusela, *et al.*, 1996). Thus, at high concentrations of H^+ it is possible that adenine and guanine bases of DNA are protonated at their N3 and N7 centres, making them more susceptible to leave the structure by the rupture of the *N*-glycosidic bond. It is also expected that a metal ion bearing a positive charge (such as Cd^{2+}) can replace a proton. Coordination of the metal ion to one of the ring nitrogens of the purine bases would cause electronic perturbation in the heterocyclic ring systems such that the electron density at the N9 atom is lowered. The result would be a weakening of the phosphodiester bonds leading to the unimolecular rupture of the *N*-glycosidic bond (Kuusela, *et al.*, 1996). It is also likely that metal ions can promote hydrolysis of phosphodiester bonds since they can function as Lewis acid, polarising phosphorous-oxygen bond to facilitate bond breakage (Barton, 1994).

It is also likely that the DNA damage may be caused by O_2 especially in presence of increased H^+. Redox active metal ions such as those of copper or iron may undergo Fenton or Haber-Weiss reaction (Tanaka, *et al.*, 1999) producing hydroxide radical ($^{\bullet}OH$) which is a powerful and non-selective oxidant that can abstract hydrogen atoms from organic molecules. It can modify the DNA bases and cause strand scission by degrading the ribose ring. This will be discussed again when the effect of antioxidants on the damage to DNA due to Cd^{2+} is considered in the next section. However, both the mechanisms require the involvement of hydrogen peroxide, which is formed in living systems. In aerobic living systems, molecular oxygen is reduced to produce superoxide anion, then to H_2O_2 and finally H_2O. Although the reduction of O_2 to H_2O_2 is thermodynamically favourable (E^o = +0.82 V), two electron transfer is difficult because of kinetic barriers imposed by spin state restrictions (Wilkins, *et al.*, 1997).
In any case, the production of hydroxide radical by either of the mechanisms is not relevant in the present study since Cd^{2+} is not redox active and that no hydrogen peroxide is present.

It should be recalled that at neutral pH, when [Cd^{2+}] was low, both Form I and Form II bands appeared and the migration rate of the bands decreased when [Cd^{2+}] was increased. It was also found that as [Cd^{2+}] was increased, there was a progressive decrease in intensity of the Form I band whilst that of Form II band first increased and then decreased slightly (Figure 7.12 (d)). The decrease in migration rate can be explained in terms of an increased amount of Cd^{2+} being bound to DNA thus causing an increase in its mass and a decrease in its overall negative charge. The decrease in intensity of the bands may be due to a partial damage and a change in conformation of the DNA.

Enzyme digestion

The difference in binding of Cd^{2+} and plasmid DNA in presence of cutting enzymes is considered. Enzyme Bam H1 naturally hydrolyses phosphodiester bonds between adjacent guanine sites, on the other hand, Ssp1 hydrolyses the adjacent adenine sites. Appearance of two bands in the case of Ssp1 digestion and one band in the case of BamH1 digestion indicates that cutting due to Ssp1 is prevented whereas that due to BamH1 is not hindered. It can therefore be concluded that Cd^{2+} forms intrastrand bifunctional AT adducts but not the GG adducts. Alternatively, if GG adducts are also formed, they need to be more labile than the AT adducts so that differential DNA damage may result. However, if it is so, the increased DNA damage in the case of BamH1 can be explained in terms of continual breaking and formation of the adducts so that BamH1 can attack the DNA at Cd^{2+}-free GG sites. This would explain why damage to DNA due to BamH1 is more pronounced at low [Cd^{2+}]. Another possible scenario could be that neither AT nor GG intrastrand adducts are formed. If that is so, Ssp1 digestion may still be prevented because of chemical modification introduced in adenine due to its binding with Cd^{2+} (see Section 8.1.2) such that the enzyme does not recognize the AT site. The idea that intrastrand AT adducts are not formed, would also fit in well with the observation that Cd^{2+} is not found to bind easily with thymine.

8.4.2 Cd^{2+}-plasmid binding in presence of antioxidants

Cd^{2+}-cysteine and plasmid

It can be seen that the cadmium-cysteine complex is more damaging to DNA than cadmium(II) nitrate (See chapter 7, section 7.7.1, page 174). The difference can be explained in terms of damage caused by ROS produced in the reaction between cysteine and molecular oxygen, in addition to the damage resulting from the combined effect of acidity and covalent binding (which are common in both cases).

The results show that the solution of cysteine is more damaging to DNA at pH 7.4 than at pH 4.0. (See chapter 7, section 7.7.1, page 174). This can be explained by considering the fact that cysteine is more stable under acidic conditions than in neutral or slightly alkaline solutions in which case it is oxidized by molecular oxygen to produce cystine and ROS (The Merck Index, 1983).

That Cd(cysteine)$_2$ is less damaging than free cysteine but more damaging than cadmium(II) nitrate, can be explained in terms of the following: Since, Cd^{2+} has a strong affinity for cysteine (which is a sulfur donor ligand), concentration of free cysteine is likely to be very small (but not zero) in 1:2 mixture between Cd^{2+} and cysteine. This means that more ROS are produced due to cysteine alone than due to the 1:2 mixture between Cd^{2+} and cysteine.

It was noted earlier that cysteine is more stable under acidic conditions than in neutral or slightly alkaline solutions in which case it is oxidized to cystine by molecular oxygen and ROS (The Merck Index, 1983). When these experiments were repeated under a nitrogen atmosphere, Forms I and II bands were observed that remained unchanged in intensity with the change in concentration of the cadmium-cysteine complex (Figure 7.14 (d) page 178). From the above results, the following conclusions can be made: (1) Molecular oxygen can cause some damage to DNA, (2) ROS produced from interaction between molecular oxygen and the 1:2 mixture between cadmium(II) nitrate and cysteine must be the main contributor to DNA damage. It was stated earlier that the highly acidic solution of cysteine obtained by dissolving cysteine hydrochloride caused total damage to DNA even when the concentration of cysteine was very low.

It appears that in the case of cysteine hydrochloride solution (which has a pH of about 1.50), extensive damage to DNA is mainly due to acid-catalysed depurination of the DNA (Kuusela, *et al.*, 1996) whereas in the case of the cadmium(II) nitrate and cysteine mixture (which has a pH of about 4.0), the observed damage is mainly due to ROS produced from the reaction between cysteine and molecular oxygen. The increase in the extent of damage to DNA with the increase in pH from 4.0 to 7.4 (observed for cysteine solutions as well as cadmium(II) nitrate and cysteine mixtures) is simply the result of increased production of ROS due to the reduced stability of cysteine under those pH conditions.

Cd^{2+}-glutathione and plasmid

The extensive damage to DNA caused by glutathione at the pH of the solution is believed due its high acidity (pH 2.5). Thus the reason why a solution of glutathione caused an extensive damage to DNA whereas a mixture of cadmium(II) nitrate and glutathione did not, is also believed to be due to the difference in their acidity (pH 2.5 for glutathione and 4.0 for the mixture). As stated earlier, indeed the damaging effect of glutathione was gone when its pH was adjusted to 7.4. It was observed at pH 7.4, the mobility of the bands was less in the case of the mixture than in the case of glutathione alone and in both cases, the mobility increased as the concentration of the mixture or glutathione decreased. The reduced mobility in the case of mixture is believed to be due to the covalent binding of $Cd(glutathione)_2$ with DNA. The fact that a decrease in concentration of glutathione also caused an increase in mobility of the bands suggests the occurrence of some kind of association between glutathione and DNA.

Cd^{2+}-ascorbate and plasmid

As stated earlier, Littlefield *et al.* (Littlefield, *et al.*, 1995) also observed that the damage to double stranded DNA (of human lymphocyte cells) due to Cd^{2+} and other heavy metal ions was accentuated by ascorbate. In their study, they allowed the human lymphocyte cells first to interact with cadmium(II) chloride (50 μM) for four hours at the end of which ascorbate (500 μM) was added to achieve a cadmium(II) chloride to ascorbate molar ratio of 1:10. Then, the incubation was continued for a further period of time. In the present study, plasmid DNA was allowed to interact with mixtures of cadmium(II) acetate and ascorbate where the concentration of each was varied from 0 to 100 mM. It was found that maximum damage occurred when cadmium(II) acetate and ascorbate were mixed at 1:1 molar ratio.

The results suggest that damage to DNA is potentiated when cadmium(II) acetate and ascorbate are present together at 1:1 molar ratio. Thus, whereas neither cadmium(II) acetate nor ascorbate alone (up to 100 mM concentration) caused any significant damage to DNA, when present together at 1:1 molar ratio, only 1 mM concentration of each caused total damage to DNA. It should be noted here that, as reported by Littlefield *et al.* (Littlefield, *et al.*, 1995), damage to DNA in presence of ascorbate was greater for cadmium(II) chloride than for nickel(II) chloride. Nickel is a redox active metal and may produce reactive free radical which in turn can cause oxidative damage to DNA. But that this metal is found in most cases to cause less damage to DNA is probably because of the fact that nickel acts on the heterochromatin fibre and hence does not act directly on the DNA molecule (Littlefield, *et al.*, 1995). On the other hand, cadmium is more damaging to DNA because of its direct action on DNA molecule (Littlefield, *et al.*, 1995).

