9

Metals in Medicine

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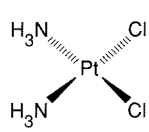
I. INTRODUCTION AND OVERVIEW

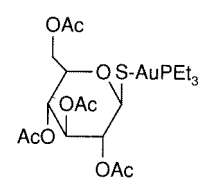
Metal ions are required for many critical functions in humans. Scarcity of some metal ions can lead to disease. Well-known examples include pernicious anemia resulting from iron deficiency, growth retardation arising from insufficient dietary zinc, and heart disease in infants owing to copper deficiency. The ability to recognize, to understand at the molecular level, and to treat diseases caused by inadequate metal-ion function constitutes an important aspect of medicinal bioinorganic chemistry.

Metal ions can also induce toxicity in humans, classic examples being heavy-metal poisons such as mercury and lead. Even essential metal ions can be toxic when present in excess; iron is a common household poison in the United States as a result of accidental ingestion, usually by children, of the dietary supplement ferrous sulfate. Understanding the biochemistry and molecular biology of natural detoxification mechanisms, and designing and applying ion-specific chelating agents to treat metal overloads, are two components of a second major aspect of the new science that is evolving at the interface of bioinorganic chemistry and medicine.

Less well known than the fact that metal ions are required in biology is their role as pharmaceuticals. Two major drugs based on metals that have no known natural biological function, Pt (cisplatin) and Au (auranofin), are widely used



cis-diamminedichloroplatinum(II)
(cisplatin, or *cis*-DDP)



2,3,4,5-tetra-O-acetyl-1-1-β-D-
thioglucose(triethylphosphine)gold(I)
(auranofin)

for the treatment of genitourinary and head and neck tumors and of rheumatoid arthritis, respectively. In addition, compounds of radioactive metal ions such as ^{99m}Tc and complexes of paramagnetic metals such as Gd(III) are now in widespread use as imaging agents for the diagnosis of disease. Many patients admitted overnight to a hospital in the U.S. will receive an injection of a ^{99m}Tc compound for radiodiagnostic purposes. Yet, despite the obvious success of metal complexes as diagnostic and chemotherapeutic agents, few pharmaceutical or chemical companies have serious in-house research programs that address these important bioinorganic aspects of medicine.

This chapter introduces three broad aspects of metals in medicine: nutritional requirements and diseases related thereto; the toxic effects of metals; and the use of metals for diagnosis and chemotherapy. Each area is discussed in survey form, with attention drawn to those problems for which substantial chemical information exists. Since there is only a primitive understanding at the molecular level of the underlying biochemical mechanisms for most of the topics, this field is an important frontier area of bioinorganic chemistry. The major focus of this chapter is on the platinum anticancer drug cisplatin, which is presented as a case study exemplifying the scope of the problem, the array of methodologies employed, and the progress that can be made in understanding the molecular basis of a single, if spectacular, metal complex used in medicine today.

II. METAL DEFICIENCY AND DISEASE[1]

A. Essential Metals

Four main group (Na, K, Mg, and Ca) and ten transition (V, Cr, Mn, Fe, Co, Ni, Cu, Zn, Mo, and Cd) metals are currently known or thought to be required for normal biological functions in humans. Table 9.1 lists these elements, their relative abundances, and the medical consequences of insufficient quantities where known. The nutritional requirements for selected members of the essential metals are discussed in the following sections.

B. Anemia and Iron[2]

Anemia results from insufficient oxygen supply, often because of a decrease in hemoglobin (Hb) blood levels. Approximately 65 to 70 percent of total body iron resides in Hb. In the U.S., many foods, especially those derived from flour, are enriched in iron. In third-world countries, however, scarcity of dietary iron is a major contributor to anemia. This information illustrates one important fact about disease that results from metal deficiency, namely, the need for an adequate supply of essential metals in food. A related aspect, one of greater interest for bioinorganic chemistry, is the requirement that metals be adequately absorbed by cells, appropriately stored, and ultimately inserted into the proper environment to carry out the requisite biological function. For iron, these tasks,

Table 9.1
Essential metals and medical consequences resulting from their deficiency.[a]

Metal	Abundance: Sea Water mg/l (ppm)	Abundance: Earth's Crust mg/l (ppm)	Diseases resulting from metal deficiency
Na	1.05×10^4	2.83×10^4	
K	380	2.59×10^4	
M-	1.35×10^3	2.09×10^4	
Ca	400	3.63×10^4	bone deterioration
V	2×10^{-3}	135	
Cr	5×10^{-5}	100	glucose tolerance (?)
Mn	2×10^{-3}	950	
Fe	1×10^{-2}	5.00×10^4	anemia
Co	1×10^{-4}	25	anemia
Ni	2×10^{-3}	75	
Cu	3×10^{-3}	55	brain disease, anemia, heart disease
Zn	1×10^{-2}	70	growth retardation, skin changes
Mo	1×10^{-2}	1.5	
Cd	1.1×10^{-4}	0.2	

[a] Data taken from E.-i. Ochiai, *Bioinorganic Chemistry*, Allyn & Bacon, 1977, p. 6.

among others, are performed by specific iron-chelating agents, the storage protein ferritin and the transport protein transferrin, the bioinorganic chemistry of which is extensively discussed in Chapter 1.

Another cause of anemia exists in individuals who have a mutant variety of hemoglobin, HbS, in which valine has been substituted for glutamic acid in the sixth position of the β subunits.[3] Interestingly, extensive studies have shown that this phenomenon, which leads to sickling of the red blood cells, does not result from failure of the protein to bind heme or from changes in the O_2 binding constant of the iron atom. Rather, deoxy HbS polymerizes into soluble, ordered fibrous structures that lower the ability of blood to carry oxygen effectively to the tissues. These results illustrate the importance of structural features remote from the metal-binding domain in determining the functional characteristics of a metalloprotein.

C. Causes and Consequences of Zinc Deficiency[4–6]

The average adult contains ~ 2 g of zinc and requires a daily intake of 15 to 20 mg, only half of which is absorbed, to maintain this level. Although food in many technologically advanced societies contains sufficient zinc to afford this balance, zinc deficiencies occur in certain populations where there is either an unbalanced diet or food that inhibits zinc absorption. An especially interesting example of the latter phenomenon is found in certain villages in the Middle East

where phytates, organic phosphates present in unleavened bread, chelate zinc ion and render it inaccessible. Zinc deficiency produces growth retardation, testicular atrophy, skin lesions, poor appetite, and loss of body hair. Little is known about the biochemical events that give rise to these varied consequences, although the three most affected enzymes are alkaline phosphatase, carboxypeptidase, and thymidine kinase. About 30 percent of zinc in adults occurs in skin and bones, which are also likely to be affected by an insufficient supply of the element. Zinc deficiency is readily reversed by dietary supplements such as $ZnSO_4$, but high doses (>200 mg) cannot be given without inducing secondary effects of copper, iron, and calcium deficiency.

D. Copper Deficiency[7]

More copper is found in the brain and heart than in any other tissue except for liver, where it is stored as copper thionein and released as ceruloplasmin or in the form of a complex with serum albumin. The high metabolic rate of the heart and brain requires relatively large amounts of copper metalloenzymes including tyrosinase, cytochrome *c* oxidase, dopamine-β-hydroxylase, pyridoxal-requiring monamine oxidases, and Cu-Zn superoxide dismutase. Copper deficiency, which can occur for reasons analogous to those discussed above for Fe and Zn, leads to brain disease in infants, anemia (since cytochrome oxidase is required for blood formation), and heart disease. Few details are known about the molecular basis for copper uptake from foods.

E. Summary

From the above anecdotal cases, for which similar examples may be found for the other metals in Table 9.1, the biological consequences of metal deficiency are seen to result from a breakdown in one or more of the following steps: adequate supply in ingestible form in foodstuffs; absorption and circulation in the body; uptake into cells; insertion into critical proteins and enzymes requiring the element; adequate storage to supply needed metal in case of stress; and an appropriate mechanism to trigger release of the needed element under such circumstances. Only for iron, and to a lesser extent copper and zinc, is there a reasonably satisfying picture of the molecular processes involved in this chain of events. The elucidation of the detailed mechanisms of these phenomena, for example, the insertion of iron into ferritin, remains an exciting challenge for the bioinorganic chemist (see Chapter 1).

III. TOXIC EFFECTS OF METALS

A. Two Classes of Toxic Metal Compounds

As intimated in the previous section, the presence of excess quantities of an essential metal can be as deleterious as insufficient amounts. This situation can

arise from accidental ingestion of the element or from metabolic disorders leading to the incapacitation of normal biochemical mechanisms that control uptake and distribution phenomena. These possibilities constitute one major class of metal toxicity. The other broad class results from entry of nonessential metals into the cell through food, skin absorption, or respiration. The toxicities associated with this latter class have received much recent attention because of the public health risks of chemical and radioisotopic environmental pollutants.

In this section, we survey examples of both categories, and discuss ways in which bioinorganic chemistry can contribute to the removal of toxic metals and restoration of normal function. One way involves chelation therapy, in which metal-specific chelating agents are administered as drugs to complex and facilitate excretion of the unwanted excess element. The use of desferrioxamine to treat iron poisoning is one example of this approach. A second role of bioinorganic chemistry is to identify fundamental biological mechanisms that regulate metal detoxification, and to apply the principles that emerge to help control the toxic effects of metal ions in the environment. Recent studies of mercury resistance and detoxification in bacteria provide an elegant example of the way in which biochemistry and molecular biology can be used to elucidate events at the molecular level. This work, which has uncovered the existence of metalloregulatory proteins, is described in some detail in Section III.F below. It represents a benchmark by which other investigations into the mechanisms of metal-detoxification phenomena may be evaluated.

B. Copper Overload and Wilson's Disease[8]

Wilson's disease results from a genetically inherited metabolic defect in which copper can no longer be tolerated at normal levels. The clinical manifestations are liver disease, neurological damage, and brown or green (Kayser-Fleischer) rings in the cornea of the eyes. Patients suffering from Wilson's disease have low levels of the copper-storage protein ceruloplasmin; the gene and gene products responsible for the altered metabolism have not yet been identified. Chelation therapy, using $K_2Ca(EDTA)$, the Ca^{2+} ion being added to replenish body calcium stores depleted by EDTA coordination, 2,3-dimercaptopropan-1-ol (BAL, British Anti-Lewisite), or d-penicillamine to remove excess copper, causes the symptoms to disappear. The sulfhydryl groups of the latter two compounds presumably effect removal of copper as Cu(I) thiolate complexes. Wilson's disease offers an excellent opportunity for modern methodologies to isolate and clone the gene responsible for this altered Cu metabolism, ultimately providing a rational basis for treatment.

C. Iron Toxicity[9]

Chelation therapy is also used to treat iron overload. Acute iron poisoning, such as that resulting from accidental ingestion of $FeSO_4$ tablets, results in corrosion of the gastrointestinal tract. Chronic iron poisoning, or hemochromatosis, arises

from digestion of excess iron usually supplied by vessels used for cooking. A classic case of the latter is siderosis induced in members of the Bantu tribe in South Africa, who consume large quantities of beer brewed in iron pots and who suffer from deposits of iron in liver, kidney, and heart, causing failure of these organs. The chelating agent of choice for iron toxicity is the siderophore desferrioxamine, a polypeptide having a very high affinity for Fe(III) but not for other metals. Ferrioxamine chelates occur naturally in bacteria as iron-transport agents. Attempts to mimic and improve upon the natural systems to provide better ligands for chelation therapy constitutes an active area of bioinorganic research (see Chapter 1).

D. Toxic Effects of Other Essential Metals[10,11]

When present in concentrations above their normal cellular levels, most of the other metals listed in Table 9.1 are toxic. Calcium levels in the body are controlled by vitamin D and parathyroid hormones. Failure to regulate Ca^{2+} leads to calcification of tissue, the formation of stones and cataracts, a complex process about which little is understood (see Chapter 3). Chronic manganese poisoning, which can occur following ingestion of metal-oxide dust, e.g., among miners in Chile, produces neurological symptoms similar to Parkinson's disease. Neuron damage has been demonstrated. Although Zn toxicity is rare, it can lead to deficiencies in other essential metals, notably calcium, iron, and copper. Cobalt poisoning leads to gastrointestinal distress and heart failure. Metal poisoning by those elements has been treated by chelating agents, most frequently $CaNa_2$(EDTA), but the selectivity offered by the ferrioxamine class of ligands available for iron has not even been approached. Fortunately, there are few cases involving these metals.

E. Plutonium: A Consequence of the Nuclear Age[12]

Some of the chelating agents developed to treat iron toxicity have found application as therapeutics for plutonium poisoning. Diethylenetriaminepentaacetic acid (DTPA) salts and siderophores are especially effective. Some improvement over the naturally occurring chelates has been made by tailoring the ligand to encapsulate completely the eight-coordinate Pu(IV) center. Although few individuals have been affected, ingestion of ^{239}Pu, for example, as small particles of PuO_2, at nuclear-reactor sites can have dire consequences. ^{239}Pu emits high energy α particles, leading to malignancies of bone, liver, lung and lymph nodes, to which tissues it is transported by transferrin. With a maximum tolerated dose of only 1.5 μg, plutonium is among the most toxic metals known. We turn now to other, more classic examples of such industrial pollutants.

F. Mercury Toxicity[13] and Bacterial Resistance[14–17]

Mercury is released into the environment as Hg(II) ions through weathering of its most common ore, HgS, red cinnabar. Organomercurials of general formula

RHgX used in agriculture have also entered the environment as toxic waste. Both RHgX and HgX_2 compounds bind avidly to sulfhydryl groups in proteins, which can lead to neurological disease and kidney failure. Metallothionein is a favored protein target, which may help to limit mercury toxicity. A highly publicized case occurred in 1953 at Minimata, Japan, where 52 people died after eating mercury-contaminated fish and crustaceans near a factory waste outlet. The volatile, elemental form of mercury, Hg(0), is reportedly nontoxic, but its conversion to alkylmercury compounds by anaerobic microorganisms utilizing a vitamin B-12 biosynthetic pathway constitutes a serious health hazard.

Because of the high affinity of mercury for sulfur-donor ligands, mercury poisoning is treated by BAL; N-acetylpenicillamine has also been proposed. Recently, a very interesting natural detoxification system has been discovered in bacteria resistant to mercury; this system, when fully elucidated, might provide important strategies for treating heavy-metal poisoning in humans.

Presumably under environmental pressure, bacteria have developed mechanisms of resistance to HgX_2 and RHgX compounds in which mercury is recycled back to Hg(0). At least five gene products are involved in the bacterial mercury-resistance mechanism. MerT and MerP mediate the specific uptake of mercury compounds. MerB, organomercury lyase, and MerA, mercuric reductase, catalyze two of the reactions, given in Equations (9.1) and (9.2). Plasmids encoding the genes for these two proteins have been isolated. A typical arrangement of genes in the *mer* operon

$$RHgX + H^+ + X^- \xrightarrow[\text{lyase}]{\text{organomercury}} HgX_2 + RH \tag{9.1}$$

$$Hg(SR)_2 + NADPH + H^+ \xrightarrow[\text{reductase}]{\text{mercuric}} Hg(0) + NADP^+ + 2RSH \tag{9.2}$$

region of these plasmids is shown in Figure 9.1. The most thoroughly studied gene product is MerR, a metalloregulatory protein that controls transcription of the *mer* genes. In the absence of Hg(II) the MerR protein binds to DNA as a repressor, preventing transcription of the *merT*, *P*, *A*, and *B* genes (Figure 9.1) and negatively autoregulating its own synthesis. When Hg(II) is present, transcription of these genes is turned on. Interestingly, the MerR protein remains bound to the same site on DNA whether acting as an activator in the presence

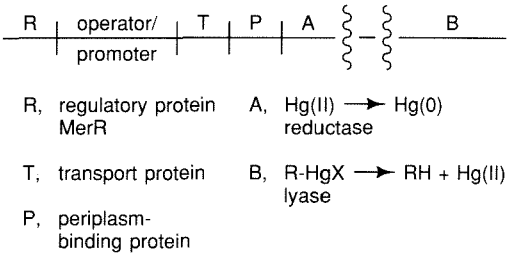
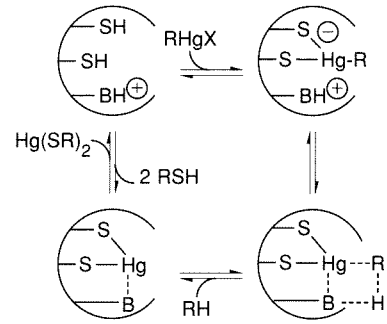


Figure 9.1
Arrangement of genes in *mer* operon of a gram negative bacterium (adapted from Figure 1, Reference 14).

of Hg(II) or as a repressor in its absence. Random and site-specific mutagenesis studies implicate several cysteine residues in the carboxyl terminal region of the protein as candidates for the mercury-binding site.

Organomercury lyase, encoded by the *merB* gene, achieves the remarkable enzymatic step of breaking Hg-C bonds (Equation 9.1). It is a 22-kDa protein with no metals or cofactors. Two cysteine-sulfhydryl groups on the protein have been postulated to effect this chemistry, as depicted in Equation (9.3). Stereochemical studies of the Hg-C bond cleavage revealed retention of configuration, indicating that cleavage of the Hg-C bond probably does not proceed by a radical pathway. A novel concerted S_E2 mechanism has been suggested. The enzyme turnover numbers, ranging from 1 min^{-1} for CH_3HgCl to 240 min^{-1} for butenylmercuric chloride, although slow, are $\sim 10^5$–10^8-fold faster than the nonenzymatic rate.