The present study shows that although ascorbate is a powerful antioxidant that can protect DNA from oxidative damage, in presence of heavy metal ions such as Cd^{2+}, it enhances damage to DNA. Blasiak *et al.* (Blasiak, *et al.*, 2000) also found that there was a synergistic effect of vitamin C on damage to DNA (in human lymphocytes) due to Cd^{2+}. It is also well known that in presence of oxidants such as molecular oxygen and redox active metal ions, biological reductants such cysteine or ascorbate can cause a greater damage to DNA probably because of the production of free radicals. In the case of redox active metal ions such as those of nickel, chromium, iron and copper, the generation of free radicals may follow a Fenton-type reaction. According to Littlefield *et al. in vitro* damage to d/s DNA by cadmium(II) chloride is due to the induction of a Fenton-type reaction in which ascorbate is oxidised producing hydroxyl radicals (•OH) which can cause damage to DNA (Littlefield, *et al.*, 1995). Likewise, Blasiak *et al.* also concluded that the damage to DNA of human lymphocytes due to Cd^{2+} in presence of vitamin C is due to participation of cadmium in a Fenton reaction (Blasiak, *et al.*, 2000). Although it was not clearly stated, it was implied that in the reaction cadmium undergoes redox cycling. It is difficult to see how such a mechanism is possible for Cd^{2+} since it is not redox active at least under the conditions available in the living systems, although it can explain the DNA damage due to chromate in presence of ascorbate (Kortenkamp, *et al.*, 1994). Hence, a different mechanism must operate in the case of the mixture of cadmium(II) acetate and ascorbate. Since Cd^{2+} is expected to bind covalently with nucleobases in DNA, the observed much pronounced DNA damage due to a 1:1 mixture of cadmium(II) acetate and ascorbate can be due to a combination of the following factors: (1) Perturbation of the electron density in heterocyclic ring of nucleobases due to binding with Cd^{2+} resulting into a weakening of *N*-glycosidic bond between the nucleobase and the pentose sugar (2) Cd^{2+} playing a catalytic role in the oxidation of ascorbate by biological oxidants (molecular oxygen in the present experiments). That the greatest damage is observed for the 1:1 combination may also suggest that there is a one-to-one interaction between Cd^{2+} and ascorbate that results into the production of reactive oxygen species in presence of molecular oxygen. When cadmium(II) acetate is allowed to react with ascorbate in solution in water, UV-difference spectral results show that Cd^{2+} indeed reacts with ascorbate forming 1:1 adduct in solution (Figure 7.11 page 165). Noriko *et al.* (Noriko, *et al.*, 1999) found that even in the absence of heavy metal ions, L-ascorbic acid is oxidised by molecular oxygen to produce superoxide anion. It is also known that in presence of heavy metal ions and redox-active transition metal ions, ascorbate can undergo autooxidation producing highly reactive ascorbate radical (Noriko, *et al.*, 1999). It is proposed that 1:1 complexation of Cd^{2+} with ascorbate activates the ascorbate anion such that it is more susceptible to autooxidation and/or more susceptible to react with molecular oxygen (Hossain and Huq, 2002b). Irrespective of the actual mechanisms that may be operating, it is clear from above, the biological antioxidants such as ascorbate that play a protective role in our body under normal physiological conditions, in presence of harmful xenobiotics such as Cd^{2+} or CrO_4^{2-}, these may play a more damaging role due to the production of free radicals. Thus, Liehr, *et al.* points out that there is a dynamic balance between oxidation and antioxidation that is constantly on going within the cell (Liehr, *et al.*, 1989).

Since, the biological half-life of cadmium is long (5 to 15 years for liver, 10 to 30 years for kidney and over 30 years for muscle); continued exposure to the metal ion will invariably result into an increased body burden due to cadmium. This in turn may have an impact over normal functioning of the cell eg it may increase the concentration of circulating Cd^{2+} in the blood. If the increased DNA damage due to cadmium in presence of ascorbate that was observed in *in vitro* experiments, is also applicable to *in vivo* situation, clearly at high concentrations of Cd^{2+}, ascorbate rather than protecting DNA, may actually promote DNA damage. However, it should be noted that the situation in biological systems is more complex due to the presence of other Cd^{2+} sequestering agents (eg proteins, peptides and amino acids) and free radical scavengers such as superoxide dismutase. Thus, in the absence of conclusive *in vivo* results, it is difficult to decide whether regular intake of Vitamin C should be contra-indicated for persons who have a continued exposure to cadmium (eg smokers).

Summary

In presence of antioxidants (such as ascorbate and cysteine) and Cd^{2+}, there is a pronounced damage to DNA. This is believed to be caused by reactive oxygen species produced in the interaction between molecular oxygen and antioxidants, with the metal ion playing a catalytic role in the reaction. In the case of Cd^{2+} and ascorbate mixture, it is found that damage is most pronounced when Cd^{2+} and ascorbate are present in 1:1 molar ratio. Indeed it has been found that Cd^{2+} binds with ascorbate forming predominantly 1:1 adduct. Thus, it has been concluded that ascorbate is activated due to its binding with Cd^{2+} so that it is more susceptible for reaction with molecular oxygen.

8.5 Interaction between Cd^2+ and genomic DNA

8.5.1 Interaction between cadmium(II) acetate and salmon sperm DNA (ssDNA) in absence or in presence of ascorbate

The results of submarine gel electrophoresis show that Cd^{2+} reacts with ssDNA to give a single DNA band whose mobility decreases as the concentration of Cd^{2+} is increased. There is also a corresponding decrease in the intensity of the band with the increase in concentration of Cd^{2+}. The decrease in mobility of the band indicates increased binding of Cd^{2+} to the DNA. The decrease in intensity of the band especially at high concentrations of Cd^{2+} indicates the occurrence of a partial damage to DNA, that is believed to be the result of covalent binding between nucleobases in DNA and Cd^{2+}. In some respects, the results are similar to that was observed in the interaction between Cd^{2+} and plasmid DNA where it was found that the mobility and intensity of Form I and Form II DNA bands decreased as the concentration of Cd^{2+} was increased.

Ascorbate alone at pH 7.4 is found not to cause total damage to plasmid DNA for all concentrations of ascorbate ranging from 0 to 100 mM. However, it is found that as the concentration of ascorbate is increased, the intensity of the Form I band decreases and that of Form II band increases. The results indicate that in the presence of an increasing amount of ascorbate, a greater number of supercoiled plasmid DNA molecules are partially damaged to produce singly-nicked relaxed Form II plasmid DNA. Damage to ssDNA due to ascorbate is found to be less than that in the case of plasmid DNA. The single ssDNA band is observed for all concentrations of ascorbate from 0 to 100 mM. However, there is a slight decrease in the mobility of the band as the concentration of ascorbate is increased unlike that in the case of plasmid DNA where there is an increase in the mobility of the bands. The decrease in mobility in the case of ssDNA points to some kind of associative interaction between ssDNA and ascorbate, resulting into an increase in the effective molecular mass of the DNA and/or a change in its conformation. The increase in mobility of the Form I and Form

II bands of plasmid DNA can be explained only in terms of a change in conformation of the DNA (eg nature of super-coiling of the Form I DNA).

When 1:1 mixtures of Cd^{2+} and ascorbate are allowed to interact with ssDNA, it is found that the DNA is completely damaged when the concentration of the mixture is $\geq$ 25 mM. A partial damage to DNA occurs in the concentration range: 1 to12.5 mM. It is also found that in the concentration range: 1–12.5 mM there is a sharper decrease in mobility of the band as the concentration of the mixture is increased. The decrease in mobility indicates increased binding of Cd^{2+} with the DNA. The total damage to DNA occurring in the case of mixture of Cd^{2+} and ascorbate (concentration $\geq$ 25 mM) but not in the case of Cd^{2+} or ascorbate (present alone), indicates the catalytic effect of the metal ion on the oxidation of the ascorbate. It has been noted before that Cd^{2+} binds with ascorbate at 1:1 molar ratio and that the resulting complex is more susceptible to oxidation than free ascorbate. In fact, it has been found that the mixture of Cd^{2+} and ascorbate is more damaging to plasmid DNA than ssDNA (total damage of plasmid DNA occurred at concentration $\geq$ 1 mM whereas total damage to ssDNA occurred at concentration $\geq$ 25 mM). Similarly it is found that damage to calf thymus DNA is less than that in the case of plasmid DNA (considered later). The results suggest that as compared to plasmid DNA, genomic DNA is more protected from the onslaught of free radicals. As to the reasons why that is so, the following may be cited. Since plasmid is a prokaryotic DNA its polymeric conformation is less organised and less compact so that it is easily susceptible to attack by ROS (especially the nucleobases of the DNA). On the other hand, linear ssDNA (which is eukaryotic) has a more compact structure and hence less susceptible to attack by ROS.

8.5.2 Interaction between cadmium(II) acetate and calf thymus DNA in absence or in presence of ascorbate

The results of submarine gel electrophoresis show that calf thymus DNA appears as a single streaking band which indicates that the molecular mass of the DNA is distributed over a range of values – an observation which supports the more complex nature of the DNA as compared to ssDNA or plasmid DNA. When it is allowed to interact with Cd^{2+}, it is generally true to say that the intensity of the band gets brighter as the concentration of Cd^{2+} is increased. A more careful analysis shows that as concentration of Cd^{2+} is increased above 50 μM, the intensity of the tail part of the band increases and that of the head part decreases such that at 100 mM concentration of Cd^{2+}, the head part is almost missing. It is also found that the mobility of the band decreases as concentration of Cd^{2+} is increased. The decrease in mobility of the band indicates increased binding of Cd^{2+} to the DNA. The decrease in intensity of head part of the band especially at high concentrations of Cd^{2+} indicates the occurrence of a partial damage to DNA, that is believed to be the result of covalent binding between nucleobases in DNA and Cd^{2+}.

The increase in intensity of the tail part at high concentration of Cd^{2+} is believed to be associated with a change in conformation of the corresponding DNA fraction (because of increased covalent binding with Cd^{2+}) such that more of ethidium bromide intercalates within DNA.