(9.3)

Mercuric ion reductase, the FAD-containing *merA* gene product, has several pairs of conserved cysteines. From site-specific mutagenesis studies, cysteine residues in the sequence 134-Thr-Cys-Val-Asn-Val-Gly-Cys-140 are known to comprise a redox-active disulfide group; in addition, a redox-inactive pair of cysteines near the carboxyl terminus is also required for the selective reduction of Hg(II). Exactly how the enzyme achieves the chemistry shown in Equation (9.2) is currently uncertain, but the redox activities of the flavin and disulfide/thiol centers are undoubtedly involved. This enzyme serves both to detoxify mercury supplied directly from the environment as Hg(II) salts and to complete clearance of Hg^{2+} generated by the MerB protein from RHgX compounds. Clearly, Nature has invented a remarkable system to detoxify mercury in this fascinating class of Hg-resistant bacteria.

G. Cadmium and Lead Toxicity[18]

Gastrointestinal, neurological, and kidney toxicity are among the symptoms experienced by acute or chronic exposure to these heavy metals. The use of unleaded gasoline and the removal of lead-containing pigments from paint have substantially diminished the quantity of this element released to the environment each year. Cadmium sources include alkaline batteries, pigments, and plating.

Lead poisoning can be treated by chelation therapy using $CaNa_2(EDTA)$ (acute) or penicillamine (chronic). Although both Cd(II) and Pb(II) bind to sulfhydryl groups in thionein, we have little information at the molecular level on the mechanisms by which these elements induce toxicity.

H. Metals as Carcinogens[19,20]

Although most metal ions have been reported to be carcinogenic, the three most effective cancer-causing metals are Ni, Cr, and, to a lesser extent, Cd. Nickel subsulfide, Ni_2S_3, found in many nickel-containing ores, has been extensively studied and shown to be carcinogenic in humans and other animals. In short-term bioassays including mutagenesis, enhanced infidelity of gene replication *in vitro* and altered bacterial DNA repair were observed. Chromium is most carcinogenic as chromate ion (CrO_4^{2-}), which enters cells by the sulfate uptake pathway and is ultimately reduced to Cr(III) via a Cr(V)-glutathione intermediate species. The latter complex binds to DNA to produce a kinetically inert and potentially damaging lesion. Despite the fact that much information is available about metal-DNA interactions, molecular mechanisms of metal-induced carcinogenesis have not been elucidated. Two aspects of the problem are tumor initiation and tumor development, which are likely to involve different pathways. As new methods become available for studying the molecular events responsible for cancer (oncogenesis), it should be possible for bioinorganic chemists to unravel details of how metals act as carcinogens and as mutagens. Since cancer has genetic origins, metal/nucleic-acid chemistry is likely to be prominent in such mechanisms. As discussed later, metal-DNA interactions are an important aspect of the antitumor drug mechanism of *cis*-$[Pt(NH_3)_2Cl_2]$.

I. Summary

Toxicity can arise from excessive quantities of either an essential metal, possibly the result of a metabolic deficiency, or a nonessential metal. Both acute and chronic exposure can be treated by chelation therapy, in which hard-soft acid-base relationships are useful in the choice of chelating agent. Since chelates can also remove essential metals not present in toxic amounts, ligands with high specificity are greatly desired. The design and synthesis of such ligands for chelation therapy remains an important objective for the medicinal bioinorganic chemist. Until recently, studies of the toxic effects of metals and their removal, sometimes categorized under "environmental chemistry," have been empirical, with little insight at the molecular level. Application of the new tools of molecular biology to these problems has the potential to change this situation, as illustrated by rapid progress made in cloning the genes and studying the gene products of the mercury-resistance phenotype in bacteria. The discovery of such resistance phenomena in mammalian cells, and even the remote prospect of transferring Hg-resistant genes from bacteria to humans, are exciting possibilities for the future.

IV. SURVEY OF METALS USED FOR DIAGNOSIS AND CHEMOTHERAPY

A. Radiodiagnostic Agents[21,22]

Metal complexes having radioactive nuclei find many applications in medicine, such as in tumor, organ, and tissue imaging. Early detection of cancer, for example, by selective uptake and imaging of the tumor using a radioactive metal compound can facilitate surgical removal or chemotherapeutic treatment before the disease reaches an advanced stage. Ideally, radioisotopes used for diagnostic purposes should be short-lived, emit low-energy γ photons, and emit no α or β particles. Table 9.2 lists the radionuclides most commonly employed for this purpose in nuclear medicine. Among these, ^{99m}Tc is perhaps the most desirable,[23] for it gives off a 140-keV γ ray that is readily detected by scintillation cameras and produces clear images. This radionuclide is prepared from an alumina column loaded with $^{99}MoO_4^{2-}$, which decays to form $^{99m}TcO_4^-$, which in turn may be selectively eluted from the column with saline solution, owing to its lower charge. Subsequent treatment with a reducing agent in the presence of the appropriate ligands produces technetium radiopharmaceuticals with desired water solubility, stability, and tissue-distribution properties. Such complexes may be injected at concentrations of 10^{-6}–10^{-8} M. For example, isocyanide complexes such as $[Tc(CNR)_6]^+$ (R = t-Bu, $CH_2CO_2Bu^t$, etc.) have been found to be taken up selectively into heart tissue and thus have the potential to be used as heart-imaging agents. Figure 9.2 displays skeletal bone as imaged by a ^{99m}Tc bone agent. The dark portions correspond to surface areas of high metabolic activity, which can be used to diagnose injury or disease. One

Table 9.2
Radionuclides most commonly employed in diagnostic nuclear medicine.[a]

Radionuclide	Half-Life	Energy (keV)
^{57}Co	271 d	836
^{67}Ga	78 h	1,001
^{99m}Tc	6 h	140
^{111}In	67 h	172, 247
^{113m}In	104 m	392
^{123}I	13 h	1,230
^{169}Yb	32 d	207
^{197}Hg	64 h	159
^{201}Tl	72 h	135, 167

[a] Data are from Table of the Isotopes in D. R. Lide, ed., *CRC Handbook of Chemistry and Physics*, CRC Press, 71st ed., 1990–91, pp. 11–33 ff.

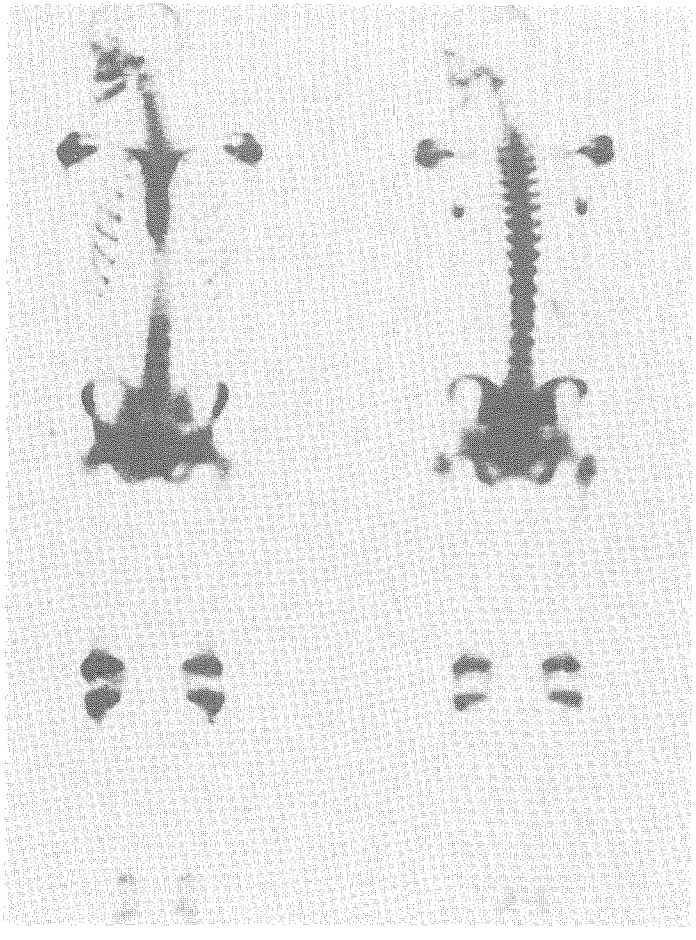


Figure 9.2
Human skeleton (bone) imaged with ^{99m}Tc. Both anterior (left) and posterior (right) views are shown.

goal of research in this field is to provide real-time images of myocardial infarcts or clogged arteries for physicians who can watch the patient's heart on a video monitor during surgery. Although chemical details responsible for the selective tissue uptake of Tc isocyanide, phosphine, and other complexes are largely unknown, synthetic modifications are possible and have provided many new compounds for clinical evaluation.

Among the few molecules known to be absorbed selectively by tumor cells is the antitumor antibiotic bleomycin (BLM),[24,25] the structure of which is portrayed in Figure 9.3. Bleomycin binds most radioactive metal ions, but the ^{57}Co(III) complex has the best tumor-to-blood distribution ratio. Unfortunately, the long ^{57}Co half-life (Table 9.2) has limited its clinical utility. Attempts to prepare stable ^{99m}Tc complexes of BLM with selective uptake properties approaching that of the cobalt complex have not yet been successful, although the

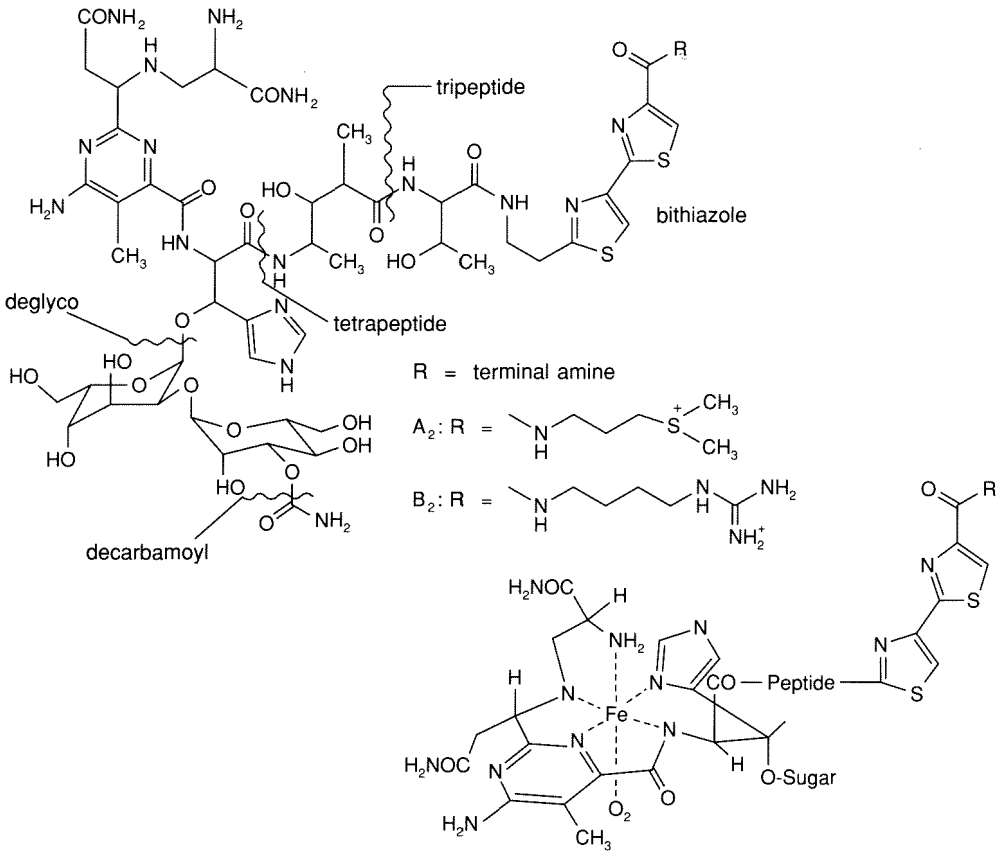


Figure 9.3
Structure of bleomycin and its proposed iron complex (reproduced by permission from Reference 25).

target molecule would be a most valuable radiodiagnostic agent. One imaginative solution[26] to this problem was achieved by covalent attachment of an EDTA moiety to the terminal thiazole ring of BLM (Figure 9.3). The resulting Co(III) BLM-EDTA molecule was radiolabeled with $^{111}In^{3+}$ and found to be useful for diagnosis of cancer in humans. Also used for tumor imaging are ^{99m}Tc and ^{67}Ga citrate complexes, the latter being the agent of choice for many applications. Again, there is little known at the molecular level about the mechanism of tumor-cell specificity.

An alternative approach to radionuclide-based tumor-imaging agents for diagnosis of disease is to modify, with metal chelating agents, antibodies raised against a biological substance, such as a tumor-cell antigen, hormone, or other target. Antibodies are proteins that are synthesized by specialized cells of the immune system in response to an external stimulant, or antigen. The high specificity and affinity of antibodies for the antigen can be used to target the antibody to a particular biological site, such as a site on the membrane of a particular cell type. Chelating agents are now routinely attached to antibodies and

used to bind radioactive metal ions. The resulting radionuclide-labeled products are currently under extensive study in diagnostic medicine.[26]

B. Magnetic Resonance Imaging (MRI)[27]

Nuclear magnetic resonance (NMR) spectroscopy can be used to image specific tissues of biological specimens because of differences in the relaxation times of water proton resonances, usually brought about by paramagnetic metal ions. An early, pioneering example was the demonstration that Mn(II) salts localize in normal heart-muscle tissue in dogs rather than in regions affected by blocked coronary arteries. Since the paramagnetism of the d^5 Mn(II) ions alters the relaxation rate of nearby water protons, the normal and diseased tissue could be distinguished. Of the various metal ions surveyed in attempts to provide clinically useful NMR images in humans, Gd(III), Fe(III), and Mn(II) were found to give the best proton-relaxation enhancements. The gadolinium complex $[Gd(DTPA)(H_2O)]^{2-}$, an agent currently used in the clinic, has been successfully employed to image brain tumors. Ferric chloride improves gastrointestinal tract images in humans and, as already mentioned, manganous salts can be used for heart imaging. NMR imaging methodologies have advanced to the stage where increases as small as 10 to 20 percent in T_1^{-1}, the inverse nuclear-spin relaxation time, can be detected. As with radionuclide labeling, the complexes must be soluble and stable in biological fluids and relatively nontoxic, and are of greatest value when able to target a specific tissue. Even more important than targeting, however, is that proton relaxivity be maximally enhanced, an objective that depends not only upon the local binding constant but also upon large magnetic moments, long electron-spin relaxation (T_{1e}) values, access to and the residence lifetime in the inner and outer coordination spheres by water molecules, and the rotational correlation time of the complex at its binding site. An obvious advantage of paramagnetic NMR over radioisotopic imaging agents is that there is no possibility of radiation damage; on the other hand, the need for 10–100 μM concentrations at the site of imaging is a distinct drawback. Both methods are likely to continue to be used in the future, and both will benefit from the design of new stable chelates that are selectively absorbed by the tissue to be diagnosed.

C. Lithium and Mental Health[28–31]

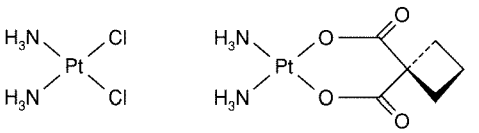
One in every 1,000 people in the United States currently receives lithium, as Li_2CO_3, for the treatment and prophylaxis of manic-depressive behavior. Doses of 250 mg to 2 g per day are administered in order to maintain a 0.5 to 2.0 mM concentration window, outside of which the drug is either toxic or ineffective. The detailed molecular mechanism by which Li^+ ion brings about its remarkable chemotherapeutic effects is largely unknown, but there are various theories. One theory proposes that lithium binds to inositol phosphates, inhibiting their breakdown to inositol, and so reducing inositol-containing phospholipids. A consequence of this chain of events would be disruption of the neurotransmis-

sion pathway based on inositol 1,4,5-triphosphate and 1,2-diacylglycerol, reducing neuronal communication, which is most likely hyperactivated in the manic state. This theory does not account for the antidepressive action of the drug, however. An alternative explanation is that lithium inhibits cyclic adenosine monophosphate (AMP) formation, again interfering with neurotransmission by intercepting this key intracellular signaling molecule. Recent experiments indicate that lithium affects the activation of G-proteins, a class of guanosine triphosphate (GTP)-binding proteins involved in information transduction. Possibly these effects result from displacement by Li^+ of Mg^{2+} from GTP and/or from protein-binding sites normally required for activation. Use of ^7Li NMR spectroscopy to study lithium transport in human erythrocytes suggests that it might be possible to apply this method to unravel details of the bioinorganic chemistry of lithium associated with the management of manic depression.

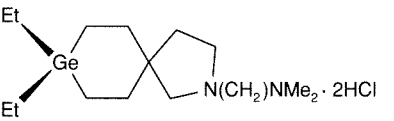
D. Gold and Rheumatoid Arthritis[23,32,33]

Gold compounds have been used in medicine for centuries, an application known as chrysotherapy. Since 1940, however, complexes of gold have been used most successfully to treat arthritic disorders in humans and other animals. Au(I) compounds are currently the only class of pharmaceuticals known to halt the progression of rheumatoid arthritis.

Until recently, gold compounds used to treat arthritis were painfully administered as intramuscular injections. Included were colloidal gold metal, colloidal gold sulfides, $Na_3[Au(S_2O_3)_2]$ (Sanocrysin), gold thiomalate and its sodium and calcium salts (Myochrisin), and polymeric gold thioglucose (Solganol, approved by the FDA). It was discovered, however, that triethylphosphinegold(I) tetra-O-acetylthioglucose (auranofin, Figure 9.4, approved by the FDA) was equally effective against rheumatoid arthritis and could be orally administered. The availability of this compound has sparked many studies of its biodistribution, stability, and possible metabolism that lead to antiarthritic activity. The mode



cis-DDP (cisplatin) carboplatin



spirogermanium

Figure 9.4
Structures and trivial names of metal-based antitumor drugs.

of action of antiarthritic gold drugs is largely unknown, but it may involve binding of Au(I) to protein thiol groups, a process that inhibits the formation of disulfide bonds, and could lead to denaturation and subsequent formation of macroglobulins.

E. Anticancer Drugs

1. Platinum ammine halides[34,35]

The discovery that *cis*-diamminedichloroplatinum(II), *cis*-DDP or cisplatin (Figure 9.4), has anticancer activity in mice, and its subsequent clinical success in the treatment of genitourinary and head and neck tumors in humans, constitutes the most impressive contribution to the use of metals in medicine. Given in combination chemotherapy as an intravenous injection together with large amounts of saline solution to limit kidney toxicity, cisplatin treatment results in long-term (>5 yr) survival for more than 90 percent of testicular cancer patients. In a typical course, ~ 5 mg/kg body weight of the drug is administered once a week for four weeks. Extensive studies of platinum ammine halide analogues led to a series of empirical rules governing their chemotherapeutic potential. Specifically, it was concluded that active compounds should:

(1) be neutral, presumably to facilitate passive diffusion into cells;
(2) have two leaving groups in a *cis* configuration;
(3) contain nonleaving groups with poor trans-labilizing ability, similar to that of NH_3 or organic amines;
(4) have leaving groups with a "window of lability" centered on chloride.

These early structure-activity relationships have had to be modified somewhat, however, since chelating dicarboxylate ligands such as 1,1-dicarboxylatocyclobutane can replace the two chloride ions, and since cationic complexes with only one labile ligand, specifically, *cis*-$[Pt(NH_3)_2Cl(4\text{-X-py})]^+$, where X = H, Br, CH_3, etc., showed activity in some tumor screens. The two compounds shown in Figure 9.4, cisplatin and carboplatin (Figure 9.4), were the first to be approved for clinical use. Of particular interest to the bioinorganic chemist is that complexes having a *trans* disposition of leaving groups are inactive *in vivo*. This difference suggests the presence of a specific cellular receptor that, when identified, should facilitate the design of new, metal-based anticancer drugs. Present evidence strongly points to DNA as being the relevant cellular target molecule. Section V of this chapter expands on this topic in considerable detail.

2. Metallocenes and their halides: Ti, V, Fe[36,37]

Several compounds in this category, including $[(C_5H_5)_2TiX_2]$ (X = Cl, Br, O_2CCl_3), $[(C_5H_5)_2VCl_2]$, $[(C_5H_5)_2NbCl_2]$, $[(C_5H_5)_2MoCl_2]$, and $[(C_5H_5)_2Fe]^+$ salts, exhibit significant activity against experimental animal tumors. Higher quantities

(200 mg/kg) of these compounds than of *cis*-DDP can be tolerated with fewer toxic side effects, but their failure in two mouse leukemia screens commonly used to predict the success of platinum anticancer agents appears to have delayed their introduction into human clinical trials. Studies of Ehrlich ascites tumor cells treated with $[(C_5H_5)_2VCl_2]$ *in vitro* revealed selective inhibition of incorporation of radiolabeled thymidine, versus uridine or leucine, indicating that the complex blocks DNA replication. Unlike cisplatin, however, metallocene halides undergo rapid hydrolysis reactions in aqueous media, forming oxo-bridged and aqua complexes that may have a higher affinity for phosphate oxygen atoms than the heterocyclic nitrogen atoms of the bases in DNA.[38] Exactly how the ferrocenium ion might bind to DNA is even more obscure, although partial metallointercalation and groove binding are more likely than covalent attachment of the chemically unmodified cation. From the limited information available, metallocenes and their halides appear to behave fundamentally differently from platinum antitumor compounds. As a class, they provide a promising new opportunity to expand the scope of metal complexes used in cancer chemotherapy.

3. Gold and other metal phosphines[39]

Following the successful entry of the soluble gold-phosphine complex auranofin (Figure 9.4) into the metal-based pharmaceuticals industry, several gold-phosphine complexes were examined for possible anticancer activity. Although auranofin itself was active in only a small fraction of the mouse tumor models tested, biological activity approaching that of cisplatin was discovered for many analogues, most notably the diphosphine bridged complex $[ClAu(PPh_2CH_2CH_2PPh_2)AuCl]$. Attempts to replace the phosphine with As or S donor ligands, to increase or decrease the length of the 2-carbon bridge, or to replace the phenyl with alkyl groups all led to diminished activity. Most noteworthy is that the diphosphine ligands themselves have activity very similar to that of their gold complexes, and that Ag(I) and Cu(I) analogues are also effective. These results strongly imply that the phosphine ligands are the chemical agents responsible for the anticancer properties of these compounds. Coordination to a metal presumably serves to protect phosphines against oxidation to the phosphine oxides, which independent investigations have proved to be ineffective. A possible role for the metal in the cytotoxicity of the compounds cannot be ruled out, however.

4. Other main group and transition-metal compounds[36,40,41]

Several main group metal complexes exhibit anticancer activity. Gallium(III) nitrate is active against human lymphomas, but with dose-limiting side effects on the kidneys and gastrointestinal tract. Tin complexes of general formula $R_2L_2SnX_2$, where R = alkyl or phenyl, L_2 = py_2, bpy, or phen, and X_2 = two *cis*-oriented halide or pseudohalide leaving groups, are active against the mouse P388 leukemia tumor. The *cis* disposition of the leaving groups suggests a possible mechanism analogous to that of cisplatin (see below). Organo-

germanium compounds are also active, notably the derivative spirogermanium shown in Figure 9.4. Nothing is known about the mechanism of action of any of these compounds.

Following the discovery of activity for cisplatin, several thousand platinum and nearly 100 other transition-metal complexes have been screened in various tumor model systems in the hope of achieving better activity against a broader range of tumors. Among the classes of nonplatinum compounds showing some activity are ruthenium complexes *cis*-$[RuCl_2(DMSO)_4]$, $[Ru(NH_3)_5(Asc)](CF_3SO_3)$, where Asc is ascorbate dianion, and *fac*-$[Ru(NH_3)_3Cl_3]$, all of which are believed to bind to DNA; binuclear rhodium complexes $[Rh_2(O_2CR)_4L_2]$; octahedral Pd(IV) complexes such as *cis*-$[Pd(NH_3)_2Cl_4]$; and such miscellaneous molecules as the iron(II) complex of 2-formylpyridine thiosemicarbazone, the site of action of which is thought to be ribonucleotide reductase. These examples illustrate the broad scope encompassed by this field, which has a potential for developing fundamental information about metal-biomolecule interactions as well as novel anticancer drugs. Much remains to be explored.

F. Miscellaneous Metals in Medicine

Numerous other anecdotal and some fairly elaborate studies have been reported for metal complexes as medicinal agents. The use of zinc applied topically to promote the healing of wounds dates back to around 1500 B.C., and silver is now commonly applied to prevent infection in burn patients.[42,43] Osmium carbohydrate polymers have been reported to have antiarthritic activity.[44] Transition-metal complexes have a long history of use as antibacterial and antiviral agents; for example, Zn^{2+} is used to treat herpes, possibly by inhibiting the viral DNA polymerase.[45] Early transition-metal (e.g., tungsten) polyoxoanions have been employed to treat AIDS patients.[46] Numerous reports have appeared detailing the anti-inflammatory, antiulcer, and analgesic activities of copper carboxylate complexes.[7] As in the previous section, these reports and others like them require more serious attention from bioinorganic chemists to elucidate the molecular events responsible for such a fascinating menu of biologically active metal complexes.

G. Summary and Prospectus

The clinical successes of platinum anticancer and gold antiarthritic drugs have changed the attitudes of many who doubted that heavy-metal compounds, notorious for their deleterious effects on human health, would ever play a serious role in chemotherapy. Indeed, we have seen that Hg^{2+}, Pb^{2+}, and Cd^{2+} are toxic elements. Even essential metals can be highly toxic if present in excess, either because of chronic or acute poisoning or because of metabolic defects that deregulate their control in the cell. An important common theme running throughout this discussion is selectivity. For a drug to be effective, it must be selectively toxic to diseased tissue while leaving normal tissue alone; or it must selectively kill harmful microorganisms at levels where it fails to deplete helpful

ones. For a chelating agent to be useful in limiting the toxic effects of metals, it must bind as selectively as possible to the deleterious ion while coordinating only weakly, if at all, to others. For a diagnostic metal complex to be useful, it must be taken up (or excluded) selectively from diseased cells relative to normal ones, or to one tissue type versus another. Rarely has such selectivity been designed in advance of the discovery of a useful metal-based pharmaceutical, although spectacular advances in biology, such as monoclonal antibodies, may be hastening the day when such an objective might be common. Interestingly, the successes of such unlikely compounds as *cis*-$[Pt(NH_3)_2Cl_2]$ and $[(Et_3P)Au(OAc_4$-thioglucose$)]$ in chemotherapy were driven by the personal involvement of individuals like B. Rosenberg for the former and B. Sutton for the latter. Like Hollywood producers, these men mustered every conceivable resource to promote the compounds for testing, introduction into human clinical trials, and eventually approval by the FDA. Such zeal requires years, usually more than a decade, of sustained personal effort, and may be the reason why other promising metal complexes, such as those mentioned above, have not had the impact of a cisplatin or an auranofin. On average, only one of 7,000 such compounds makes it from the laboratory bench to the patient, at an average cost of 250 million dollars and a time interval of 13 years.

Another significant component of the evolving field of metals in medicine, however, is that, once a compound has proved its utility in the clinic, how does it work? This question is deceptively simple, for coordination chemistry *in vivo*, and the ability of cells to respond to unnatural external stimuli such as metal complexes, are complex matters about which we are only just beginning to learn. As progress is made in this latter area, it should become possible to design drugs in a rational way to achieve the required selectivity.

The remainder of this chapter focuses on a single case study where some progress in unraveling the molecular mechanism of a metal-based drug, cisplatin, is being made. If nothing else, this discussion will elucidate strategic guidelines that may be employed to attack similar questions about other chemotherapeutic metal compounds discussed earlier in this section. Unfortunately, there is very little information available about the molecular mechanisms of these other complexes. At this major transition point in our discussion, we move from general considerations to a specific, in-depth analysis. The reader must here take time to become familiar with the biological aspects of the new material.

V. PLATINUM ANTICANCER DRUGS: A CASE STUDY

A. History of the Discovery[47]

cis-$[Pt(NH_3)_2Cl_2]$, a molecule known since the mid-19th century, has been a subject of considerable importance in the development of inorganic stereochemistry and substitution reaction kinetics.[48] Its biological activity was discovered by accident. In the mid-1960s, biophysicist Barnett Rosenberg, at Michigan State University, was studying the effects of electric fields on the growth of

Escherichia coli cells in culture. They had hypothesized that, during cell division, the orientation of the mitotic spindle might be affected by local electric fields which they hoped to perturb. Instead, they observed growth without cell division, the result being elongated, spaghetti-like bacterial filaments approaching 1 cm in length. After much detective work, they realized that small amounts of platinum from the electrodes used to apply the electric fields had reacted with NH_4Cl in their buffer to produce various platinum ammine halide compounds. Two of these, *cis*-$[Pt(NH_3)_2Cl_2]$ and *cis*-$[Pt(NH_3)_2Cl_4]$, were capable of inducing filamentous growth in the absence of any electric field. Since chemicals that produce filamentation in bacteria had been known to exhibit antitumor activity, Rosenberg was eager to have his platinum compounds tested. Unable to convince existing agencies like the National Cancer Institute (who to their credit later spearheaded the development of cisplatin) that a heavy-metal complex could actually be beneficial to animals, the Michigan State group set up their own animal-tumor screens. The results were nothing short of spectacular. Injection of *cis*-DDP directly into the abdominal cavity of mice into which a solid Sarcoma-180 tumor had been implanted led within a few days to a blackening (necrosis), reduction in size, and eventual disappearance of the tumor (Figure 9.5). The cured mouse enjoyed a normal lifespan. From these and other animal

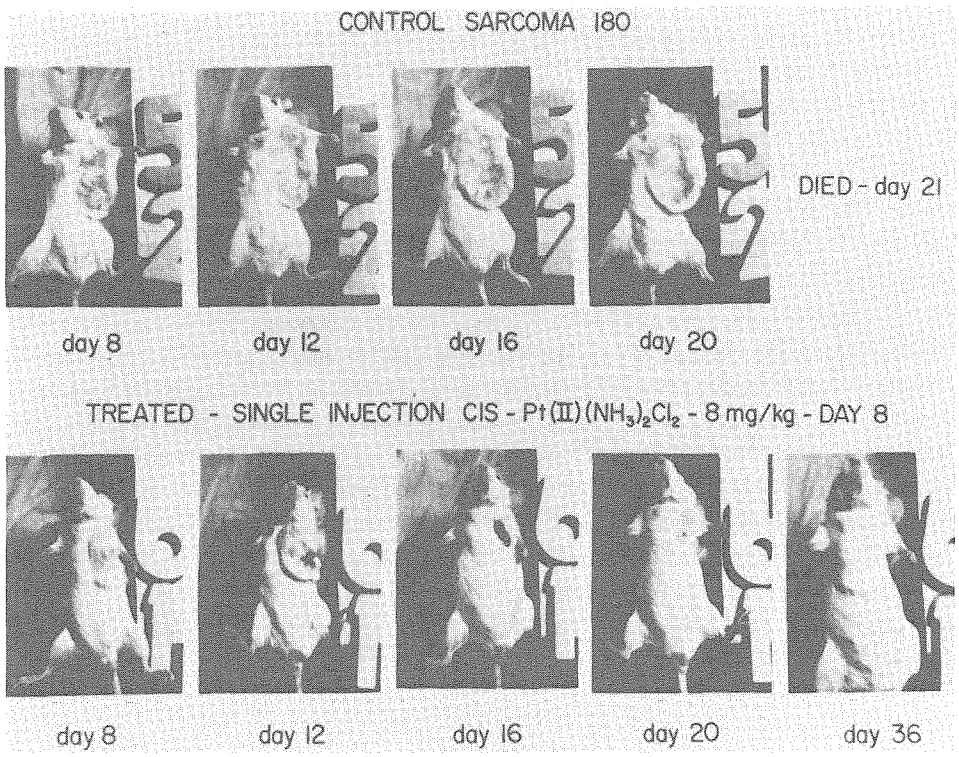


Figure 9.5
Photographic demonstration of the dramatic ability of cisplatin to cure a Sarcoma-180 murine tumor (reproduced by permission from Reference 47).

studies, physicians became convinced that administering platinum compounds to cancer patients might be worthwhile, and a new field involving bioinorganic chemistry and cancer chemotherapy was born. The drug, marketed as Platinol with the generic name cisplatin, received FDA approval in 1979 and is today one of the leading anticancer agents.

B. Principles that Underlie Drug Development

1. Strategic considerations

There are two general routes to the development of inorganic complexes, and indeed most chemical compounds, as drugs. One, illustrated by cisplatin, arises from an empirical observation of biological activity followed by attempts to optimize the clinical efficacy through investigations of structure-activity relationships (SAR). The goals are to minimize toxicity, to develop cell culture and animal screens for testing related compounds, and ultimately to elucidate the molecular mechanism. Knowledge of the molecular mechanism might eventually lead to a rational strategy for designing better drugs.

The second general approach to drug design begins with known biochemistry. For example, ribonucleotide reductase is required in the first committed step in the biosynthesis of DNA, the conversion of ribo- to deoxyribonucleoside diphosphates. The mammalian enzyme contains a binuclear non-heme iron center required for activity. Compounds that would selectively inhibit this enzyme by destroying this center are potentially useful as antiviral or antitumor agents. Another example is the enzyme reverse transcriptase, encoded by the HIV (AIDS) virus and required for its integration into the genome of the host cell. Compounds like 3'-azidothymidine (AZT) are accepted by the enzyme as substrates but, when added to the growing DNA chain, cannot be linked to the next nucleotide. Chain termination therefore occurs and the replication process becomes permanently interrupted. Attempts to find organic molecules or inorganic complexes that are more effective chain terminators than AZT constitute a rational strategy for developing new anti-AIDS drugs.

In the remainder of this chapter we describe research that has evolved following the discovery of biological activity for cisplatin. Although the initial breakthrough was serendipitous, subsequent studies have revealed many aspects of the molecular mechanism. From this known biochemistry we may one day be in a position to design more effective anticancer drugs and therapies based upon the fundamental bioinorganic chemistry of cisplatin.

2. Pre-clinical and clinical trials[49]

Predicting the chemotherapeutic potential of an inorganic compound such as *cis*-DDP prior to its introduction into human cancer patients is an important objective. Compounds are most easily tested for their cytotoxic effects on bacterial or mammalian cells in culture. Shown in Figure 9.6 are results for the

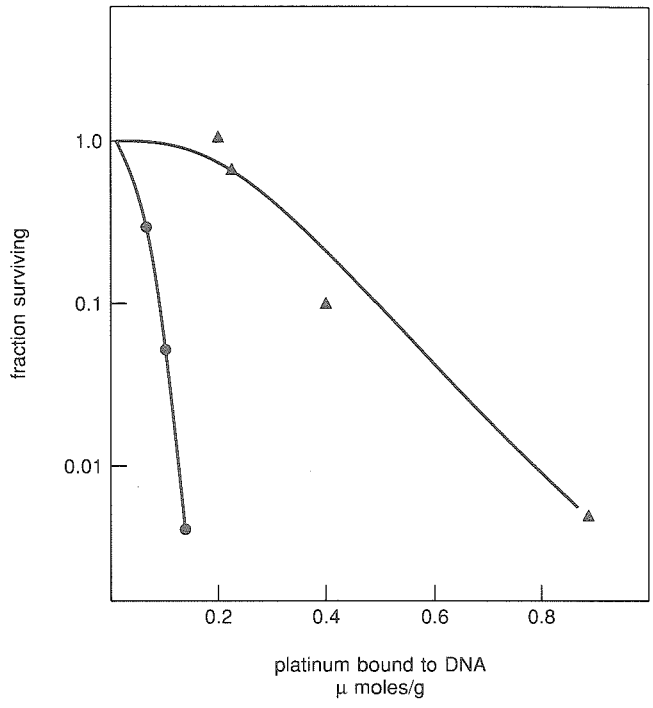


Figure 9.6
Differential toxicity of *cis*- (●) and *trans*-DDP (▲) on HeLa cells growing in culture (reproduced by permission from Reference 51).

survival of cultured L1210 cells in the presence of increasing amounts of *cis*- or *trans*-DDP.[50,51] The data reveal the markedly greater toxicity of the *cis* isomer, which is a much better anticancer agent than its stereoisomer. Unfortunately, no single assay has yet been found that can predict the chemotherapeutic potential of platinum compounds in humans. The best that can be obtained are results relative to those for *cis*-DDP, in which case toxicity at low dose is usually scored positive.