In presence of ascorbate also, calf thymus DNA appears as a single streaking band. The mobility of the band is found to remain unchanged with the change in concentration of the ascorbate from 0 to 100 mM. As the concentration of ascorbate is increased, the intensity of the band increases uniformly to reach a maximum at 50 μM. Beyond 50 μM, the intensity of the band gradually decreases as the concentration of ascorbate is increased. When the changes in intensity of ssDNA and calf thymus DNA bands are compared due to their interaction with ascorbate, it is found that the changes are more pronounced in calf thymus DNA than ssDNA, although both are genomic in nature. Perhaps the observed changes in intensity of the calf thymus DNA band are a direct consequence of changes in conformation of the DNA (rather than actual damage) such that the degree of intercalation of ethidium bromide molecules changes. This is especially true at low concentrations of ascorbate. At higher concentrations however, a partial damage to the DNA may take place and as a result a decrease in intensity can occur. It is found that the widths of the bands are greater in presence of ascorbate than in the presence of Cd^{2+}. Possibly it is associated with the difference in the nature of their interaction with the DNA – whereas Cd^{2+} binds covalently, ascorbate is more likely to undergo noncovalent interactions.

When 1:1 mixtures of Cd^{2+} and ascorbate are allowed to interact with calf thymus DNA, total damage to DNA is found to occur when the concentration of the mixture is ≥ 25 mM. It is also found that, for all concentrations, the DNA band is fainter than that observed in the interaction of calf thymus DNA with cadmium(II) acetate.

8.6 Interaction between Ag^+ and plasmid or genomic DNA

8.6.1 (a) Interaction between silver(I) acetate and plasmid DNA

When plasmid was allowed to interact with silver(I) acetate, two bands were observed for all concentrations of Ag^+ ranging from 0 to 50 mM. There was no observable change in the intensity of the bands with the increase in concentration of the metal ion. However, the mobility of the bands decreased slightly as [Ag^+] was increased. The decrease in mobility is believed due to binding of Ag^+ ions to DNA, thus reducing its overall negative charge and increasing its molecular mass. The decrease in mobility could also be due to a change in conformation of the DNA as it is well known that binding of metal ions to nucleobases in DNA can change its conformation (Lippard, 1994, Rossetto, *et al.*, 1994). The results show that Ag^+ ions within the concentration range (0 to 50 mM) do not cause any observable damage to pBR322 plasmid DNA at pH 7.4. The results are different from those observed in the interaction of cadmium(II) acetate with plasmid DNA where an increasing damage to DNA occurred, as [Cd^{2+}] was increased (Figure 7.12 (d) page 170). It can therefore be concluded that Cd^{2+} is more damaging to plasmid DNA than Ag^+. It has been proposed that Ag^+ forms three types of complexes with DNA (type I, type II and type III) depending on the concentration of Ag^+ and pH of the solution [Arakawa, *et al.*

2001]. In type I complex, Ag^+ binds to N7 positions of adenine and guanine in a single strand. In type II complex, the metal ion forms bifunctional interstrand AT and GC adducts whereas in type III complexes (which are found in the solid state) the mode of binding is less clear-cut. Type I complexes are formed at low $[Ag^+]$:[nucleotide] ratio ($r < 0.2$). Type II complexes are formed when r ranges from 02 to 0.5 and type III complexes may be formed when r is greater than 0.5 when the major binding sites for type I and type II complexes have become already saturated. In this experiment, the r value ranges from about 0.40 to 800 indicating that type II and type III will predominate. Unlike Ag^+ which binds strongly to nucleobases but not to phosphate groups, Cd^{2+} can bind both to nucleobases and phosphate groups. Whereas at low $[Cd^{2+}]$, the metal ion may bind significantly to phosphate groups, at high $[Cd^{2+}]$ it may bind more to N7 positions of adenine and guanine. That there was no observable damage to pBR322 plasmid DNA with the increase in $[Ag^+]$ unlike that in the interaction with Cd^{2+} (where there was a progressive decrease in the intensity of both Form I and Form II bands with the increase in concentration of the metal ion) can be explained in terms of the difference in the nature of binding of the two metal ions. Whereas type I binding destablizes DNA, type II binding actually does the opposite by tying the two DNA strands together. Hence, in the concentration range used in the experiment, in the absence of other complicating factors, one would expect an overall increase in stabilization of DNA as $[Ag^+]$ is increased. On the contrary, in the case of Cd^{2+}, there will be a progressive destabilization and even damage of DNA (because of the weakening of phosphodiester bonds resulting into hydrolysis) as $[Cd^{2+}]$ is increased since no bifunctional interstrand adducts (similar to type II adducts associated with Ag^+) are formed.

8.6.1 (b) Interaction between silver(I) acetate, ascorbate and plasmid DNA

It was found that when ascorbate alone at pH 7.4 was allowed to interact with plasmid DNA, two bands were observed for all concentrations of ascorbate from 0 to 100 mM (Figure 7.16 (a) page 183). However, the intensity of the Form II band was found to increase and that of Form I band decrease as the concentration of ascorbate was increased. The changes in intensity of the bands (decrease for Form I and increase for Form II) indicated the occurrence of a partial damage to supercoiled DNA to produce more of the singly-nicked Form II plasmid DNA. It is believed that the observed DNA damage is due to some free radicals produced from the reaction between ascorbate and molecular oxygen.

When 1:1 mixtures of silver(I) acetate and ascorbate were allowed to interact with plasmid DNA, total damage to DNA did not occur at any concentration over the range 12.5 μM to 100 μM and it was found that most significant damage occurred not at the highest concentration but at a lower concentration namely 12.5 mM (Figure 7.19 (b) page 198). At this concentration a faint band with a much greater electrophoretic mobility was observed. As in the other experiments, two bands corresponding Form I and Form II were observed for unreacted PBR322 plasmid DNA. The Form I band was found to be broader (than Form II band) which almost separated into two bands corresponding to Form I and Form III when the concentration of the mixture was 50 μM. For the concentration range: 100 μM – 6.25 mM, two narrow DNA bands corresponding to Form II and Form III were observed. At 50 mM concentration, only the Form II band was observed whereas at 25 mM concentration both Form II and Form III bands (with Form II band being weaker in intensity than Form III band) were found. The results show that 1:1 mixtures of silver(I) acetate and ascorbate are much more damaging to pBR322 plasmid DNA than silver(I) acetate or ascorbate alone. However, when the results are compared with those observed for the interaction of PBR322 plasmid DNA with mixtures of cadmium and ascorbate (Hossain and Huq, 2002b), it is found that the 1:1 mixtures of silver(I) acetate and ascorbate are less damaging than 1:1 mixtures of cadmium(II) acetate and ascorbate (whereas not even 50 mM concentration caused extensive damage to DNA in the former, 1 mM concentration caused extensive damage in the latter. To explain the much more pronounced damage to DNA due to 1:1 mixtures of cadmium(II) acetate and ascorbate (as compared to that due to cadmium(II) acetate or ascorbate alone), it was suggested that Cd^{2+} was playing a catalytic role in the oxidation of ascorbate and that free radicals were produced in the reaction which then damaged DNA. Based on continuous variation studies using UV-difference spectral measurements, it was found that Cd^{2+} formed 1:1 complex with ascorbate (Figure 7.11, page 162). It was suggested that the formation of the complex activated ascorbate such that it was more susceptible to attack by molecular oxygen. In this study, it has been found that Ag^{+} also forms 1:1 complex with ascorbate in solution in 0.10 M $NaNO_3$. It is thus, suggested that Ag^{+} may also be playing a catalytic role in the oxidation of ascorbate, although not as efficiently as Cd^{2+} (More about that will be considered in section 8.8).

The reason why a mixture of silver(I) acetate and ascorbate at 12.5 mM concentration caused a greater damage to pBR322 plasmid DNA than other mixtures at both higher and lower concentrations appear to be intriguing. However, a clue to the mystery can be found when we note at 50 mM concentration only Form II band was observed whereas at 25 mM Form III band as well as a faint Form II band were observed and that Ag(I) acetate on its own did not cause any damage to pBR322 plasmid DNA (instead it might have stabilised the DNA by interstrand binding). It is therefore proposed that (in the concentration range used in the experiment) when present together both ascorbate and Ag^{+} may be playing two opposing roles. For Ag^{+}, these are: (1) stabilsation of DNA by interstrand binding and (2) induction of DNA damage by promoting the production of free radicals by acting as a catalyst in the oxidation of ascorbate (Hossain and Huq, 2002c). For ascorbate, these are its functioning as both: (1) a prooxidant that enhances DNA damage and (2) an antioxidant that prevents or reduces DNA damage. If the prooxidant behaviour of ascorbate increases with the increase in concentration to reach a plateau whereas its antioxidant behaviour continues to increase linearly, then maximum damage will occur not at the highest concentration of ascorbate but at an intermediate concentration where the two plots intersect (i.e., the point at which the two tendencies balance each other).

8.6.2 (a) Interaction between silver(I) acetate and ssDNA

Submarine gel electrophoresis of ssDNA at pH 7.4 gave a single band. When it was electrophoresed after its interaction with silver(I) acetate, only a slight decrease in the mobility of the band was observed as the concentration of Ag^+ was increased from 0 to 50 mM. However there was a gradual decrease in the intensity of the band as the concentration of Ag^+ was increased (Figure 7.20 (a)). The decrease in mobility of the band indicates increased binding of Ag^+ to the DNA. The decrease in intensity of the band is believed to one or both of the following two reasons: (1) a change in conformation of the DNA due to its binding with Ag^+ such that less of ethidium bromide can intercalate within DNA (as metal ions including Ag^+ are known to induce cooperative conformational transitions in DNA) and (2) some damage to DNA brought about by its covalent binding with Ag^+. Sorokin *et al.* (Sorokin, *et al.*, 2000) stated that Ag^+ undergoes both 'strong' and 'weak' binding to DNA. The authors suggested that the strong binding was to N7 positions of guanine whereas the weak binding was due to the cooperativity of the transition of DNA to a new double-helical conformation.

8.6.2 (b) Interaction between silver(I) acetate, ascorbate and ssDNA

When ascorbate at pH 7.4 was allowed to interact with ssDNA, a single band was observed for all concentrations of ascorbate from 0 to 100 mM although there was a slight decrease in mobility of the band at higher concentrations (50 M to 100 mM) (Figure 7.17 (b) page 190). There was no detectable change in the intensity of the band. The decrease in mobility is believed to be due to a change in the conformation of ssDNA as a result of its interaction with ascorbate.