The next level of testing, often employed directly without first examining cell-culture results, involves animal (usually mammals, excluding human) screens.[49] Among the most popular measures of the chemotherapeutic activity of platinum compounds has been their ability to prolong the survival of mice bearing the L1210 or P388 leukemia. A suspension of cells is inoculated intraperitoneally (i.p., in the abdominal cavity), producing a leukemia that eventually progresses to the generalized disease. In one commonly used protocol, platinum compounds are dissolved in physiological saline (0.85 percent NaCl) or sterile H_2O and injected i.p. 24 h, 5, 9, and 13 days after inoculation of the leukemia cells. Several indices of antitumor activity and toxicity have been defined. The percent I.L.S., or increased lifespan, measures the mean survival of treated versus control animals that were given no platinum drug. A related index

is the median survival, percent T/C (Test/Control), which is 100 + percent I.L.S. The LD_{50} value measures toxicity as mean lethal dose, the amount of drug (usually in mg/kg body weight) required to kill half the animals. Potency is defined by ID_{90}, the inhibiting dose at which 90 percent of the tumor cells are killed. From these values, a therapeutic index (TI) = LD_{50}/ID_{90} is sometimes defined, which should be substantially greater than one. Typical values for *cis*-DDP are 85 percent I.L.S. at 8 mg/kg for the L1210 tumor, 13.0 mg/kg LD_{50}, and 1.6 mg/kg ID_{90} resulting in a TI of 8.1.

In addition to being tested in mice, cisplatin and related compounds have been screened in other mammals, specifically dogs and monkeys, mainly to look for possible dose-limiting side effects. Severe vomiting, once thought to be an insurmountable obstacle, was monitored by using ferrets. None of the animal screens can substitute for the ultimate test, however, which is human clinical trials. In 1972, such trials were initiated using terminally ill cancer patients. It was determined that intravenous (i.v.) injection, rather than i.p. or oral administration, was the preferred method for giving the drug. Further details of the clinical development of cisplatin are discussed in a later section.

From the animal screens emerged the set of structure-activity relationships enumerated earlier (Section IV.E.1). Both cisplatin and carboplatin conform to these rules, and to date no compounds with demonstrably better antitumor activity have been tested in humans. The decision to move an experimental drug into the clinic is a difficult one, however, and it may be that molecules such as *cis*-$[Pt(NH_3)_2(4\text{-Br-py})Cl]Cl$ (see Section V.D.7.c) would be effective for tumors that are refractive to cisplatin chemotherapy. In any case, the foregoing chain of events, from studying the effects of a compound on cells in culture through animal screens and eventually to humans, constitutes the principal route for introducing a new anticancer drug. The process can take more than a decade.

3. Mechanism of action studies

Once a class of compounds has been identified as biologically active, studies to elucidate the molecular mechanism of action can be undertaken. A first step is to identify the major cellular target or targets responsible for the chemotherapeutic properties of the drug. These investigations must also focus on chemical transformations that might take place in the solutions being administered and in the biological fluids that transport the drug to its ultimate target site. The next major step is to characterize the adduct or family of adducts made with the biological target molecule. The structure and kinetic lifetime of these adducts need to be investigated. Once this information is in hand, the effect of the adducts on the structure, stability, and function of the biological target molecule must be studied. Here many powerful new methodologies of modern molecular biology, genetics, and immunology can be brought to bear on the problem. The ultimate goals are to translate the molecular events elucidated into a realistic mechanism for how the drug molecule brings about its toxic effects selectively at the sites responsible for the disease and to use this information to design even better drugs.

Having progressed this far, we next need to bridge the gap between fundamental knowledge gained in studies of the mechanism of action and the SAR gleaned through pre-clinical and clinical trials. Whether such a happy situation can be reached for cisplatin remains to be seen, but there are encouraging signs, as we hope to demonstrate in the following discussion.

C. Clinical Picture for Cisplatin and Carboplatin[49,52]

1. Responsive tumors and combination chemotherapy

It was an early observation that the best responses to cisplatin occurred in patients with genitourinary tumors. For testicular cancer, once a leading cause of death for males of age 20–40, cisplatin cures nearly all patients with stage A (testes alone) or B (metastasis or retroperitoneal lymph nodes) carcinomas. Platinum is usually given in combination with other drugs, commonly vinblastine and bleomycin for testicular cancer. This combination chemotherapy, as it is known, has several objectives. Some tumors have a natural or acquired resistance to one class of drugs and, by applying several, it is hoped that an effective reduction in tumor mass can be achieved. In addition, various drugs are known to affect different phases of the cell cycle, so several are applied simultaneously to allow for this possibility. Finally, synergism, where the response is greater than expected from simple additive effects, can occur, although it is rare. In addition to testicular cancer, platinum chemotherapy has produced responses in patients with ovarian carcinomas (>90 percent), head and neck cancers, non-small-cell lung cancer, and cervical cancer. Cisplatin is also effective when combined with radiation therapy.

2. Dose-limiting problems; toxicology

An early and quite worrisome adverse side effect of cisplatin was kidney toxicity. This problem, not commonly encountered with the older cancer drugs, nearly prevented its widespread use and eventual FDA approval. The major breakthrough here was made by E. Cvitkovic, who, while working at Sloan-Kettering Memorial Hospital in New York, administered large quantities of water by intravenous injection to patients, together with an osmotic diuretic agent such as D-mannitol. The rationale was that such hydration could ameliorate kidney toxicity by flushing out the heavy-metal complex. This simple idea worked, and the dose of the platinum compound could be increased threefold without accompanying nephrotoxicity. Hydration therapy is now commonly employed when cisplatin is administered. Among the other toxic effects encountered in cisplatin chemotherapy are nausea and vomiting, but this problem has also been controlled by use of antiemetic agents. Patients have also been known to experience bone-marrow suppression, a ringing in the ears, and occasional allergic reactions.

More recently, attempts have been made to extend cisplatin treatment to other broad classes of tumors by raising the dose above the ~ 5 mg/kg body

weight levels given by i.v. injection every few weeks. Direct injection into the peritoneal cavity has been employed for refractory ovarian tumors. These more aggressive therapeutic protocols have been frustrated by drug resistance, a phenomenon by which cells learn to tolerate a toxic agent and for which many mechanisms exist, and by the return of the usual cisplatin side effects, most notably kidney toxicity and neurotoxicity. In order to combat toxic effects to the kidneys, chemoprotector drugs have been introduced. Based on the known affinity of platinum(II) complexes for sulfur-donor ligands, sodium diethyldithiocarbamate (DDTC) has been given both to experimental animals and to humans by i.v. infusion over about an hour following cisplatin administration.[53] DDTC inhibits many of the toxic side effects, particularly to the kidneys and bone marrow, without itself producing long-term side effects or apparently inhibiting the antitumor properties of *cis*-DDP. Similar efforts have been made to reduce the toxic effects of cisplatin with other sulfur-containing compounds including thiosulfate and the naturally occurring biomolecules glutathione, cysteine, and methionine. The relative amounts of the latter three molecules can be controlled by drugs that affect their normal cellular concentrations.

Another approach to reducing cisplatin toxicity is to develop new classes of platinum drugs or different routes of their administration. Carboplatin (Figure 9.4) is one result of these efforts. The bidentate chelating dicarboxylate leaving group in carboplatin presumably retards the rates of reactions leading to toxicity, but does not adversely interfere with the chemistry required for antitumor activity. Recently, promising platinum compounds for oral administration have been developed.[54] In Pt(IV) complexes of the kind *cis*, *trans*, *cis*-$[Pt(NH_3)(C_6H_{11}NH_2) \cdot (O_2CCH_3)_2Cl_2]$, where $C_6H_{11}NH_2$ is cyclohexylamine, have been found to be effective in preclinical screens. The greater kinetic inertness of these complexes apparently renders them sufficiently stable to the chemically harsh environment of the gastrointestinal tract. Once absorbed into the bloodstream, these compounds are metabolized to the Pt(II) analogues, *cis*-$[Pt(NH_3)(C_6H_{11}NH_2)Cl_2]$, which are presumed to be the active form of the drug. The Pt(IV) compound has recently entered clinical trials.

Although impressive inroads have been made in the management of human tumors by platinum chemotherapy, the fact remains that, apart from testicular and to a lesser extent ovarian cancer, the median survival times are measured in months. Clearly, there is much room for improvement.

3. Pharmacology[49,52]

Solutions of cisplatin are usually given in physiological saline (NaCl), since hydrolysis reactions occur that can modify the nature of the compound and its reactions *in vivo* (see below). Cisplatin is rapidly cleared from the plasma after injection, 70–90 percent of the platinum being removed within the first 15 minutes. It has been found that more than half the platinum binds to serum proteins and is excreted. Most of the platinum exits the body via the urine within a few days. These results account for the use of multiple-dose chemotherapy at inter-

vals of several weeks. Animal studies employing *cis*-DDP labeled with ^{195m}Pt, a 99 keV γ-emitter with a 4.1-day half-life, reveal retention half-times in various tissues of 8.4 (kidney), 6.0 (ileum), 4.1 (liver), 2.8 (tumor), and 1.9 (serum) days following a single dose. Platinum distributes widely to all tissue, with kidney, uterus, liver, and skin having the most, and muscles, testes, and brain the least amount of the compound. There is no evidence for selective uptake into normal versus tumor cells.

D. Bioinorganic Chemistry of Platinum Anticancer Drugs; How Might They Work?

The material in this section constitutes the major portion of this chapter. One important goal of the discussion is to illustrate, by means of an in-depth analysis of a single case history, the questions that must be addressed to elucidate the molecular mechanism of an inorganic pharmaceutical. Another is to introduce the techniques that are required to answer these questions, at least for the chosen case. The inorganic chemist reading this material with little or no biological background may find the experience challenging, although an attempt has been made to explain unfamiliar terms as much as possible. It is strongly advised that material in Chapter 8 be read before this section. Toward the end of this section, the results obtained are used to speculate about a molecular mechanism to account for the biological activity of the drug. Experiments directed toward evaluating the various hypotheses are delineated. Once the mechanism or mechanisms are known, it should be possible to design new and better antitumor drugs which, if successful, would be the ultimate proof of the validity of the hypotheses. This topic is discussed in the next and final section of the chapter. Such an analysis could, in principle, be applied to probe the molecular mechanisms of the other metals used in medicine described previously. In fact, it is hoped that the approach will prove valuable to students and researchers in these other areas, where much less information is currently available at the molecular level.

The material in this section has been organized in the following manner. First we discuss the relevant inorganic chemistry of platinum complexes in biological media. Next we summarize the evidence that DNA is a major target of cisplatin in the cancer cell, responsible for its antitumor activity. The chemical, physical, and biological consequences of damaging DNA by the drug are then described, followed by a presentation of the methodologies used to map its binding sites on DNA. The detailed structures of the DNA adducts of both active and inactive platinum complexes are then discussed, together with the way in which the tertiary structure of the double helix can modulate these structures. Finally, the response of cellular proteins to cisplatin-damaged DNA is presented, leading eventually to hypotheses about how tumor cells are selectively destroyed by the drug. Together these events constitute our knowledge of the "molecular mechanism," at least as it is currently understood.

1. Reactions of *cis*-DDP and related compounds in aqueous, biological, and other media

cis-Diamminedichloroplatinum(II) is a square-planar d^8 complex. As such, it belongs to a class of compounds extensively investigated by coordination chemists.[55] Typically, such compounds are relatively inert kinetically, do not usually expand their coordination numbers, and undergo ligand substitution reactions by two independent pathways with the rate law as given by Equation (9.4). The rate constants k_1 and k_2 correspond to first-order (solvent-assisted) and second-order (bimolecular) pathways;

$$\text{Rate} = (k_1 + k_2[Y])\,[\text{complex}] \tag{9.4}$$

$[Y]$ is the concentration of the incoming ligand. Usually, $k_1 << k_2$ by several orders of magnitude. In biological fluids, however, the concentration of a potential target molecule could be $\sim 10^{-6}$ M, in which case $k_1 \gtrsim k_2[Y]$. Substitution of ligands in *cis*-DDP, required for binding to a cellular target molecule, is therefore likely to proceed by the solvent-assisted pathway. Such a pathway is assumed in the ensuing discussion.

For the hydrolysis of the first chloride ion from *cis*- or *trans*-DDP,

$$[Pt(NH_3)_2Cl_2] + H_2O \rightleftharpoons [Pt(NH_3)_2Cl(OH_2)]^+ + Cl^- \tag{9.5}$$

the k_1 values at 25°C are 2.5×10^{-5} and $9.8 \times 10^{-5}\ s^{-1}$, respectively.[55] These hydrolyzed complexes can undergo further equilibrium reactions, summarized by Equations (9.6) to (9.9).

$$[Pt(NH_3)_2Cl(OH_2)]^+ \rightleftharpoons [Pt(NH_3)_2Cl(OH)] + H^+ \tag{9.6}$$

$$[Pt(NH_3)_2Cl(OH_2)]^+ + H_2O \rightleftharpoons [Pt(NH_3)_2(OH_2)_2]^{2+} + Cl^- \tag{9.7}$$

$$[Pt(NH_3)_2(OH_2)_2]^{2+} \rightleftharpoons [Pt(NH_3)_2(OH_2)(OH)]^+ + H^+ \tag{9.8}$$

$$[Pt(NH_3)_2(OH_2)(OH)]^+ \rightleftharpoons [Pt(NH_3)_2](OH)_2] + H^+ \tag{9.9}$$

The formation of dimers such as $[Pt(NH_3)_2(OH)]_2^{2+}$ and higher oligomers can also occur,[56,57] but such reactions are unlikely to be important at the low platinum concentrations encountered in biological media. Reactions (9.5) to (9.9), which depend on pH and the chloride-ion concentrations, have been followed by ^{195}Pt ($I = \frac{1}{2}$, 34.4 percent abundance) and ^{15}N (using enriched compounds) NMR spectroscopy. The latter method has revealed for the *cis*-diammine complexes pK_a values of 6.70 ± 0.10 at 25°C for Reaction (9.6) and of 5.95 ± 0.1 and 7.85 ± 0.1 at 5°C for Reactions (9.8) and (9.9), respectively.[58]

The effects of pH and Cl^- ion concentration on the species distribution of platinum compounds have been used to fashion the following plausible argument for the chemistry of *cis*-DDP *in vivo*.[59] With the use of thermodynamic data for the ethylenediamine (en) analogue $[Pt(en)Cl_2]$, the relative concentrations of hydrolyzed species at pH 7.4 were estimated (see Table 9.3) for blood plasma and cytoplasm (Figure 9.7). The higher chloride ion concentration in

Table 9.3
Distribution of various adducts formed between *cis*-DDP or [^{3}H][Pt(en)Cl_2][a] and DNA *in vitro* and *in vivo*.[118–122]

D/N ratio	Total incubation time	Adducts formed				Remaining platinum[c]
		cis-[PtA_2{d(pGpG)}][b]	*cis*-[PtA_2{d(pApG)}][b]	*cis*-[PtA_2{d(GMP)}$_2$][b]	Mono-functional adducts	
In vitro						
0.055[c]	5 h (50°C)	47—50%	23—28%	8—10%	2—3%	10%
0.022[d]	5 h (50°C)	60—65%	20%	~4%	~2%	9—14%
0.01[e]	16 h (37°C)	62%	21%	7%	—	10%
[ef]	30 m (37°C)	36%	3%	8%	40%	13%
[ef]	2 h (37°C)	54%	9%	9%	14%	14%
[ef]	3 h (37°C)	57%	15%	9%	4%	15%
In vivo						
[dg]	1 h (37°C)	35.9 ± 4.7%[h]	<34%[i]	3.1 ± 1.6%[h]	38.5%[i]	~22%
[dg]	25 h (37°C)	46.6 ± 6.8%[h]	<48%[i]	3.0 ± 0.9%[h]	<14.5%[i]	~50%

[a] A radiolabeled analogue of *cis*-DDP, [^{3}H]dichloroethylenediamineplatinum(II).
[b] A_2 represents either $(NH_3)_2$ or ethylenediamine.
[c] By difference.
[d] Percentage of adducts based on total amount of platinum eluted from the separation column.
[e] Percentage of adducts based on total amount of radioactivity eluted from the separation column.
[f] Not given.
[g] Chinese hamster ovary cells treated with 83 μM *cis*-DDP.
[h] Results from ELISA.
[i] Results from AAS. Where the signal was too weak for reliable quantitation, the maximal amount possible is given. Adapted from Table I in Reference 81.

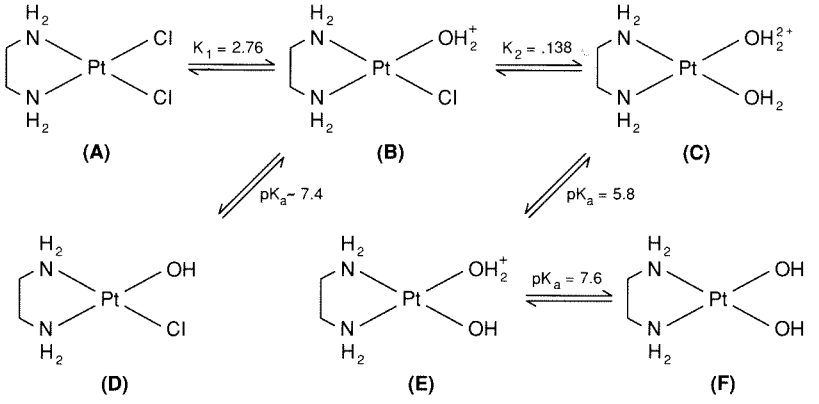


Figure 9.7
Hydrolysis reactions of antitumor platinum complexes and an estimate of the species present in plasma and cytoplasm (reproduced by permission from Reference 51).

plasma preserves the complex as the neutral molecule *cis*-DDP, which passively diffuses across cell membranes. The lower intracellular chloride ion concentration facilitates hydrolysis reactions such as Equations (9.5) to (9.9), thereby activating the drug for binding to its biological target molecules. There is, of course, a reasonable probability that *cis*-DDP and species derived from it will encounter small molecules and macromolecules *in vivo* that divert it from this route to the target. We have already seen such cases; cisplatin binds to serum proteins, and there is good evidence that intracellular thiols react with the drug.[60] Glutathione, for example, is present in millimolar concentrations in cells. How, one might ask, does cisplatin swim through such a sea of sulfur donors to find its target in the tumor cell? Is it possible that a modified form of the drug, in which a Pt-Cl bond has been displaced by thiolate to form a Pt-S bond, is the actual species responsible for its activity? Although these questions have not yet been satisfactorily answered, there is reason to believe that such reactions are not directly involved in the molecular mechanism of action. As evident from structure-activity relationship studies, the most active compounds have two labile ligands in *cis* positions. If Pt-S bonds were required, then compounds already having such linkages would be expected to exhibit activity and they do not. Rather, it seems most likely that the antitumor activity of cisplatin results from surviving species of the kind written in Equations (9.5) to (9.9) that find their way to the target molecule, and that the induced toxicity must arise from a significantly disruptive structural consequence of drug binding. Since only *cis* complexes are active, it is reasonable for the coordination chemist to infer that the stereochemistry of this interaction is of fundamental importance.

Reactions of platinum compounds with components in media used to dissolve them can give and undoubtedly have given rise to misleading results, both in fundamental mechanistic work and in screening studies. A particularly noteworthy example is dimethylsulfoxide (DMSO), which even recently has been

used to dissolve platinum compounds, presumably owing to their greater solubility in DMSO compared to water. As demonstrated by ^{195}Pt NMR spectroscopy, both *cis*- and *trans*-DDP react rapidly ($t_{1/2}$ = 60 and 8 min at 37°C, respectively) to form $[Pt(NH_3)_2Cl(DMSO)]^+$ complexes with chemical and biological reactivity different from those of the parent ammine halides.[61]

2. Evidence that DNA is the target

Two early sets of experiments pointed to interactions of cisplatin with DNA, rather than the many other possible cellular receptors, as an essential target responsible for cytotoxicity and antitumor properties.[62,63] Monitoring the uptake of radiolabeled precursors for synthesizing DNA, RNA, and proteins, showed that [^{3}H]thymidine incorporation was most affected by therapeutic levels of cisplatin for both cells in culture and Ehrlich ascites cells in mice. Since independent studies showed that *cis*-DDP binding to DNA polymerase does not alter its ability to synthesize DNA, it was concluded that platination of the template and not the enzyme was responsible for the inhibition of replication.

In a second kind of experiment demonstrating that DNA is a target of cisplatin, hydrolyzed forms of the drug in low concentrations were added to a strain of *E. coli* K12 cells containing a sex-specific factor F.[64,65] After free platinum was removed, these F^+ cells were conjugated with a strain of *E. coli* K12 cells lacking this factor that had previously been infected with lambda bacteriophage. Addition of *cis*-DDP, but not *trans*-DDP, directly to the latter infected F^- cells had been shown in a separate study to accelerate cell lysis. Conjugation with the platinum-treated F^+ cells produced the same effect, strongly suggesting that Pt had been transferred from the F^+ to the F^- cells. Since only DNA is passed between the F^+ and F^- strains, it was concluded that Pt was attached to the DNA and that this modification was essential for the observed lysis of the cell. Further studies showed a good correlation between cell lysis by platinum compounds and their antitumor properties.

Various other observations are consistent with the notion that platinum binding to DNA in the cell is an event of biological consequence.[66] The filamentous bacterial growth observed in the original Rosenberg experiment is one such piece of evidence, since other known DNA-damaging agents, for example, alkylating drugs and x-irradiation, also elicit this response. Another is the greater sensitivity toward *cis*-DDP of cells deficient in their ability to repair DNA. Finally, quantitation of the amount of platinum bound to DNA, RNA, and proteins revealed that, although more Pt was bound to RNA per gram biomolecule, much more Pt was on the DNA when expressed as a per-molecule basis. In the absence of any selective interaction of Pt with a specific molecule, only one out of every 1,500 protein molecules (average M.W. ~ 60 kDa) in a cell will contain a single bound platinum atom, whereas hundreds or thousands of Pt atoms are coordinated to DNA (M.W. ~ 10^{11}). If the replication apparatus cannot bypass these lesions, then cell division will not occur, and tumor growth is inhibited.

Although these and other results all point to DNA as an important cellular target of cisplatin, most likely responsible for its anticancer activity, this information does not explain why tumor cells are more affected by *cis*-DDP than non-tumor cells of the same tissue. Moreover, why is *trans*-DDP, which also enters cells, binds DNA, and inhibits replication, albeit at much higher doses (see discussion below), not an active anticancer drug? What causes cisplatin to kill cells and not merely to arrest tumor growth? The latter can be explained by DNA synthesis inhibition, but not necessarily the former. Very recent studies have begun to address these questions using powerful new methodologies of molecular and cell biology, as described in subsequent sections of this chapter. The results, although preliminary, continue to point to DNA as the most important cellular target of cisplatin.

3. Aspects of platinum binding to DNA

Given that DNA is a major target of platinum binding in cells, it is incumbent upon the bioinorganic chemist to investigate the nature of these interactions and their biological consequences. Of all the ligands studied in coordination chemistry, DNA is surely among the most complex. In the ensuing discussion, we first present experiments that delineate the chemical steps involved in *cis*- and *trans*-DDP binding to DNA as well as the chemical consequences of the adducts formed. We next describe the physical changes in the double helix that accompany platinum binding, and then we discuss the biological consequences that attend the platination of DNA. Subsequent sections describe the major adducts formed, in other words the regiospecificity of the drug, the three-dimensional structures of the adducts, and the way in which different structures within DNA can modulate platinum binding. Finally, we consider the response of the cell to Pt-DNA adducts, including studies with site-specifically modified DNA, and speculate about how this chemistry might relate to the antitumor drug mechanism.

a. Kinetics of Platinum Binding to DNA The binding of *cis*- and *trans*-DDP to DNA has been studied[67] by ^{195}Pt NMR spectroscopy with the use of isotopically enriched ^{195}Pt, which has a nuclear spin $I = \frac{1}{2}$. The DNA used in this experiment was obtained from chicken red blood cell chromosomes that had been enzymatically degraded to relatively small pieces ranging from 20 to 60 base pairs in length (molecular-weight range 13 to 30 kDa). Since the ^{195}Pt chemical shifts are very sensitive to chemical environment, this NMR study provided important details about the kinetics and mechanism of platinum binding to the biopolymer. The rate-determining step in platination of the DNA is loss of chloride ion (Equation 9.5) to form the monoaqua complex, which rapidly coordinates to a nitrogen donor on the nucleic acid. The identification of the coordinating atom as nitrogen was possible because the ^{195}Pt chemical shift is characteristic of species having one chloride and three nitrogen ligands bound to Pt(II).[67] The spectroscopic changes that accompany the formation of the family of monofunctional adducts are shown in Figure 9.8. Subsequent hydrolysis

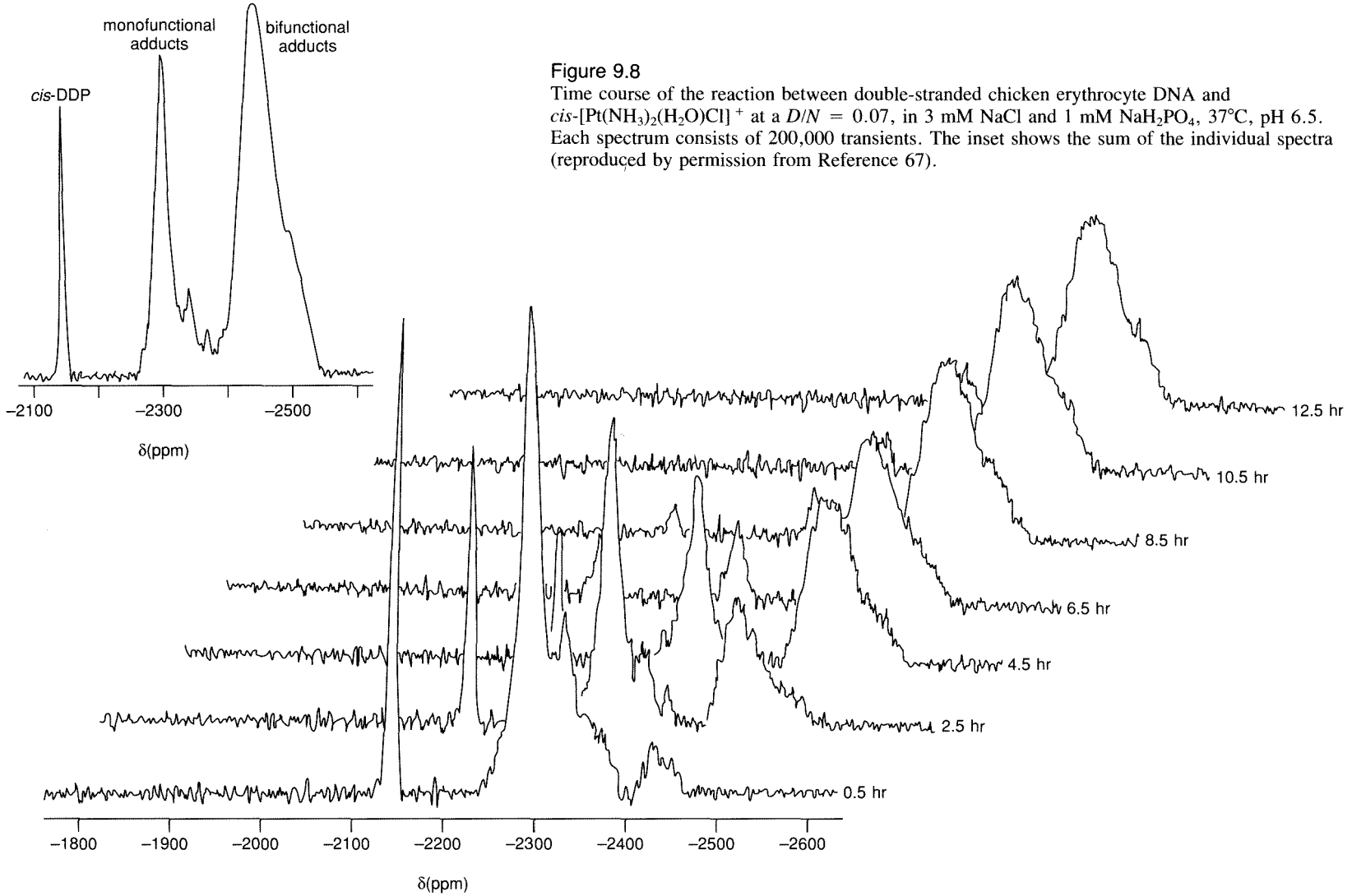
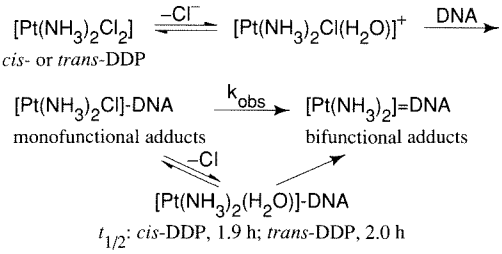


Figure 9.8
Time course of the reaction between double-stranded chicken erythrocyte DNA and *cis*-$[Pt(NH_3)_2(H_2O)Cl]^+$ at a $D/N = 0.07$, in 3 mM NaCl and 1 mM NaH_2PO_4, 37°C, pH 6.5. Each spectrum consists of 200,000 transients. The inset shows the sum of the individual spectra (reproduced by permission from Reference 67).

of the second chloride ion leads to the formation of a second bond with DNA. This sequence of events affords bifunctional adducts and is similarly accompanied by discrete ^{195}Pt spectral changes (Figure 9.8). From the ^{195}Pt chemical-shift range of the final products, it was apparent that the *cis*-$\{Pt(NH_3)_2\}^{2+}$ moiety is bound primarily to two nitrogen donors on the nucleic acid. This chemistry is summarized in Equation (9.10), together with the half-lives for the mono-



(9.10)

functional adducts. The half-lives were calculated from a kinetic analysis of the time-dependence of the ^{195}Pt spectral changes. As can be seen, the rates of closure of mono- to bifunctional adducts for the two isomers are quite similar, suggesting that their different biological properties are not a consequence of the kinetics of binding to DNA.

The next logical question to address is what donor atoms on DNA are coordinating to platinum in the mono- and bifunctional adducts. This important issue is discussed in considerable detail in Sections V.D.4 and V.D.5. As will be shown, the N7 atoms of the purine bases adenine and guanine are the principal binding sites. Alkylation of DNA at these positions facilitates depurination. Platinum binding to N7 atoms of purines (Figure 9.9), however, stabilizes the glycosidic (N9-C1′) linkage.[68–70] Presumably the positive charge is better distributed over the platinum atom and its ligands in the adduct than over a purine alkylated at N7. On the other hand, platinum binding to N7 of guanine does perturb the charge distribution in the purine ring, as evidenced by the lowering of the pK_a of N1 by ≈ 2 units from its value in the unplatinated nucleotide (usually from p$K_a \approx 10$ to p$K_a \approx 8$).[71,72] This effect has been used to assign platinum binding sites in DNA fragments, as discussed below.

What are the chemical changes at the platinum center when *cis*-DDP binds to DNA? Both chloride ions are lost from the coordination sphere, as already indicated. Platinum EXAFS studies of calf-thymus DNA modified with *cis*-DDP revealed no chlorine backscattering features characteristic of Pt-Cl bonds.[73] The spectra were consistent with the presence of four Pt-N/O linkages, since the technique is unable to distinguish between the two low-Z elements oxygen and nitrogen. Various studies reveal that, under most circumstances, the NH_3 ligands are not lost from DNA upon the binding of platinum ammine halides. For example, when ^{14}C-labeled *cis*-$[Pt(NH_2CH_3)_2Cl_2]$ was allowed to bind to T7 (47 percent GC content) or *M. luteus* (73 percent GC content) DNA, no loss of radiolabel was found to accompany platinum binding.[74] *In vivo*, however, loss of amine ligands has been observed. Injection of ^{195m}Pt and ^{14}C doubly labeled

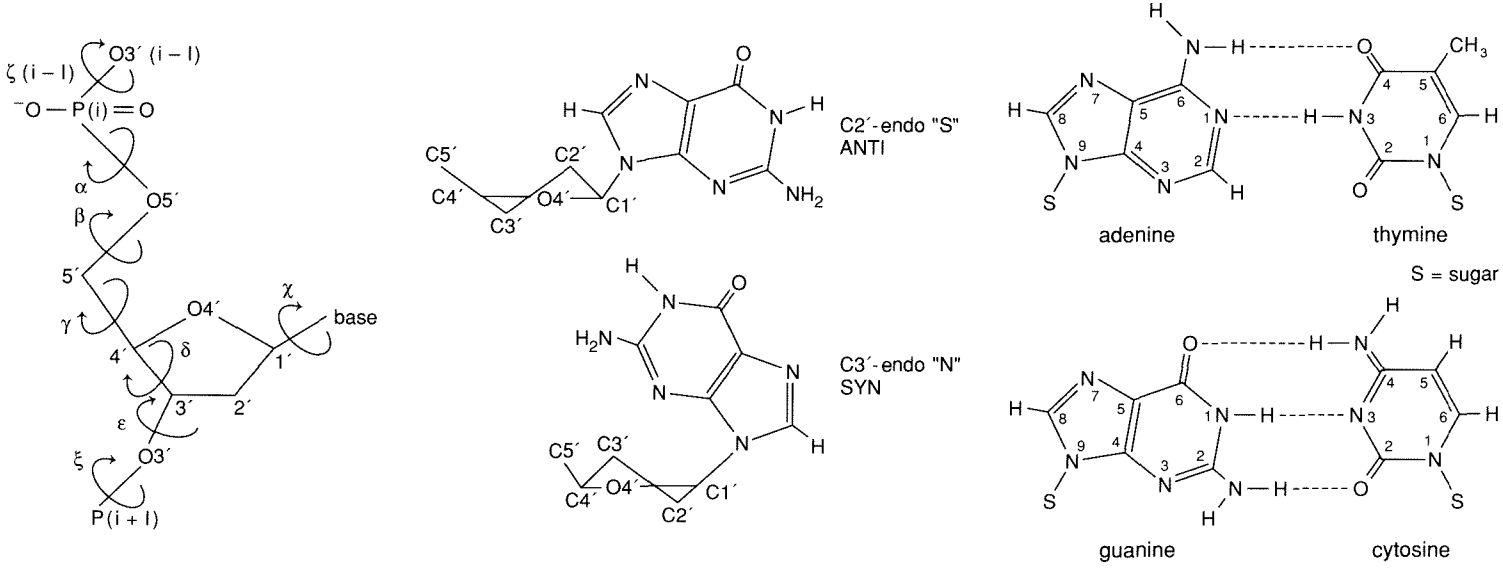


Figure 9.9
Structural components of DNA. The left panel shows a schematic of the DNA backbone, including the deoxyribose ring numbering scheme and torsion angles. The center panel gives the two main classes of sugar pucker and base orientations. The right panel gives the bases in their Watson-Crick, A-T, and G-C pairs and the base numbering scheme, viewed down the helix axis, with the major grooves pointing toward the top of the figure and the minor grooves toward the bottom.