When ssDNA was allowed to interact with 1:1 mixtures of silver(I) acetate and ascorbate the intensity of the band was found to decrease slightly as the concentration of Ag^+ was increased (Figure 7.20 (b) page 201). As the concentration of the mixture was increased, the mobility of the band remained unchanged over the concentration range: 0 to 50 μM and then increased slightly over the concentration range: 100 μM to 50 mM. The decrease in intensity and the increase in electrophoretic mobility (at high concentrations) suggest a reduction in the size of the DNA molecule due to its partial cleavage. It should however be noted that (as stated earlier) a change in DNA conformation could also cause a change in its mobility and intensity. The results show that 1:1 mixtures of silver(I) acetate and ascorbate are more damaging to ssDNA than silver(I) acetate or ascorbate alone. However, the increase in damage produced in ssDNA by the mixtures of silver(I) acetate and ascorbate was found to be less pronounced than that produced in pBR322 plasmid DNA, indicating that ssDNA is less susceptible to attack by free radicals than pBR322 plasmid DNA. It will be seen later that like ssDNA, calf thymus DNA is less susceptible to attack by free radicals than plasmid DNA. This is believed to be due to a higher level of organization of salmon sperm and calf thymus DNA than plasmid DNA.

8.6.3 (a) Interaction between silver(I) acetate and calf thymus DNA

Calf thymus DNA originally appeared as a faint streaking band at pH 7.4 (Figure 7.21 (a) page 202), indicating that the molecular mass of the DNA covered a wide range of values. When it was allowed to interact with silver(I) acetate at pH 7.4, it was found that although the unreacted DNA band was very faint, there was a pronounced increase in intensity of the band for most of the concentrations of silver(I) acetate. The actual changes in intensity of the bands with the increase in concentration of silver(I) acetate were as follows. First, it was found that (as in the case of unreacted DNA); the band at 12.5 μM concentration was very faint. The most intense band was observed at the next higher concentration, namely 25 μM. Between 100 μM to 1 mM concentrations, the bands were again faint. The next intense band was at 12.5 mM concentration. The other three bands i.e., the ones at 6.25 mM, 25 mM and 50 mM concentrations had almost the same brightness (slightly less than that at 12.5 mM concentration). The electrophoretic mobility of the band was found to decrease slightly as the concentrations of Ag^+ was increased from 0 to 50 mM (Figure 7.21 (a)), in a manner similar to that observed in the interaction of the DNA with Cd^{2+} (Figure7.12 (c) or (d) page 169 or 170). The results suggest that covalent binding of Ag^+ caused a change in the conformation of calf thymus DNA such that more of ethidium bromide intercalated and hence an increase in intensity of the band was generally observed. There was no observable damage to calf thymus DNA due to its interaction with silver(I) acetate. The interaction between calf thymus and Ag^+ had been studied previously (Rosetto, *et al.*, 1994). It was found to cause monophasic transitions to the conformation of DNA. It has been suggested that similar to its interaction with other DNAs (stated earlier) Ag^+ interacts with calf thymus DNA forming three distinct complexes marked I, II and III with progressively higher amounts of Ag^+. Complex I is considered to be formed at low $[Ag^+]$:[DNA] ratio (less than or equal to 0.3). Complex II is formed when the molar ratio lies between 0.3 to 2.0. Complex I has been assigned a modified B conformation whereas complex II reflects a novel conformation in which both the base-pair tilt and roll differ significantly from that seen in the B-conformation. It appears that less of ethidium bromide can intercalate within DNA in this conformation than in the conformation due to the formation of type II complex. It should also be noted that as Ag^+ binds covalently to nucleobases in the DNA, a spin-orbit enhancement of the phosphorescence quantum yield of the complexed bases and a reduction of the triplet lifetime are produced (Luk, *et al.*, 1975). Except for the lowest concentration of Ag^+ used in the experiments, the $[Ag^+]$:[nucleotide] ratio r is greater than 0.3 so that type II and type III will predominate. At the lowest concentration of Ag^+ used (namely 12.5 μM), r is about 0.20. At this relative concentration, in the type I complex formed, Ag^+ is believed to be bonded to guanine and adenine forming only monofunctional adducts.

8.6.3 (b) Interaction between silver(I) acetate, ascorbate and calf thymus DNA

When calf thymus DNA was allowed to interact with ascorbate at pH 7.4, again a single streaking band was observed for all concentration of ascorbate from 0 to 100 mM whose intensity first increased and then decreased slightly as the concentration of the ascorbate was increased. The bands observed at 100 μM and 100 mM concentrations were found to have the weakest intensity whereas those at 12.5 μM, 1 mM and 6.25 mM concentrations were found to have moderate intensity values. The mobility of the band was found to decrease, as the concentration of ascorbate was increased (Figure 7. 18 (b) page 194). The change in intensity of the band generally indicates a change in the amount of ethidium bromide intercalating within DNA due to a change in its conformation brought by interaction with ascorbate. However, the presence of very weak band at 50 mM concentration is believed to be due to DNA damage caused by free radicals produced in the interaction of ascorbate with molecular oxygen. Why the band observed at 50 μM concentration was also very weak in intensity is rather difficult to explain since bands were observed at both higher and lower concentrations.

When calf thymus DNA was allowed to interact with 1:1 mixtures of silver(I) acetate and ascorbate, it was found that on a relative scale the intensities of the observed bands were generally less than those observed in the interaction of the DNA with silver(I) acetate or ascorbate alone showing that mixtures were more damaging to calf thymus DNA than either silver(I) acetate or ascorbate alone (Figure 7.21 (b) page 204). The actual changes in intensity of the bands with the change in concentration of the mixtures are described as follows. The most intense band was observed at 25 μM concentration for the mixture whereas the weakest bands were found at 100 μM, 1 mM and 6.25 mM concentrations for the mixture. As the concentration of the mixture was increased, the mobility of the band was found to decrease slightly over the concentration range: 0 to 50 μM and then increase more significantly over the concentration range: 100 μM to 50 mM. The decrease in mobility is believed to be due to the binding of Ag^+ to the DNA and the increase in mobility at higher concentrations is believed to be due to a reduction in size of DNA molecules because of a partial cleavage. As stated earlier, the metal ion might be playing a catalytic role in the oxidation of ascorbate by molecular oxygen resulting into the formation of free radicals that can damage DNA. It was established based on UV-difference spectral measurements that both the metal ions (Cd^{2+} and Ag^+) formed mainly 1:1 adduct with ascorbate in aqueous solution.

Summary

Irrespective of the form of DNA (plasmid or genomic), Cd^{2+} is found to be more damaging to DNA than Ag^{+} in presence of ascorbate.

8.7 Interaction between Zn^{2+} and plasmid DNA

8.7.1 Interaction between Zn(II) acetate and plasmid DNA in absence or in presence of ascorbate

When pBR322 plasmid DNA was allowed to interact with varying amounts of zinc(II) acetate over the concentration range: 0 to 50 mM, two DNA bands corresponding to Form I and Form II were observed. However, it was found that as the concentration of Zn^{2+} was increased, the band that was believed to be due to Form I almost got separated into two bands corresponding to Form I and Form III. As $[Zn^{2+}]$ increased, the mobility of the bands remained unchanged over the range: 0 to 25 μM and decreased over the range: 25 μM to 50 mM. There was no observable change in the intensity of the bands. The decrease in mobility is believed to be associated with the binding of Zn^{2+} with DNA resulting into an increase in its molecular mass, decrease in its overall negative charge and a change in its conformation. The separation of the broad band into two distinct bands is believed to be due to changes in DNA conformation rather than any actual damage to DNA. The results show that although Zn^{2+} may be undergoing associative interaction with pBR322 plasmid DNA, this does not cause any significant damage to the DNA.

When pBR322 plasmid DNA was allowed to interact with 1:1 mixtures of zinc(II) acetate and ascorbate, it was found that there was no significant damage to DNA for all concentrations of the mixture from 0 to 50 mM. The results are very different from those observed in the interaction of pBR322 plasmid DNA with 1:1 mixtures of cadmium(II) acetate and ascorbate (where 1 mM concentration was found to cause total damage to DNA) as well as those observed in the interaction of pBR322 plasmid DNA with 1:1 mixtures of silver(I) acetate and ascorbate.

8.8 Why and how does Cd^{2+} activate ascorbate but Ag^+ and Zn^{2+} do not?

From the above discussion it is clear that 1:1 mixtures of Cd^{2+} and ascorbate (or other antioxidants such as cysteine and glutathione) are more damaging to DNA than the corresponding mixtures of ascorbate with Ag^+ or Zn^{2+}. Whereas 1:1 mixture of Cd^{2+} and ascorbate caused total damage to pBR322 plasmid DNA, there was no observable damage to the DNA in the case of mixtures of Zn^{2+} and ascorbate (for any concentration from 0 to 50 mM) and only a partial damage to DNA occurred in the case of mixtures of Ag^+ and ascorbate even at 50 mM.

To find out why DNA damage due to ascorbate was most prominent in the presence of Cd^{2+} and least in the presence of Zn^{2+}, first interaction between Ag^+ and ascorbate and that between Zn^{2+} and ascorbate in solution in 0.10 M $NaNO_3$ were followed using the technique of continuous variation following procedures similar to those used for the interaction between Cd^{2+} and ascorbate (Chapter 7 Section 7.5). Table 8.1 gives the absorbance values applying to the continuously varying mixtures of silver(I) acetate and ascorbate taken at 232 nm against ascorbate blanks and Figure 8.9 gives the corresponding absorbance versus concentration plot.

TABLE 8.1: Interaction between CH_3COOAg (1 mM) and ascorbate (1 mM) made in 0.10 M $NaNO_3$ at pH 7.4: Incubated for 24 hours in the dark at room temperature

Absorbances taken against ascorbate blanks

Solution	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S	T
mLs of CH_3COOAg	4.0	3.75	3.50	3.25	3.0	2.75	2.50	2.25	2.00	1.75	1.50	1.38	1.25	1.13	1.00	0.88	0.75	0.50	0.25	0.0
mLs of ascorbate	0.0	0.25	0.50	0.75	1.0	1.25	1.50	1.75	2.00	2.25	2.50	2.62	2.75	2.87	3.00	3.13	3.25	3.50	3.75	4.0
$[Ag^+]$ mM	1.0	0.94	0.88	0.81	0.75	0.69	0.63	0.56	0.50	0.44	0.38	0.35	0.31	0.28	0.25	0.22	0.19	0.13	0.06	0.0
[ascorbate] mM	0.0	0.06	0.12	0.19	0.25	0.31	0.37	0.44	0.50	0.56	0.62	0.65	0.69	0.72	0.75	0.78	0.81	0.87	0.94	1.0
Absorbance at 232 nm	0.0	0.0	0.009	0.015	0.032	0.058	0.072	0.086	0.096	0.085	0.073	0.064	0.043	0.034	0.024	0.009	0.009	0.0	0.0	0.0

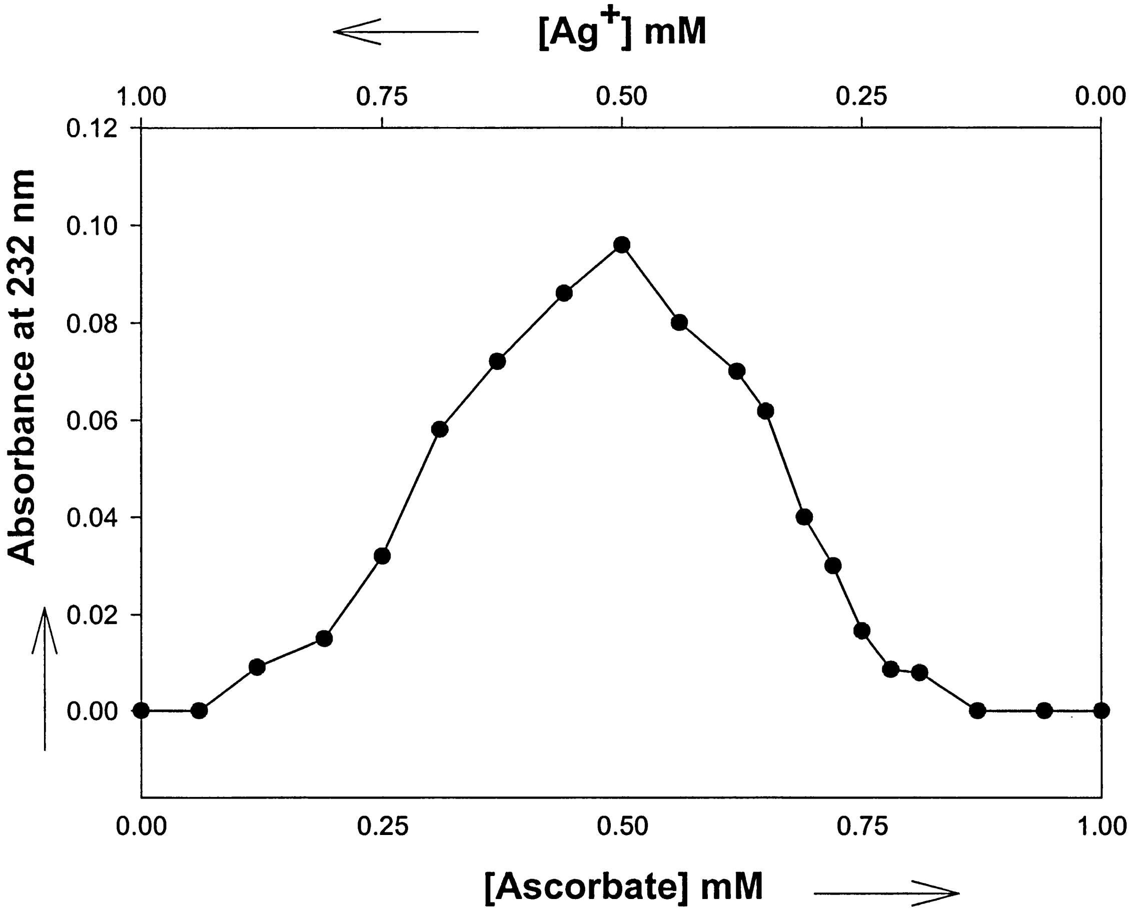


FIGURE 8.9: Interaction between Ag^+ and ascorbate: Plot of absorbance versus concentration at 232 nm indicating the formation of 1:1 adduct between Ag^+ and ascorbate

It was noted earlier that the plot of absorbance versus concentration at 266 nm applying to the continuously varying mixtures of cadmium(II) acetate and ascorbate at pH 7.4 had a sharply defined minimum corresponding to the formation of 1:1 adduct between Cd^{2+} and ascorbate. The corresponding plot applying to the continuously varying mixtures of silver(I) acetate and ascorbate at 232 nm gave a maximum (rather than a minimum) but again corresponding to the formation of 1:1 adduct. The results show that both Cd^{2+} and Ag^{+} combine with ascorbate in solution in 0.10 M $NaNO_3$ forming mainly 1:1 adduct between the metal ions and ascorbate. When similar experiments were done to investigate the interaction between zinc(II) acetate and ascorbate, there was no significant change in UV absorbance of ascorbate due to its interaction with Zn^{2+} and no clearly defined maximum or minimum was observed in the absorbance versus concentration plot (absorbance values and the corresponding plot not given). It can therefore be concluded that unlike Cd^{2+} and Ag^{+}, Zn^{2+} does not bind with ascorbate. Since Zn^{2+} does not bind with ascorbate, it cannot activate ascorbate and therefore cannot promote its oxidation, thus, providing an explanation as to why DNA damage due to ascorbate is least in presence of Zn^{2+}. To find out how the covalent binding of Cd^{2+} and Ag^{+} activate ascorbate and why the two metal ions differ in their ability to promote DNA damage due to ascorbate, the following molecular modeling analyses and pH measurements were carried out.

In one set of experiments, solutions of cadmium(II) nitrate and ascorbic acid made in mQ water as well as 0.10 M $NaNO_3$ were mixed in 1:1 molar ratio at different concentrations from 1 mM to 0.10 M and the pH of the mixtures were noted immediately after mixing and 24 h after mixing. The measured pH values were compared with the corresponding values for the components. The experiments were repeated for the interaction between silver(I) nitrate and ascorbic acid and that between zinc(II) sulfate and ascorbic acid. It was found that in the case of Cd^{2+} interacting with ascorbic acid there was a decrease in pH immediately after mixing which decreased further after 24 h. For example, when 1 mM solutions were mixed together, in solution in mQ water pH changed to 3.71 immediately after mixing and to 2.85 after 24 h, and in solution in 0.10 M $NaNO_3$ the corresponding values were 3.65

and 3.16 respectively (Table 8.2). The pH values of 1 mM cadmium(II) nitrate were found to be 5.92 in mQ water and 5.72 in 0.10 M $NaNO_3$ and the corresponding values for 1 mM ascorbic acid were 3.83 in mQ water and 3.84 in 0.10 M $NaNO_3$ and these values did not change with time. Figure 8.10 illustrates the changes in pH over time when solutions of cadmium(II) nitrate and ascorbic were mixed together.

TABLE 8.2: pH changes due to interaction between solutions of cadmium(II) nitrate/silver(1) nitrate and ascorbic acid at 20°C

Solution	Initial pH or that immediately after mixing	pH after 24 h
1 mM $Cd(NO_3)_2$ in mQ water	5.92	5.92
1 mM ascorbic acid in mQ water	3.83	3.82
1:1 mixture of 1 mM $Cd(NO_3)_2$ and 1 mM ascorbic acid (both made in mQ water)	3.71	2.85
1 mM $Cd(NO_3)_2$ in 0.10 M $NaNO_3$	5.72	5.72
1 mM ascorbic acid in 0.10 M $NaNO_3$	3.84	3.84
1:1 mixture of 1 mM $Cd(NO_3)_2$ and 1 mM ascorbic acid (both made in 0.10 M $NaNO_3$)	3.65	3.16
100 mM $Cd(NO_3)_2$ in 0.10 M $NaNO_3$	4.08	4.08
100 mM ascorbic acid in 0.10 M $NaNO_3$	2.80	2.80
1:1 mixture of 100 mM $Cd(NO_3)_2$ and 100 mM ascorbic acid (both made in 0.10 M $NaNO_3$)	2.48	2.22
1 mM $AgNO_3$ in mQ water	6.42	6.41
1:1 mixture of 1 mM $AgNO_3$ and 1 mM ascorbic acid (both made in mQ water)	3.76	3.59
1 mM $AgNO_3$ in 0.10 M $NaNO_3$	6.13	6.12
1:1 mixture of 1 mM $AgNO_3$ and 1 mM ascorbic acid (both made in 0.10 M $NaNO_3$)	3.71	3.56