$[Pt(en)Cl_2]$ into tumor-bearing mice resulted in unequal distribution of the two labels in various biochemical fractions, but there is no reason to believe that this result is relevant to the antitumor mechanism.[75] Metabolic inactivation of the drug could occur in a variety of ways unrelated to anticancer activity. The best evidence that ammine loss does not occur at the critical biological target of cisplatin is the finding, by using antibodies specific for *cis*-$\{Pt(NH_3)_2\}^{2+}$ nucleotide complexes (see Section V.D.4.c), that DNA, extracted from cells in culture or from human cancer patients treated with *cis*-DDP and subsequently degraded, contains intact Pt-NH_3 linkages.[76]

Once bonds are made between Pt and its targets on DNA, they are relatively inert kinetically. Platinum-DNA complexes can be subjected to various physical methods of separation and purification, including gel electrophoresis, ethanol precipitation, centrifugation, and chromatography, as well as to enzymatic and even chemical degradation procedures that digest the DNA, without releasing the platinum. Platinum can be removed, however, either by use of cyanide ion, to form the very stable ($K \sim 10^{41}$) $[Pt(CN)_4]^{2-}$ complex, or by excess thiourea.[77,78] These properties have proved to be extremely valuable in facilitating localization and characterization of the major *cis*- and *trans*-DDP binding sites on DNA.

Although Pt-DNA linkages are, generally speaking, kinetically inert, sometimes a particular adduct will rearrange into a more stable linkage isomer. One interesting example is the product of the reaction of *trans*-DDP with the dodecanucleotide 5'-d(TCTACGCGTTCT).[79] Initially the platinum coordinates to the two guanosine residues, forming a *trans*-$[Pt(NH_3)_2\{d(GCG)\}]$ 1,3-intrastrand crosslink. This complex rearranges to a more stable *trans*-$[Pt(NH_3)_2\{d(CGCG)\}]$ 1,4-intrastrand crosslink with a half-life of 129 h at 30°C or 3.6 h at 62°C. In this rearrangement product the platinum is coordinated to a cytosine and a guanosine residue.

As just described, the binding of bifunctional platinum complexes to DNA proceeds in a stepwise fashion. The second step is sufficiently slow (a few hours), however, that various reagents such as NH_3, nucleobases, and low concentrations of thiourea can coordinate to the fourth site and trap the monoadducts. Generally speaking, however, given sufficient time both *cis*- and *trans*-DDP will bind DNA in a bifunctional manner. As such, they bear some resemblance to organic alkylating agents, such as the nitrogen mustards, which have been employed as anticancer agents.[80]

b. Crosslinking Reactions of Platinum Complexes There are three broad classes of DNA adducts that can be made by bifunctional platinum complexes. As illustrated for *cis*-DDP in Figure 9.10, they are DNA-protein crosslinks, interstrand DNA-DNA crosslinks, and intrastrand crosslinks.[81] A fourth possibility for platinum complexes is bidentate chelate ring formation utilizing two donor atoms on a nucleotide. For many years, a favored such postulated mode of binding was chelation by the N7-O6 positions of the guanine base (Figure 9.9), since this structure could be formed only by *cis*- and not by *trans*-DDP.[82,83]

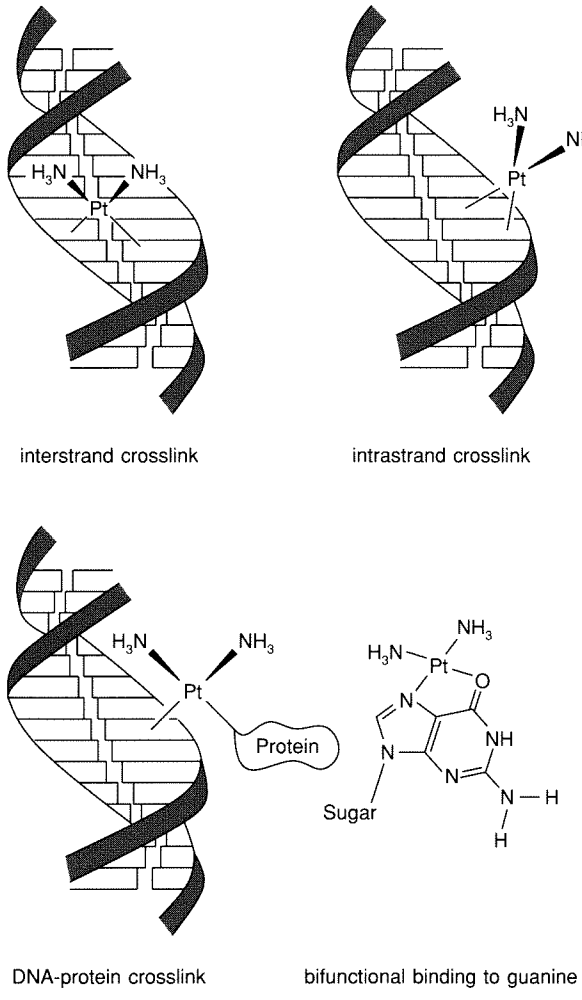


Figure 9.10
Possible bifunctional binding modes of *cis*-DDP with DNA (reproduced by permission from Reference 81).

Such a structure has never been observed for *cis*-DDP binding to DNA, however. DNA-protein and interstrand crosslinks formed by platinum complexes have been the focus of many attempts to explain cytotoxicity and antitumor behavior.[84] The technique of alkaline elution, in which crosslinked DNA-DNA strands or DNA-protein molecules bind to filter paper following denaturation under basic conditions, sensitively and easily reveals such adducts. *trans*-DDP forms such adducts more rapidly than the *cis* isomer, perhaps because of its faster chloride-ion hydrolysis rates (see above) and a more favorable geometry, but they also seem to be repaired more rapidly in cells. As will be shown, interstrand and DNA-protein crosslinks are a small minority of adducts formed by cisplatin, and their contribution to the cytotoxic and anticancer properties of

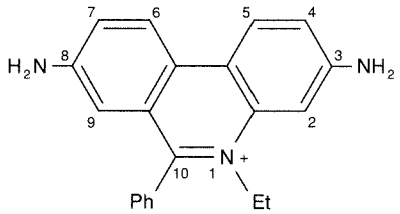
the drug remains to be established. In studies of SV40 replication *in vivo*, DNA-protein crosslinking by *cis*- and *trans*-DDP was shown not to be correlated with the inhibition of DNA replication.[85]

What proteins form crosslinks to DNA? One possibility is the histones that make up the spools around which DNA is wound when packaged into chromatin in the nucleus. Studies[86] of *cis*- and *trans*-DDP binding to nucleosome core particles (each particle made up of eight histone proteins; around each particle is wound a 146-bp piece of DNA in a shallow superhelix of 1.75 turns) revealed the DNA binding to be little affected by the protein core. Both DNA-protein and specific histone crosslinked species were observed; from the latter it was suggested that DDP complexes might be useful crosslinking probes of biological structures. Other proteins likely to form crosslinks to DNA in the presence of platinum complexes are DNA-processing enzymes, or enzymes requiring a DNA template for normal function. In the *in vivo* SV40 study, for example, T antigen was one of the proteins found to be crosslinked to SV40 DNA by cisplatin.[85] Other nuclear proteins such as the high-mobility group (HMG) class are also crosslinked to DNA in the presence of *cis*-DDP. In all cases so far, DNA-protein crosslinking has occurred when platinum was added to cells. There is as yet no evidence that transfection of platinated DNA into cells results in such crosslinking or that crosslinks form during *in vitro* enzymatic digestions of platinated DNAs.

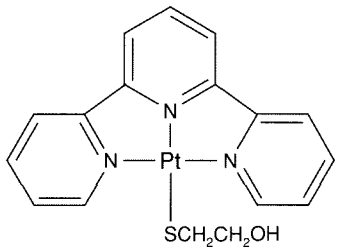
c. Physical Effects of Platinum-DNA Binding

(i). Unwinding, shortening, and bending of the double helix Early studies of *cis*- and *trans*-DDP binding to DNA employed closed and nicked circular plasmids.[87] As was described in more detail in Chapter 8, closed circular DNAs are topologically constrained such that any change in the number of helical turns must result in an equal and opposite number of superhelical turns. Any reagent that unwinds the double helix reduces the number of helical turns. Consider, for example, a stretch of DNA that is 360 base pairs (bp) long. Normal B-DNA has $\approx$10.5 bp per turn or a helical winding angle of $\approx$ 34.3° per bp. Suppose the DNA is unwound, so that there are now 12 bp per turn or a winding angle of $\approx$ 30°. Instead of 34.3 helical turns (360 $\div$ 10.5), the DNA now has only 30 (360 $\div$ 12). If this DNA molecule were in the form of a covalently closed circle, the helical unwinding of -4.3 turns would be accompanied by a superhelical winding of $+4.3$ turns.

Planar organic dyes such as ethidium bromide (EtdBr) and inorganic complexes such as $[Pt(terpy)(HET)]^+$ (Figure 9.11) bind to DNA by intercalation, inserting between the base pairs and unwinding the double helix by $\sim$ 26° per molecule bound (Figure 9.12).[88] This unwinding can be measured by monitoring changes in the superhelicity of closed circular DNA. This kind of DNA is subjected to certain topological constraints that lead to the formation of supercoils and superhelical winding that dramatically alter the hydrodynamic properties of the DNA. Either gel electrophoresis or analytical ultracentrifugation can be used to measure this phenomenon. The platinum complexes *cis*- and *trans*-DDP also



ethidium
(Etd)



$[Pt(terpy)(HET)]^+$, PtTS

Figure 9.11
Organic (top) and inorganic (bottom) intercalators.

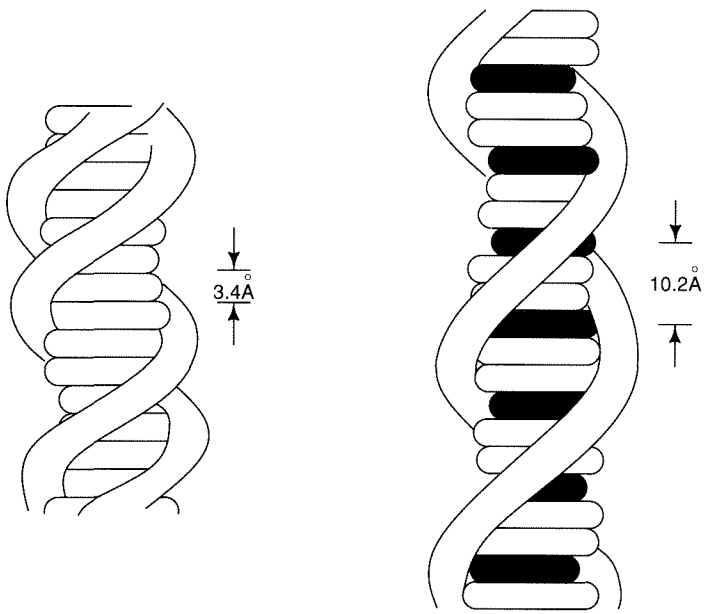


Figure 9.12
Schematic representation of double-stranded DNA without (left) and with (shaded area, right) a bound intercalator (reproduced with permission from Reference 51).

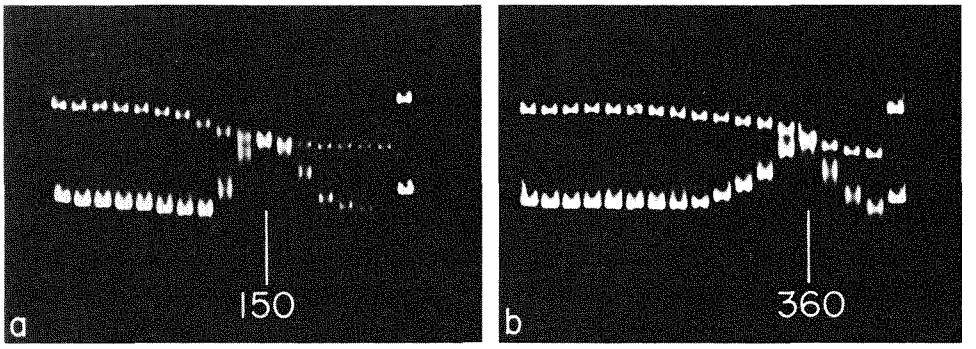


Figure 9.13
Electrophoresis in 1 percent agarose gels of nicked and closed circular PSM1 DNA incubated with (a) *cis*- and (b) *trans*-$[Pt(NH_3)_2Cl_2]$ as function of time. After electrophoresis, gels were stained with ethidium bromide. Reproduced with permission from Reference 51.

produce changes in the superhelix density when bound to closed circular DNA.[87] As shown in Figure 9.13, increasing concentrations of platinum bound per nucleotide on the DNA first retard its mobility and then increase its mobility through the gel. These interesting alterations in gel mobility occur because the negatively coiled superhelix unwinds first into an open, or untwisted, form and then into a positively supercoiled form. The conformational changes, which are depicted in Figure 9.14, are directly proportional to the drug-per-nucleotide, or $(D/N)_b$, ratio. In addition to superhelical winding, both platinum complexes increase the mobility of nicked circular DNA in the gels (Figure 9.13). Nicked DNA has one or more breaks in the sugar-phosphate backbone, which relieve the topological constraint and prohibit the DNA from twisting into superhelical structures.

What could be the cause of these physical changes in the DNA structure upon *cis*- or *trans*-DDP binding? Intercalation can be excluded, not only because the compounds do not have the aromatic character normally associated with intercalators (Figure 9.11), but also through studies of the manner by which these and other platinum complexes inhibit the intercalative binding of EtdBr to DNA.[89,90] Platinum metallointercalators such as $[Pt(terpy)(HET)]^+$ are competitive inhibitors of EtdBr binding, as measured by fluorescence Scatchard plots, whereas the non-intercalators *cis*- and *trans*-DDP are not. Moreover, intercalation tends to lengthen and stiffen the double helix, whereas the mobility changes of nicked circular DNAs upon binding of *cis*- or *trans*-DDP were shown by electron microscopy experiments to arise from a pronounced shortening of the DNA with increased Pt binding.

One manner by which *cis*- or *trans*-DDP might produce these physical alterations in DNA structure is by kinking the double helix at or near the binding site. Such an effect could be produced by the bidentate attachment of platinum; the monofunctional $[Pt(dien)Cl]^+$ complex does not have these pronounced ef-

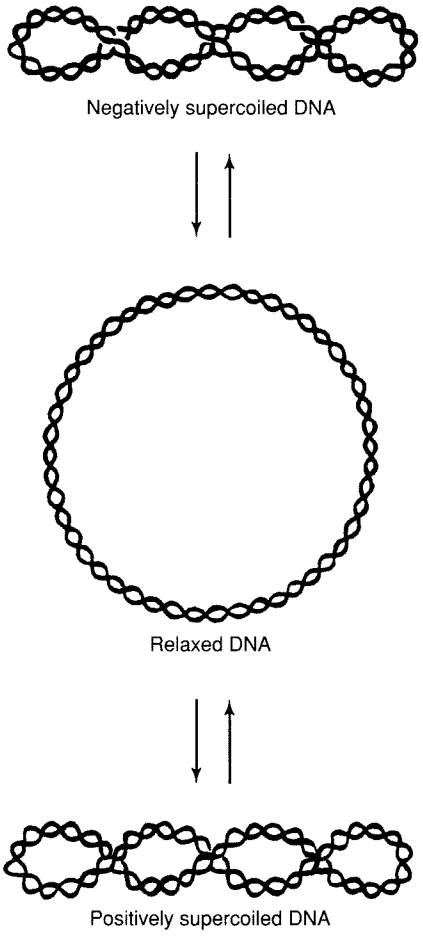


Figure 9.14
Topological forms of closed circular DNA.

fects on DNA secondary structure.[91] Recently, it has been demonstrated that *cis*-DDP binding to DNA does indeed produce a pronounced bend in the helix axis.[92,93] The proof employed a gel-electrophoretic method of analysis that had previously been used to study DNA bending at naturally occurring specific sequences called A-tracts, consisting of five or six adenosine nucleosides in a row followed by about the same number of thymidine residues.[94] When these $d(A_5T_5)_2$ sequences are positioned in the center of a DNA restriction fragment of, say, 150 bp, the mobility of the DNA through polyacrylamide electrophoresis gels is greatly retarded compared to that of a similar DNA fragment where the A-tract is at the end. For the former fragment, the bent molecules presumably cannot snake their way through the pores of the polyacrylamide as well as the molecules whose bends are at the ends and have little effect on the linear structure. It was further shown that A-tracts bend the duplex toward the minor groove of

the DNA. Moreover, in a DNA containing multiple A-tracts, the bends must be separated by integral numbers of helical turns (~ 10.5 bp) or else the effect will cancel and the gel mobility will be that of normal DNA of similar length. This latter phenomenon has been referred to as phasing.

With this background information in mind, we can now discuss the experiments with *cis*-DDP that demonstrated bending.[92,93] By methods described in Section V.D.8, a 22-bp oligonucleotide (22-mer) containing self-complementary overhanging ends (''sticky ends'') was synthesized with a single *cis*-diammineplatinum(II) moiety linking adjacent guanosine residues (Figure 9.15A). A 22-mer was chosen since it has approximately two helical turns, accounting for some platinum-induced unwinding, and will thus have phased bends when polymerized. This platinated DNA was then labeled with ^{32}P and treated with the enzyme DNA ligase, which seals the ends, producing oligomers of the 22-mer having lengths 22, 44, 66, 88, 110, etc., bp. In these oligomers, the platinum atoms are spaced apart approximately by integral numbers of helical turns. As shown in Figure 9.15, studies of this family of oligomers by gel electrophoresis revealed a pronounced retardation compared to the mobility of unplatinated DNA oligomers of comparable size (line P22 in Figure 9.15B). The plots in this figure show the relative mobilities (R_L) of the different length multimers, compared to a control in which the top strand is not platinated, as a function of the length in base pairs. From the resulting curves may be extracted the extent of cooperative bending. When oligomers of a platinated DNA fragment in which the metal atoms were spaced apart by 27 bp were examined, their relative mobilities were found to be nearly the same as unplatinated control molecules (line P27 in Figure 9.15B). These experiments unequivocally established that platinum kinks the double helix. As with A-tract-induced bends, the platinum atoms must be phased in order to induce cooperative bending. Comparison of the magnitudes of the gel mobility changes made it evident that *cis*-$\{Pt(NH_3)_2\}^{2+}$ binding produces a bend comparable to that of two A-tracts, $\approx 34°$.

In a related series of experiments,[93] the platinated 22-mer was copolymerized with various A-tract-containing 11-mers to produce ladders of oligomers in which the phasing of Pt with respect to the center of the A-tract was varied, but the Pt atoms were always in phase. The results of these studies showed that maximum gel-mobility retardation occurred when the Pt and A-tract center were spaced apart by half-integral numbers of helical turns (Figure 9.15C). Since A-tracts bend the DNA into the minor groove, this result implies that platinum bends the DNA into the major groove. Only when phased by $n/2$ (n = integer) turns will copolymers of species situated alternatively in the major and minor grooves of DNA exhibit such cooperative bending. It will be shown later that helix bending of *cis*-DDP-DNA adducts into the major groove is in accord with their known structures.

The ability to prepare site-specifically platinated oligonucleotides (see Section V.D.8) has provided a means for measuring the extent to which *cis*-DDP produces local unwinding of the double helix.[95] When the platinum atoms are positioned with respect to one another, or phased, by exactly integral numbers

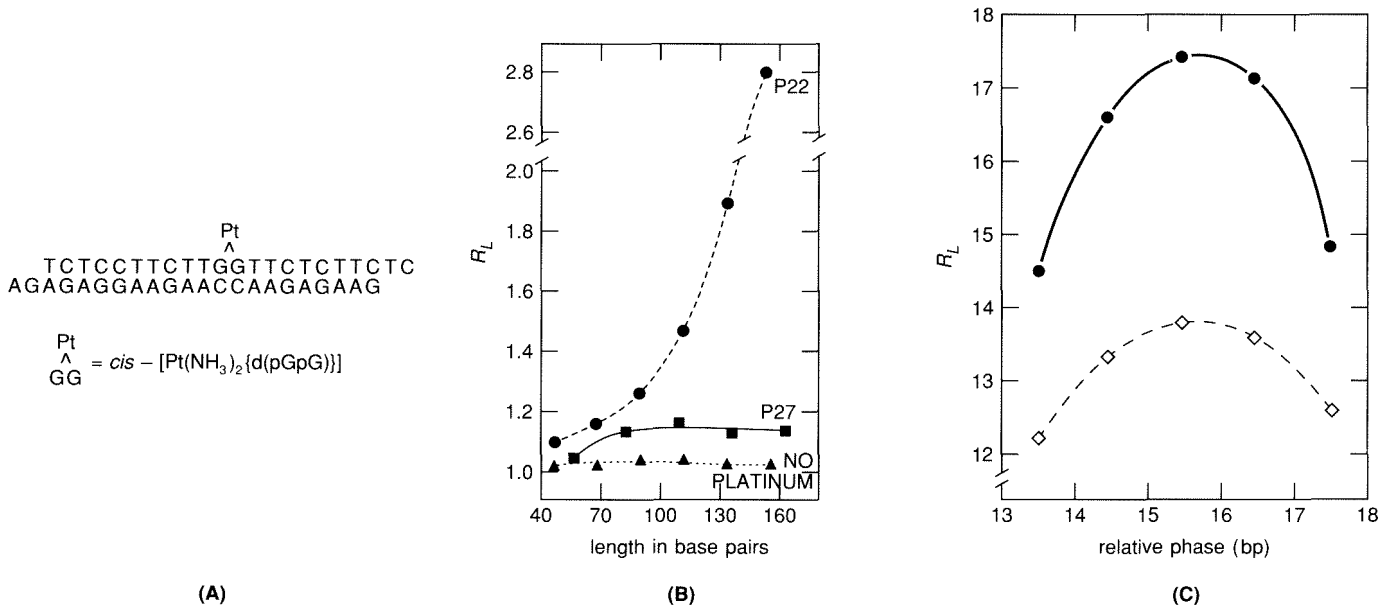


Figure 9.15
Experiment to demonstrate that *cis*-[$Pt(NH_3)_2${d(pGpG)}] intrastrand crosslinks bend duplex DNA by ≈34°. Panel A shows the platinated 22-mer sequence, panel B the effect of platination on the gel-electrophoresis mobility of the 22-mer (P22) and a control 27-mer (P27) oligomers, and panel C the mobility of copolymerized DNAs containing *cis*-DDP and A-tract induced bends (●, 128-bp Pt + A-tract DNA; ◇, 96-bp Pt + A-tract DNA) that maximize at approximately half-integral helical turns corresponding to their phasing. For more detail consult Reference 93.

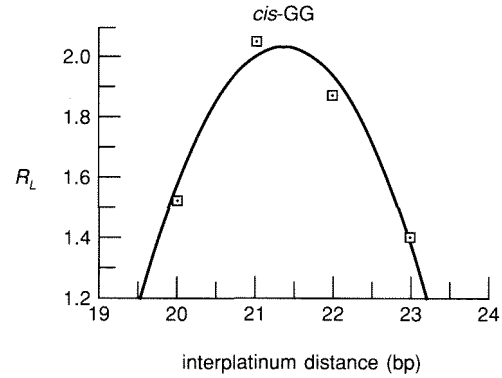


Figure 9.16
Plot showing the relative mobility (R_L) versus actual length curves for the oligomers *cis*-GG-20, *cis*-GG-21, *cis*-GG-22, and *cis*-GG-23, denoted as 20, 21, 22, and 23, respectively.

of helical turns, the retardation of the DNA multimers in the gel is maximized. This phenomenon is illustrated in Figure 9.16, where the R_L values are plotted as a function of the interplatinum spacing for oligonucleotides containing the *cis*-{$Pt(NH_3)_2d(GpG)$} intrastrand crosslink. When the resulting curve was analyzed, the maximum was found to occur at 21.38 bp. Since normal B-DNA has a helical repeat of 10.5 bp, one can compute the effect of platination from the expression [(21.38 − 2(10.5)] bp = 0.38 bp. From the fact that one helical turn of DNA comprises 360° and 10.5 bp, the unwinding of the DNA double helix due to the presence of a single *cis*-{$Pt(NH_3)_2d(GpG)$} intrastrand crosslink can be calculated as

$$(0.38/10.5) \times 360 = 13°.$$

Similar studies of DNA platinated with *trans*-DDP have been carried out. In these, oligonucleotides containing the 1,3-*trans*-{$Pt(NH_3)_2d(GpNpG)$} intrastrand crosslink were examined. The electrophoresis gels of polymerized 15-mers and 22-mers containing this adduct showed cooperative bending. This result indicates that bends at the sites of platination by *trans*-DDP are not phase sensitive, and has been interpreted to imply the formation of a "hinge joint" at these positions.[92,95] The directed bends and local unwinding of DNA produced by cisplatin could be an important structural element that triggers a response by cellular proteins. This subject is discussed in greater detail in Section V.D.7.d.

d. Biological Consequences of Platinum-DNA Binding

(i). Inhibition of replication Binding of *cis*-DDP to DNA inhibits replication both *in vivo* and *in vitro*, as shown by a variety of assays. Inhibition of replication of SV40 viral DNA in African green monkey cells as a function of the concentration of added *cis*-DDP is shown in Figure 9.17. When SV40 virus infects monkey cells, it does not integrate its DNA into the genome of the host.

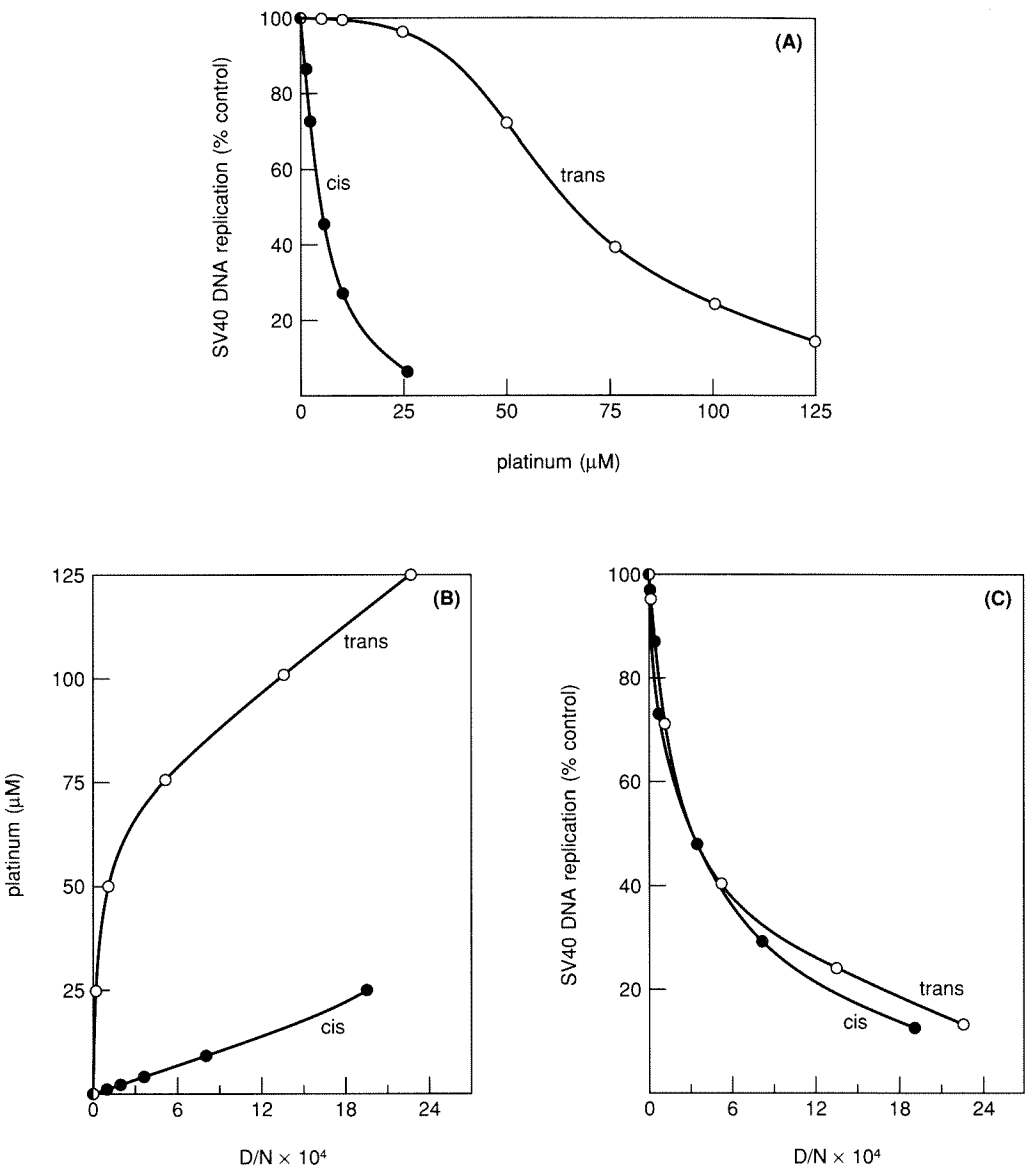


Figure 9.17
SV40 DNA replication in CV-1 cells as a function of platinum concentration in the medium (panel A) or *D/N* (panel C). In panel B, *D/N* is plotted as a function of platinum concentration in the medium. SV40-infected cells were treated with *cis*-DDP (●) or *trans*-DDP (o) at the indicated concentrations for 40 h. SV40 DNA replication relative to control (untreated) cells was measured by incorporation of [^{3}H]thymidine, added after the first 24 h of platinum treatment, and Pt in isolated SV40 chromosomes was measured by AAS. The data shown are from a representative experiment. Experiments were carried out in quadruplicate. Reproduced by permission from Reference 85.

Instead, it forms its own chromosomes in the cell nucleus. These so-called mini-chromosomes consist of $\approx$ 20 nucleosomes, fundamental chromosome building blocks. SV40 has its own life cycle, using virally encoded and cellular proteins to replicate and, ultimately, reassemble virus particles before lysing the cell and departing to infect neighboring cells.

In the experiment shown in Figure 9.17, the SV40-infected cells were treated with cisplatin. After 24 h, [^{3}H]thymidine was added and, after 24 more hours, the cells were harvested, and SV40 DNA was isolated; the amount of DNA synthesis was recorded by comparing incorporated radiolabel with results from control experiments where no platinum was present. The data show that, when 25 μM platinum was present, SV40 DNA replication was reduced to about 5 percent of control. Quantitation reveals that, at $\approx$2 platinum atoms bound per thousand nucleotides (drug-per-nucleotide, or $(D/N)_b$, = 0.002), synthesis is only 10 percent that of control.

Recently, a related series of experiments has been carried out that can monitor DNA synthesis from templates platinated *in vitro*.[96] In this work, DNA plasmids containing the SV40 origin of replication are added to cellular extracts prepared from human kidney cells previously infected with adenovirus. In the presence of large T antigen, a virally encoded protein required for replication, SV40 DNA is synthesized from the plasmid templates. Synthesis can be conveniently monitored by [^{32}P]dATP incorporation. At a $(D/N)_b$ ratio of only 1.7×10^{-3}, DNA synthesis is about 5 percent of control, in agreement with the results of the *in vivo* study.

The binding of *cis*-DDP to DNA has also been measured for normal and tumor cells implanted in nude mice and in cells obtained from the ascites fluid of patients with ovarian carcinoma 24 h after their last dose.[97] The data for mouse bone marrow and a human pancreatic tumor xenograft show that, at a dose of 10 mg/kg, $(D/N)_b$ platinum binding levels of 3.3×10^{-6} and 1.82×10^{-6} reduce survival to 20 and 10 percent of control, respectively. These ratios are in good accord with platinum levels required to inhibit DNA synthesis in mammalian cells, as revealed by various studies, but substantially less than that needed for replication inhibition in the SV40 experiments described above. The difference can be readily explained, illustrating an important point. The SV40 genome, like most other DNAs of viral or plasmid origin, consists of only 15,000 nucleotides whereas the nuclear DNA of mammals has about 10^9 nucleotides. Thus, $(D/N)_b$ levels of $\sim 10^{-6}$ would leave 99 out of 100 SV40 DNA molecules with no platinum at all, and replication would hardly be affected. For the mammalian genome, $(D/N)_b$ values of 10^{-6} place 10^3 platinum atoms on each DNA genome, sufficient to inhibit replication and reduce cell survival. Platinum-DNA binding levels of this magnitude are found for ovarian ascites cells taken from patients receiving cisplatin chemotherapy.[97]

(ii). Mutagenesis and repair Apart from inhibition of DNA synthesis, what are the other biological consequences of cisplatin binding to DNA? One such consequence is mutagenesis, in which a normal base in the sequence is replaced

by a different base. This phenomenon has been demonstrated for *cis*-DDP-treated cells in a variety of studies. What brings about such mutagenesis? There are several possibilities. One is that errors are introduced in DNA strands during attempts of the replication apparatus to synthesize past a platinum lesion. Another is that the platinum-damaged DNA is recognized by cellular repair systems that, in attempting to eliminate the platinated stretch of DNA, incorporate one or more incorrect nucleotides. Platinum-induced mutagenesis can lead to deleterious long-term health problems in patients treated with cisplatin. It is therefore important to understand the mechanism by which cellular DNA becomes mutated following platination, and to devise strategies for minimizing or eliminating this mutagenesis.