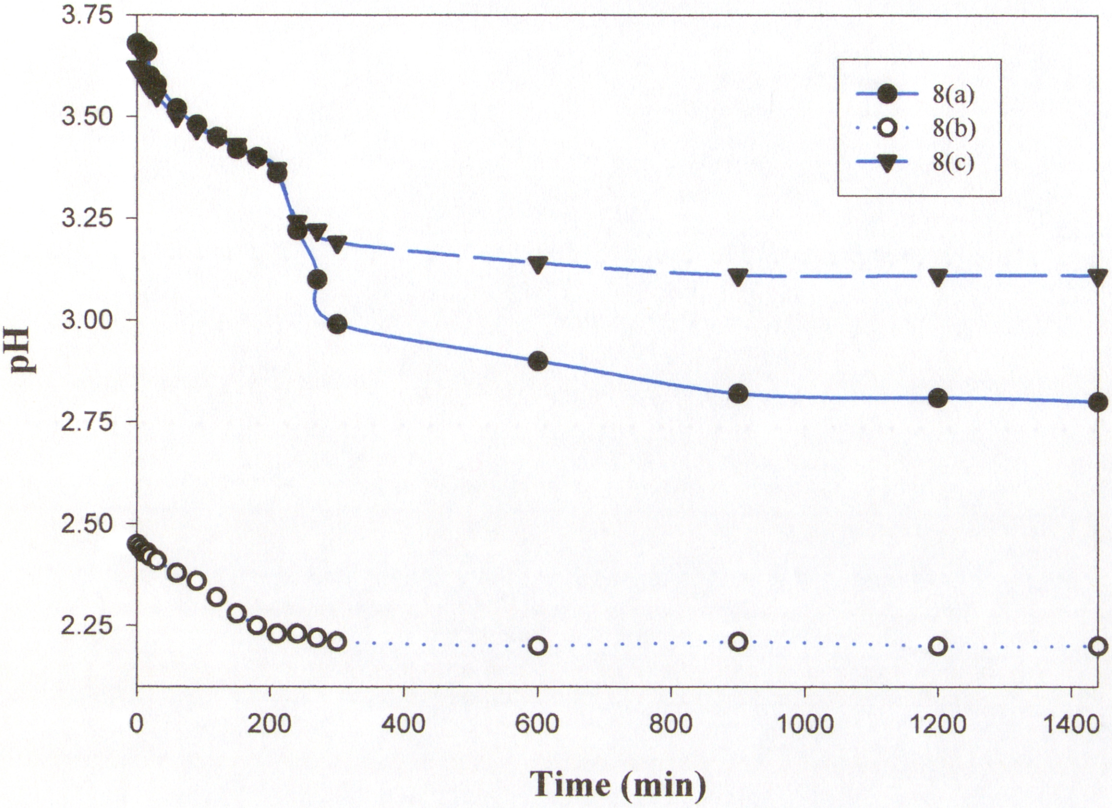


FIGURE 8.10 Changes in pH over time when solutions of cadmium(II) nitrate and ascorbic acid were mixed together: (a) 1:1 mixture of 1 mM $Cd(NO_3)_2$ and 1 mM ascorbic acid, both made in mQ water, (b) 1:1 mixture of 100 mM $Cd(NO_3)_2$ and 100 mM ascorbic acid, both made in 0.100 M $NaNO_3$, (c) 1:1 mixture of 1 mM $Cd(NO_3)_2$ and 1 mM ascorbic acid, both made in 0.1 M $NaNO_3$,

In the case of Ag(I) nitrate interacting with ascorbic acid also, for 1 mM solutions in mQ water, pH immediately after mixing was 3.76 (as against 6.42 for 1 mM $AgNO_3$ and 3.82 for 1 mM ascorbic acid) which decreased to 3.59 after 24 h. When the solutions were made in 0.10 M $NaNO_3$, the pH of 1 mM $AgNO_3$ was 6.13 and that of 1:1 mixture with 1 mM ascorbic acid in 0.10 M $NaNO_3$ was 3.71 immediately after mixing which decreased to 3.56 after 24 h.

Figure 8.11 illustrates changes in pH over time of 1:1 mixture of 1 mM silver(I) nitrate and 1 mM ascorbic, both made in mQ water

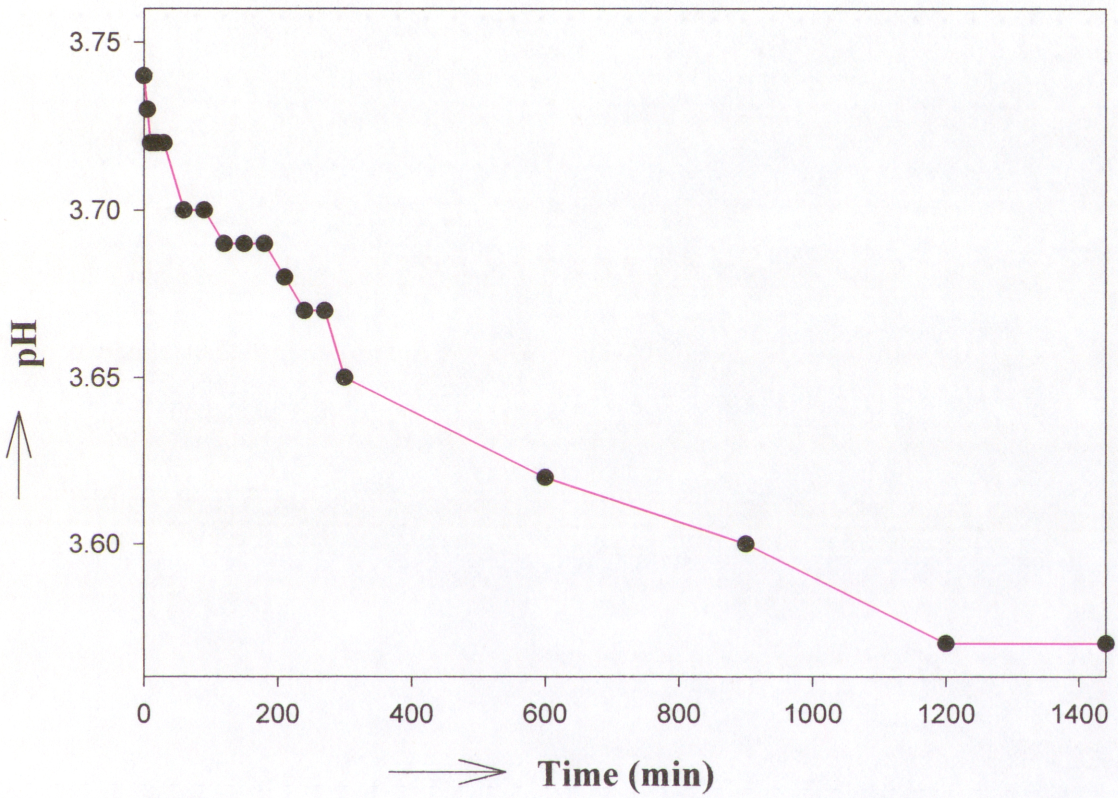


FIGURE 8.11 Changes in pH over time of 1:1 mixture of 1 mM silver(I) nitrate and 1 mM ascorbic acid, both made in mQ water

In the case of 1 mM zinc(II) sulfate interacting with 1 mM ascorbic acid (both made in mQ water), it was found that the pH of the mixture was 4.25 after mixing which remained essentially unchanged after 24 h. This pH was in between that of 1 mM zinc(II) sulfate in mQ water (5.99) and that of 1 mM ascorbic acid in mQ water

(3.83). The results show that Cd^{2+} and to some extent Ag^{+} (but not Zn^{2+}) increased the extent of dissociation of ascorbic acid to produce more of ascorbate anion, which bound covalently with the metal ions. To explore the nature of binding and its consequence on charge distribution in the coordinated ascorbate ligand, molecular mechanics and semi-empirical calculations using MM+ and ZINDO/1 programs respectively were carried out. Since it is known that in ascorbic acid the OH group attached to C3 position is most acidic (pK_a is about 4.2), this was deprotonated and the metal ion was allowed to form a coordinate covalent bond with the deprotonated oxygen atom. In the case of Cd^{2+}, the metal ion was considered to be bonded in addition to five water molecules (Figure 8.12) whereas in the case of Ag^{+}, the metal ion was considered to be bonded to one water molecule as well. When the resulting structures were optimised, it was found that when coordinated to Cd^{2+}, there was a significant change in electron density distribution such that the OH group attached to C2 position became more polarised (for the free acid, the charges on the H and O atoms were +0.216 and –0.234 units respectively whereas for the ascorbate ion coordinated to Cd^{2+} these were +0.324 and –0.460 respectively). The change was much smaller in the case of ascorbate coordinated to Ag^{+} (for the free acid, the charges on the H and O atoms were +0.216 and –0.234 units respectively whereas for the ascorbate ion coordinated to Ag^{+} these were +0.225 and –0.232 respectively).

It should be noted that the results are in agreement with a large decrease in pH observed in the interaction of ascorbic acid with Cd^{2+} and a smaller decrease in pH observed in the interaction of ascorbic acid with Ag^{+}.

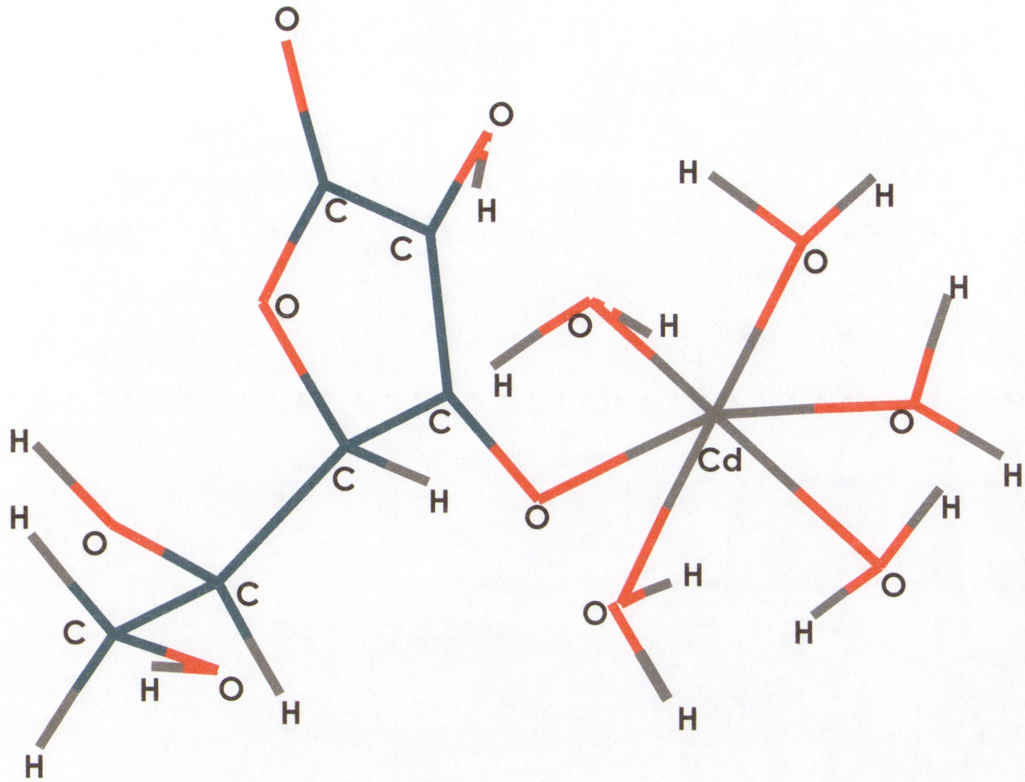


FIGURE 8.12: Proposed initial structure of 1:1 adduct between Cd^{2+} and ascorbate in which Cd^{2+} is believed to be bonded to deprotonated OH group attached to C3 position and five water molecules

To explain the decrease in pH produced when solutions of cadmium(II) nitrate and ascorbic acid were mixed together, guided by the large increase in bond polarity seen in molecular modeling calculations it is suggested that in solutions in 0.10 M $NaNO_3$ and at concentration less than or equal to 1 mM, the hydroxyl group attached to C2 was also partially deprotonated (by about 38% after 24 h) followed by its coordination to the metal ion (Figure 8.13).

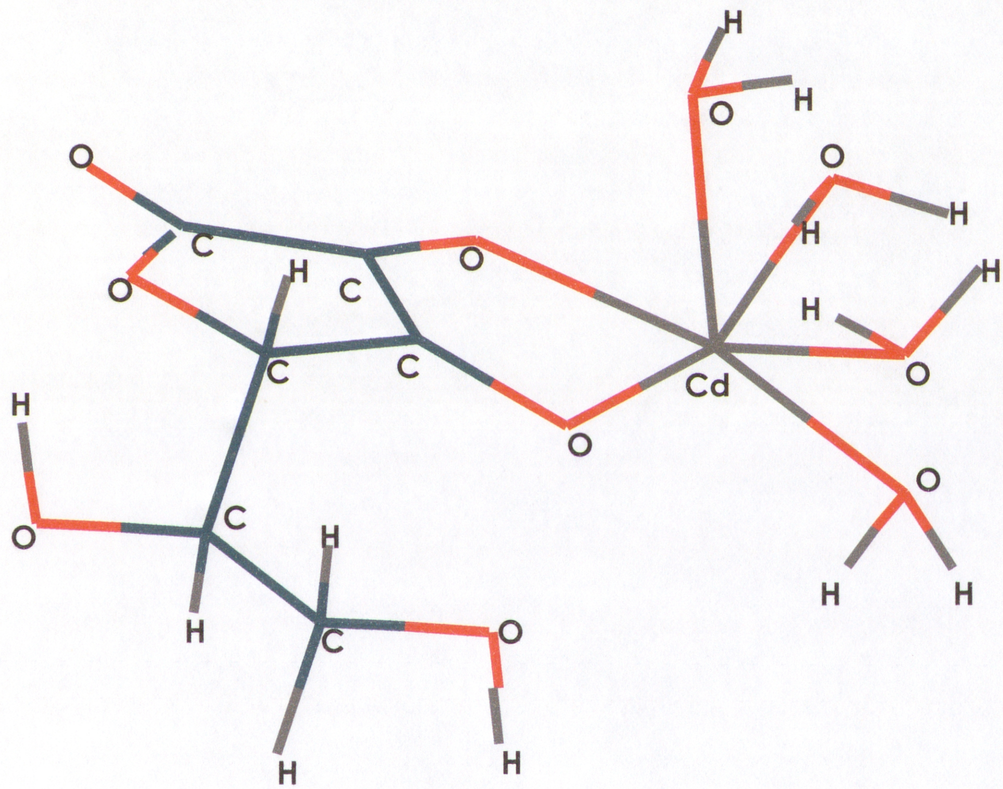


FIGURE 8.13: Proposed initial structure of 1:1 adduct between Cd^{2+} and ascorbate in which Cd^{2+} is believed to be bonded to deprotonated OH groups attached to C3 and C2 positions and four water molecules

When solutions were made in mQ water instead of 0.10 M $NaNO_3$, it was found that the decrease in pH was even greater such that complete ionisation of the second hydroxyl group as well (i.e., release of two protons altogether from ascorbic acid) was not enough to account for the observed decrease in pH. For example, whereas 1 mM ascorbic acid and 1 mM cadmium(II) nitrate both made in mQ water were found to have a pH of 3.83 and 5.92 respectively, when equal volumes of the two solutions were mixed together, the pH changed to 3.71 immediately after mixing and to 2.85 after 24 h. A third proton was thus made free by allowing the internal ester bond to hydrolyse followed by dissociation of the resulting carboxyl group. The deprotonated carboxyl group also was considered to bind to the metal ion. Thus, in the resulting adduct, the trideprotonated ascorbate ion acted as a tridentate ligand bonded to Cd^{2+}through the two deprotonated hydroxyl oxygens and the deprotonated carboxyl group (Figure 8.14).

The schematic of reactions that took place between Cd^{2+} and ascorbic acid in solution in mQ water is given in Scheme 1, where H_3A represents ascorbic acid, H_2A^- represents monodeprotonated ascorbate ion (commonly known as simply the ascorbate), HA^{2-} represents dideprotonated ascorbate ion and A^{3-} represents trideprotonated ascorbate ion obtained by hydration followed by deprotonation of HA^{2-}. In solution in 0.10 M $NaNO_3$, we believe that only the first two stages of reactions occurred after 24 h.

However, as stated earlier, the increase in acidity of ascorbic acid solution due to its binding with Cd^{2+} could not be responsible for the observed DNA damage as pH was kept unchanged at 7.4 using buffers (although this could be relevant in some *in vivo* situations at the cellular level). In addition, the acetate ion from cadmium(II) acetate that was used as the source of Cd^{2+} could also act as a proton acceptor. It will be seen later that Ag^+ also forms monodentate adduct with ascorbate but it causes a much smaller potentiation of DNA damage due to ascorbate as compared to that in presence of Cd^{2+}. Hence, it is concluded that the most likely cause for the increased DNA damage in presence of Cd^{2+} was the activation of dideprotonated ascorbate ion due to its binding with Cd^{2+}, although the monodeprotonated adduct could also be activated to some extent. It should be noted that both monodeprotonated ascorbate ion and ascorbyl radical (formed from one electron oxidation of monodeprotonated ascorbate ion) have low reduction potentials so that they can be easily oxidized by biologically relevant free radicals and oxidants including ground state triplet molecular oxygen.

It is, thus, suggested that the activated monodeprotonated ascorbate ion binds with ground state triplet molecular oxygen ($^3O_2$), producing highly reactive singlet molecular oxygen ($^1O_2$) that causes direct damage to DNA. The singlet molecular oxygen can also react with water to produce H_2O_2 (Wentworth, *et al.*, 2000) that damages DNA. The idea of ascorbate activation would also explain why there was no observed DNA damage due to ascorbate in presence of zinc(II) acetate since Zn^{2+} did not appear to bind with ascorbate. A smaller DNA damage observed in presence of silver(I) acetate could also be explained in terms of a weaker activation of ascorbate by Ag^+. We found that when Ag^+ was allowed to bind to oxygen at C3 position and a water molecule and the resulting structure optimised, the polarity of the OH group at C2 position remained essentially unchanged (changes in atomic charges were from +0.216 to +0.225 for H and from –0.234 to –0.232 for O). So we conclude that, unlike that in the case of Cd^{2+}, further deprotonation of monodeprotonated ascorbate ion did not occur in presence of Ag^+ in line with the observation that the decrease in pH was much less (see Table 8.2) when solutions of silver(I) nitrate and ascorbic acid were mixed together than when solutions of cadmium(II) nitrate and ascorbic acid were mixed together. (In the case of 1 mM $AgNO_3$ interacting with 1 mM ascorbic acid, the decrease in pH was such that after 24 h, the first stage of dissociation of ascorbic acid was 63% complete in mQ water and 100% complete in 0.10 M $NaNO_3$).

As the concentration of ascorbic acid and cadmium(II) nitrate was increased above 1 mM, we found that although there was a progressive decrease in pH with the increase in concentration, the changes in pH due to their interaction became progressively smaller. It was found that at 100 mM concentration even the first stage of dissociation was not required to be completed to account for the observed change in pH. This was not unexpected since the position of each of the three equilibria in the proposed reaction scheme would be shifted to the left as the concentration of H^+ ion was increased. It could be that bi- and tridentate adducts did not form at all at low pH (Hossain and Huq, 2002d) (eg pH of 2.31 produced 3 h after mixing of 0.10 M solutions of ascorbic acid and cadmium(II) nitrate).

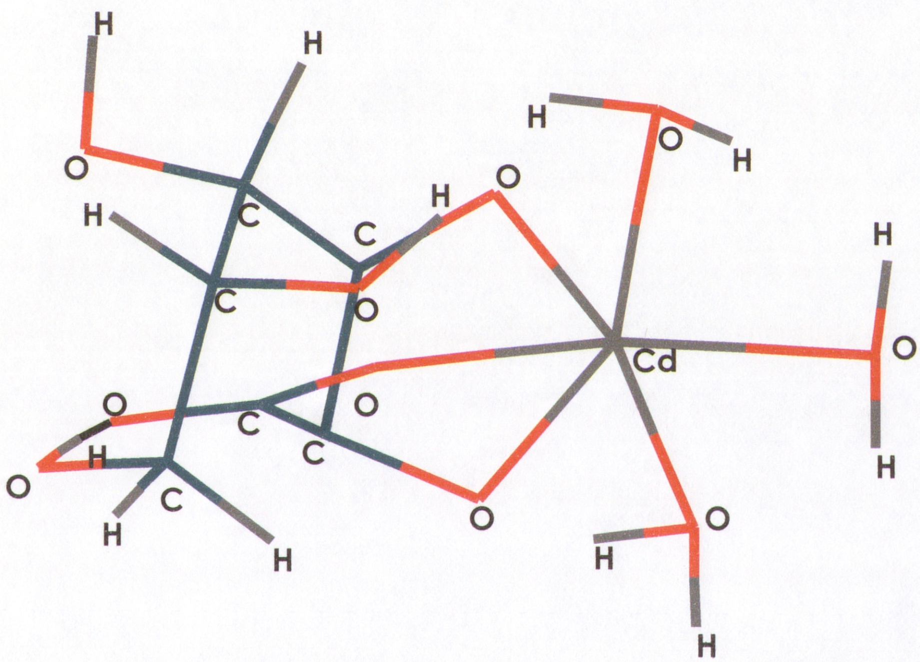


FIGURE 8.14: Proposed initial structure of 1:1 adducts between Cd^{2+} and ascorbate in which Cd^{2+} is believed to be bonded to tridentate ascorbate and three water molecules

It is possible that in presence of strong oxidising agents, the primary alcoholic OH group attached to C6 carbon can also be oxidised producing a carboxyl group.

$$Cd^{2+} + H_3A \underset{}{\overset{\text{(Step 1)}}{\rightleftharpoons}} Cd(H_2A)^{+} + H^{+}$$

$$\Big\updownarrow \text{ (Step 2)}$$

$$Cd(HA) + H^{+}$$

$$+\ H_2O \ \Big\updownarrow \ -H_2O \quad \text{(Step 3)}$$

$$Cd(A)^{-} + H^{+}$$

Scheme 1 Proposed steps of reaction between Cd^{2+} and ascorbic acid

8.9 PCR analysis of metallated plasmid

It should be recalled that it was possible to amplify the Form I band of untreated pUC8 plasmid DNA and that present in the plasmid reacted with 1 mM cadmium(II) acetate. When the concentration of cadmium(II) acetate was greater than 1 mM, although the Form I band was still present for all concentrations of Cd^{2+} up to 100 mM, it was not possible to amplify the band. This is believed to be due to a change in conformation of the DNA brought about by increased covalent binding of Cd^{2+} with the DNA so that the primers were not able to recognize the DNA sequence. It should also be recalled that Cd^{2+} was able to introduce a chemical modification in adenine and adenine nucleotides. If this also occurred in DNA, a consequence of that could be the inability of the primers to recognize the DNA sequence. At 1 mM concentration of cadmium(II) acetate, $[Cd^{2+}]$:[DNA in terms of phosphate] can be calculated to about 16.5:1 and this value will be still greater at higher $[Cd^{2+}]$. A much lower mobility of the Form I band obtained from the DNA treated with 1 mM cadmium(II) acetate as compared to that in untreated DNA, indicates that the presence of a large number of Cd^{2+} ions bound to the DNA causing a change in its conformation, an increase in its molecular mass and decreasing its overall negative charge. When pUC8 plasmid DNA was allowed to interact with cadmium(II) acetate at 200 mM concentration for the latter, a single band with mobility greater than that of Form I band was observed. This band is most likely due to doubly nicked DNA with a reduced size (i.e., Form III band with a reduced polynucleotide chain length) caused by the hydrolysis of more than two phosphodiester bonds of the supercoiled DNA. Binding experiments at other concentrations for Cd^{2+} ranging from 100 mM to 200 mM (eg 125 mM, 150 mM and 175 mM) would have provided more information in support of the explanation.

CHAPTER 9

CONCLUSION

Cadmium is a highly toxic metal of great environmental concern and has recently been classified as a category 1 (human) carcinogen. However, the molecular mechanism of cadmium-induced carcinogenesis remains largely unclear although it is believed that this may be associated with the direct and/or indirect interaction of Cd^{2+} with DNA. In this study, the interaction between Cd^{2+} and DNA and its constituents has been studied using a number of chemical and biochemical techniques. The modulation of DNA damage caused by antioxidants due to the presence of Cd^{2+} has also been investigated.

Cd^{2+} was found to bind covalently with adenine, guanine, AMP and dGMP forming a number of labile adducts which are found to dissociate in the HPLC mobile phase. The changes in retention time for adenine and adenine nucleotides suggest a structural change introduced in the nucleobase or the nucleotide as a result of their interaction with Cd^{2+}. No change in retention time is observed for guanine and guanine nucleotides. The results are different from those observed for Ni^{2+} where changes in retention time were found for guanine and guanine nucleotides but not for adenine and adenine nucleotides. It is believed that binding with Cd^{2+} causes a shift in the amino-imino tautomeric equilibrium of adenine towards the imino form followed by a more permanent change namely the hydroxylation of the imino form. That the change was permanent (i.e. once happened, the change persisted even if Cd^{2+} was removed) can be seen from the fact that removal of Cd^{2+} did not revert the retention time back to that for adenine. The presence of a broad band centred at 3467 cm^{-1} and two sharp narrow bands at 1397 cm^{-1} and 994 cm^{-1} support the presence of OH group attached to N. The shift in tautomeric equilibrium that can result into mispairing provides an explanation why Cd^{2+} is mutagenic. However, the results would not explain why cadmium is comutagenic, which, as pointed out earlier, is more likely to be associated with the inhibition of 8-oxo-dGTPase activity. Finally it should be stressed that the *in* *vivo* situations are quite different from the *in vitro* conditions so that the results reported here may or may not be quite applicable to living systems.

When cadmium(II) nitrate or cadmium(II) acetate was allowed to interact with plasmid DNA, it was found that the mobility of both Form I and Form II bands decreased with the increase in concentration of Cd^{2+}. The intensity of the bands was also found to decrease as the concentration of Cd^{2+} was increased (especially in the case of cadmium acetate). It is believed that the effects are the result of covalent binding between Cd^{2+} and guanine and adenine moieties in DNA.

When Cd^{2+} and antioxidants such as cysteine, glutathione and ascorbic acid were present together (at 1:1 molar ratio in the case of ascorbic acid), damage to DNA (pBR322 plasmid, salmon sperm and calf thymus DNAs) were found to be much greater (100 times greater in the case of pBR322 plasmid DNA) than that due to Cd^{2+} or antioxidant alone. If this result is applicable to *in vivo* situations, in presence of Cd^{2+} (eg smokers get a daily dose of Cd^{2+} that accumulate in the body resulting into a significant body burden and there are of course other environmental sources of cadmium) there may be increased damage to DNA due to ascorbate. Although it is well-known that in presence of redox active metal ions such as Cu^{2+} and Fe^{3+}, ascorbic acid which is an important antioxidant in living systems, can become a pro-oxidant, the increased damage to DNA due to ascorbate in presence of Cd^{2+} (which is not redox active) at first appeared to be intriguing. However, it has been found that Cd^{2+} binds with ascorbate forming 1:1 adduct in which ascorbate acts as a multi-dentate ligand. It is believed that it is this binding of Cd^{2+} with ascorbate that causes its molecular activation so that it is more susceptible to attack by molecular oxygen and other oxidants. Reactive oxygen species and other free radicals produced damaged DNA. Another consequence of binding of Cd^{2+} with ascorbic acid was its effective transformation into a strong polyprotic acid, clearly demonstrated by the large drop in pH when solutions of cadmium(II) nitrate and ascorbic acid were mixed together.

Prevention of hydrolysis by the enzyme Ssp1 but not by BamH1 of Cd^{2+}-treated pBR322 DNA can be explained as being due to a chemical modification of adenine moiety caused by its interaction with the metal ion so that the enzyme Ssp1 does not recognize the relevant AT site. On the other hand, no such modification occurring in guanine would mean that BamH1 digestion would not be hindered. When pBR322 plasmid DNA was allowed to interact with 1:1 mixture of ascorbate with silver(I) acetate or zinc(II) acetate, it was found that in presence of Ag^{+} and Zn^{2+}, DNA damage due to ascorbate was much less than that in presence of Cd^{2+}. Like Cd^{2+}, Ag^{+} (but not Zn^{2+}) was found to bind with ascorbate forming mainly 1:1 adduct. However, whereas ascorbate acted as a polydentate ligand in its interaction with Cd^{2+}, it behaved as a monodentate ligand in its interaction with Ag^{+}. Thus, whereas Cd^{2+} formed chelates with ascorbate and Ag^{+} formed only monodentate adduct; Zn^{2+} did not bind at all. It is suggested that it is the bidentate adduct formed between Cd^{2+} and ascorbate that is most susceptible to attack by molecular oxygen and other oxidants.

The results of PCR amplification of Form I band of untreated and cadmium-treated pUC8 plasmid DNA also support the binding of Cd^{2+} with the DNA that can cause a change in its conformation and single and double-nicking at high [Cd^{2+}]. It is found that whereas the original pUC8 consists of a mixture of Form I and Form II, on interaction with Cd^{2+} at 200 mM concentration it is totally transformed into Form III with a reduced chain length.

CHAPTER 10

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Appendix

LIST OF ABBREVIATIONS:

AT 2'-deoxyadenyl(3'→5')-2'-deoxythymidine

AMP Adenosine-5'-monophosphate

AAS atomic absorption spectrophotometry

2'-dGMP 2'-deoxyguanosine-5'-monophosphate

GG 2'-deoxyguanyl(3'→5')-deoxyguanosine

HPLC high performance liquid chromatography)

MT metallothionein

ROS reactive oxygen species

RNS reactive nitrogen species