The foregoing considerations bring up another biological consequence of *cis*-DDP binding, namely, DNA repair. Removal of platinum from DNA by cellular repair mechanisms has been demonstrated by several groups. For example, in studying *cis*-DDP-treated human fibroblast cells in culture, it was found that the amount of bound platinum per nucleotide decreased according to first-order kinetics, from $(D/N)_b$ of 2.3×10^{-5} to 3.3×10^{-6} over a six-day period. Since Pt-DNA adducts are stable with respect to dissociation from DNA under physiological conditions (see above discussion), loss of platinum was attributed to DNA repair.[98]

How does the cell remove platinum from DNA? One mechanism is by a process known as excision repair, whereby the sugar-phosphate backbone on the platinated strand is hydrolyzed (''nicked'') on either side of the damage and the remaining, unplatinated strand is used as a template for new DNA synthesis. The platinated oligonucleotide is displaced and the resulting gap filled in. In support of this picture is the fact that, in xeroderma pigmentosum (XP) human fibroblast cells, known to be deficient in excision repair, there is very little removal of platinum during post-treatment incubation.[99] Recent studies of *in vitro* repair of cisplatin-DNA adducts by a defined enzyme system, the ABC excision nuclease of *E. coli*, have provided some details at the molecular level about the process.[100,101] [^{32}P]-Labeled double-stranded DNA fragments containing $\{Pt(NH_3)_2\}^{2+}$ or $\{Pt(en)\}^{2+}$ adducts at random or defined sites were incubated with the enzyme. Cleavage of the platinated strand occurred at the 8th phosphodiester bond 5′, and the 4th phosphodiester bond 3′, to the GG or AG intrastrand crosslink. Further details about the identification and construction of such specific crosslinks will be given later in this chapter.

(iii). Drug resistance Another biological consequence of DNA-platinum interactions, probably related to the repair phenomenon, is resistance. Resistance of a cell to a chemotherapeutic agent, which can be inherent or acquired, is a phenotypical ability of the cell to tolerate doses of a drug that would be toxic to normal, or parent, cells.[102] Resistance is often acquired by prolonged exposure of cells in culture to the drug or, in patients, to repeated doses of drug therapy. There is not yet any direct proof that platinum-DNA interactions are responsible for acquired resistance to cisplatin. Studies of sensitive and resistant

tumors in rats have shown, however, that after intravenous injection of 10 mg/kg of the drug, the platinum levels were the same after an hour, but after 24 hours a larger proportion of adducts had been removed in the resistant cells.[103] Similar results have been found for studies of Pt-DNA adducts in cultured L1210 cells of varying levels of resistance to cisplatin where, in the 18 h following a 6-hour incubation with the drug, the resistant cells had up to fourfold more platinum removed than the sensitive cells.[104]

Experiments have also been carried out showing that *cis*-DDP binding to DNA inhibits transcription, the formation of RNA from a gene, and that this phenomenon is less efficiently reversed for parent versus resistant L1210 cells in culture.[105] The assay involves transfection (the process whereby free or viral DNA or RNA is taken into a cell) of pRSVcat plasmid DNA into L1210 cells. The plasmid contains the bacterial *cat* gene in a position that permits its expression in mammalian cells. The *cat* gene encodes the enzyme chloroamphenicol acyltransferase (CAT), an activity readily measured following lysis of the cells. Transfection of the *cis*-DDP-damaged plasmid into resistant L1210 cells showed that up to eight times the amount of platinum was required in the resistant versus sensitive cells to produce a mean lethal hit (63 percent reduction in activity). This result is consistent with greater repair of platinum-DNA adducts in the resistant cells.

These results should not be construed to mean that DNA repair is the only mechanism of cisplatin resistance. There is evidence that relative amounts of glutathione are increased in cisplatin-resistant cells.[106] Glutathione presumably uses its thiol moiety to coordinate platinum and diminish the amount that can bind to DNA. Reduced influx or increased efflux of a drug constitutes additional mechanisms by which cells become resistant. Further studies are required to ascertain which of these possibilities is most important for the cisplatin resistance phenomenon.

The discovery that cells can become resistant to cisplatin by repairing DNA lesions suggests a way to explain the selectivity of the drug for certain tumor tissue, and even the selective cytotoxicity of the drug for tumor versus normal cells of the same tissue. Tumor cells that cannot repair platinum-DNA adducts would be most affected by *cis*-DDP. This idea forms one of the central hypotheses about the molecular mechanism of action of *cis*-DDP, details of which can be probed by bioinorganic chemists. Specifically, it is important to inquire what DNA adducts formed by *cis*-DDP are both cytotoxic and repairable, what enzymes are responsible for such repair in mammalian cells, by what mechanisms these enzymes operate, and how this knowledge can be used to design better metal-based antitumor drugs and chemotherapeutic protocols.

(iv). DNA-protein interactions Most of the phenomena discussed in this section, inhibition of replication, DNA repair, drug resistance, and mutagenesis, probably involve interaction of a protein or group of proteins with platinated DNA. These interactions are clearly important in determining the biological consequences of DNA templates containing bound platinum. Very recent exper-

iments have uncovered the existence of proteins from a variety of mammalian sources that bind specifically to DNA platinated with *cis*- but not *trans*-DDP.[107] Identifying the nature and function of these factors may provide important clues about the mechanisms of antitumor activity, drug resistance, or repair. Study of protein-DNA-drug interactions is an essential feature of the bioinorganic chemistry of platinum chemotherapeutic agents.

4. Mapping the major adducts of *cis*- and *trans*-DDP on DNA; sequence specificity

As we have seen, the antitumor activity of cisplatin is most likely the result of its DNA-binding properties. But what are the adducts? The human genome has more than a billion nucleotides. Does platinum recognize any special regions of the DNA or any particular sequences? In other words, is binding simply random or is there at least a regioselectivity? In this section, we discuss the best strategies for answering these questions, strategies that evolved in pursuit of learning how *cis*-DDP binds to DNA. We also illustrate their power in elucidating the DNA-binding properties of other metal complexes of interest to bioinorganic chemists.

a. Early Strategic Approaches The first experiments to imply the sequence preferences of *cis*-DDP binding to DNA employed synthetic polymers.[108,109] Specifically, the buoyant density of poly(dG)·poly(dC), poly(dG·dC), and their *cis*-DDP adducts was studied in the analytical ultracentrifuge. The greatest shift in buoyant density was seen for the platinum adducts of poly(dG)·poly(dC), from which it was concluded that platinum forms an intrastrand crosslink between two neighboring guanosine nucleosides on the same strand. This interpretation was suggested by the known preference of metal ions, and especially platinum, for binding at the N7 position on the guanine base (Figure 9.9), information available from model studies of metal-nucleobase chemistry. Although other interpretations of the buoyant-density shift were possible, especially since the amount of platinum bound was not quantitated, the conclusion proved to be correct, as confirmed by later investigations. Interestingly, *trans*-DDP did not selectively increase the buoyant density of poly(dG)·poly(dC).

Following these initial experiments, the regioselectivity of *cis*-DDP binding was investigated by studying the inhibition of enzymatic digestion of platinated DNA. For example, the platinum complex inhibits the cleavage of DNA by restriction enzymes that recognize specific sequences and cut both strands of the double helix.[110] The resulting fragments are readily identified on electrophoresis gels. One such restriction enzyme is Bam HI. As shown by the arrows in Scheme (9.11), Bam HI cleaves a six-bp palindromic sequence at the phosphodiester bonds between two guanosine nucleosides. Formation of an intrastrand crosslink between the two adjacent guanosine nucleosides inhibits digestion by the enzyme. Another method, termed exonuclease mapping, involves digestion of the

strands of duplex DNA from its 3′-ends.[111,112] When the enzyme encounters a bound platinum atom, it is unable to proceed further. Analysis of the digestion products by gel electrophoresis reveals the presence of discrete bands caused by the inhibition of digestion by bound platinum at specific sequences. Results from experiments of this kind were the most definitive at this time in demonstrating the profound regioselectivity of cisplatin for adjacent guanosines, and strongly supported the earlier conclusion that the drug was making an intrastrand d(GpG) crosslink.

$$\begin{array}{l} 5' \quad \overset{\downarrow}{} \\ \quad -G\,G\,A\,T\,C\,C- \quad 3' \\ 3' \quad -C\,C\,T\,A\,G\,G- \\ \quad \qquad\qquad\quad \uparrow \quad 5' \end{array} \tag{9.11}$$

A third enzymatic strategy for exploring the regioselectivity of *cis*- and *trans*-DDP binding to DNA is outlined in Figure 9.18. Platinum is first bound to a single-stranded DNA template, in this example from bacteriophage M13mp18, to which is next annealed a short, complementary oligonucleotide termed a "primer" for DNA synthesis. Addition of the large (Klenow) fragment of *E. coli* DNA polymerase I and deoxynucleoside triphosphates, one of which bears a ^{32}P label, [α-^{32}P]dATP, initiates replication. When the enzyme encounters a platinum adduct, the chain is terminated. By running out the newly synthesized DNA strands on a sequencing gel, the sites of platinum binding can be detected by comparing the positions of the radiolabeled fragments with those obtained from sequencing ladders. The results of this procedure, which has been termed "replication mapping," confirmed that *cis*-DDP binds selectively to $(dG)_n$ ($n \geq 2$) sequences. In addition, they showed that *trans*-DDP blocks replication, in a much less regioselective manner, in the vicinity of sequences of the kind d(GpNpG), where N is an intervening nucleotide. These data afforded the first clear insight into the sequence preferences for *trans*-DDP on DNA. A control experiment run with DNA platinated by the monofunctional complex $[Pt(dien)Cl]^+$ gave the interesting result that DNA synthesis was virtually unaffected.

In yet another approach to the problem, DNA containing *cis*- or *trans*-DDP adducts was electrostatically coupled to bovine serum albumin, to enhance its

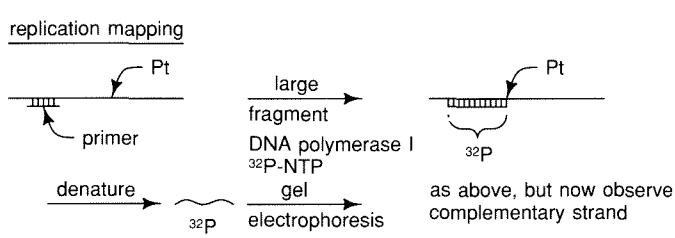


Figure 9.18
Diagram illustrating the replication mapping experiment. To a single-stranded, platinated template is annealed a short primer for DNA synthesis using DNA polymerase I (Klenow fragment) and radiolabeled nucleotides. Sites of platinum binding are revealed as bands on gel electrophoresis where chain termination occurs (see text for details).

antigenicity, and injected into rabbits.[113,114] The resulting antisera and antibodies were then studied for their ability to recognize and bind specifically to platinated DNAs having defined sequences, such as poly(dG)·poly(dC) and poly[d(GC)]·poly[d(GC)].

From experiments of this kind, the major *cis*-DDP adduct recognized by the antibody was found to be *cis*-[$Pt(NH_3)_2${d(GpG)}], in accord with the findings of the enzymatic mapping experiments. Unplatinated DNA was not recognized, nor was DNA platinated with *trans*-DDP. On the other hand, the antibody recognized DNA platinated with antitumor-active compounds [$Pt(en)Cl_2$] and [Pt(DACH)(CP)], where DACH = 1,2-diaminocyclohexane and CP = 4-carboxyphthalate. This result revealed that the antibody recognized the structural change in DNA that accompanies formation of d(GpG) intrastrand crosslinks, irrespective of the diamine ligand in the coordination sphere of the platinum atom. The antibody is also capable of distinguishing adducts formed by active versus inactive platinum complexes. Most importantly, DNA isolated from the cells of mice bearing the L1210 tumor five hours after cisplatin injection, was recognized.[113,115] Subsequent studies[116] revealed that these antibodies could detect cisplatin-DNA adducts formed in the white blood cells of patients receiving platinum chemotherapy. Thus, the antibody work linked the regiospecificity of platination chemistry *in vitro* with that occurring *in vivo* and in a clinically relevant manner.

Additional studies with monoclonal antibodies generated using DNA platinated with *cis*- or *trans*-DDP further confirmed and extended these results.[117] This later work indicated that intrastrand crosslinked d(ApG) and d(GpG) sequences possess a common structural determinant produced by *cis*-DDP platination, and that carboplatin is also capable of inducing the same DNA structure. For trans-DDP-platinated DNA, a monoclonal antibody was obtained that appeared to have the intrastrand d(GpTpG) adduct as its major recognition site. In all these studies, the primary structural determinant appears to be DNA duplex opposite the site of platination, since fairly major stereochemical changes could be made in the amine ligands with no appreciable effect on antibody binding.

b. Degradation, Chromatographic Separation, and Quantitation of DNA Adducts Experiments in which DNA platinated with *cis*-DDP is degraded to chromatographically separable, well-defined adducts have been invaluable in revealing the spectrum of products formed. In a typical experiment, platinated DNA is digested with DNAse I, nuclease P1, and alkaline phosphatase. These enzymatic digestions degrade DNA into nucleosides that can be readily separated by high-performance liquid chromatography (HPLC). Detection of the adducts can be accomplished by the UV absorption of the nucleoside bases at 260 nm or, for platinum complexes containing a radioactively labeled ligand such as [^{14}C]ethylenediamine,[118] by monitoring counts. In addition to peaks corresponding to dA, dC, dG, and dT, the chromatographic trace contains additional peaks corresponding to specific platinum nucleobase adducts such as *cis*-[$Pt(NH_3)_2(dG)_2$]. The precise nature of these adducts was established by

comparison with chemically synthesized compounds structurally characterized by NMR spectroscopy.[118–121] An alternative method for identifying the adducts employed antibodies raised against specific platinum-nucleobase complexes.[122]

This approach has revealed the relative amounts of various adducts formed by a variety of platinum complexes; selected results are summarized in Table 9.4. Usually, for cisplatin, the relative amounts of the various adducts formed varies according to the series *cis*-[$Pt(NH_3)_2${d(pGpG)}]>*cis*-[$Pt(NH_3)_2${d(pApG)}] > *cis*-[$Pt(NH_3)_2${d(GMP)}$_2$] > monofunctional adducts. Only when the total incubation time was short, less than an hour, were the monofunctional adducts more prevalent, as expected from the kinetic studies of *cis*-DDP binding to DNA discussed previously. It is noteworthy that no d(pGpA) adducts were detected. This result, which is consistent with information obtained by enzymatic mapping, can be understood on stereochemical grounds.[123] If the guanosine nucleoside N7 position is the most-preferred binding site on DNA, closure to make an N7,N7 intrastrand crosslink between two adjacent purine nucleotides is more feasible in the 5′ direction along the helix backbone (N7···N7 distance of ≈ 3 Å) than in the 3′ direction (N7···N7 distance ≈ 5 Å). In addition, molecular-me-

Table 9.4
Geometric features of the platinum coordination spheres of *cis*-[$Pt(NH_3)_2${d(pGpG)}].

Bond distances and angles[a]	Molecule 1	Molecule 2	Molecule 3	Molecule 4
Pt-N1	2.03(2)	2.01(2)	2.08(2)	2.08(2)
Pt-N2	2.03(3)	2.09(2)	2.04(3)	2.06(3)
Pt-N7A	2.01(2)	2.02(2)	1.91(3)	1.93(3)
Pt-N7B	2.05(2)	1.95(3)	2.00(3)	2.06(3)
N7A-Pt-N1	88.6(9)	90.3(9)	91.0(1)	88.4(9)
N7A-Pt-N2	179(1)	173.3(8)	178(1)	177(1)
N7A-Pt-N7B	89.1(9)	90.0(1)	85(1)	89(1)
N1-Pt-N2	92(9)	90.8(9)	91(1)	93(1)
qN1-Pt-N7B	176.5(9)	179.0(1)	173(1)	175(1)
N2-Pt-N7B	90.3(9)	89.0(1)	93(1)	89(1)

Dihedral angles[b] Molecule	3′-Gua/5′-Gua	5′-Gua/PtN_4	3′-Gua/PtN_4
1	76.2(5)	110.6(5) [3.30(3)]	86.1(5)
2	81.0(5)	110.8(5) [3.49(3)]	95.5(5)
3	86.8(6)	81.0(6)	58.0(6) [3.11(4)]
4	80.6(5)	76.6(6)	59.6(6) [3.18(4)]

[a] Bond distances are in Ångstroms and angles are in degrees.

[b] Conventions used for assigning Base/Base and Base/PtN_4 dihedral angles can be found in J. D. Orbell, L. G. Marzilli, and T. J. Kistenmacher, *J. Am. Chem. Soc.* **103** (1981), 5126. The numbers in square brackets refer to the corresponding N(ammine)···O6 distance, in Å (see text).

chanics modeling studies[124] indicate that a highly unfavorable steric clash occurs between the 6-amino group of the 3′-adenosine residue in a d(pGpA) crosslink and the platinum ammine ligand, whereas in the platinated d(pApG) sequence, the 6-oxo group forms a stabilizing hydrogen bond to this ligand. A 28 kJ mol^{-1} preference of *cis*-DDP for binding d(pApG) over d(pGpA) was calculated.

There are two likely sources of *cis*-$[Pt(NH_3)_2\{d(GMP)\}_2]$ in the spectrum of adducts. This species could arise from long-range intrastrand crosslinks, where the two coordinated guanosines are separated by one or more nucleotides. In support of this possibility is the fact that digestion of chemically synthesized *cis*-$[Pt(NH_3)_2\{d(GpNpG)\}]$, where N = C or A, led to *cis*-$[Pt(NH_3)_2\{d(Gua)\}$-$\{d(GMP)\}]$ and mononucleotides.[118,119,121] The other source of this product is interstrand crosslinked DNA, known to occur from the alkaline elution studies.

As indicated in Table 9.4, in all the experiments there was platinum that was unaccounted for in the quantitation procedures, which employed either antibodies, platinum atomic absorption spectroscopy, or a radiolabeled ethylenediamine ligand. Some of this material was assigned to oligonucleotides having high platinum content, resistant to enzymatic degradation.

Two important points emerge from the quantitation of adducts by this method. One is that intrastrand d(GpG) and d(ApG) crosslinks constitute the major adducts (>90 percent of total platination) made by cisplatin on DNA *in vivo*. Because they were identified by an antibody specific for their structures, no chemical change brought about by cellular metabolism has occurred. Secondly, the preponderance of these adducts far exceeds the frequency of adjacent guanosine or guanosine/adenosine nucleosides in DNA. This latter result implies a kinetic preference for, or recognition of, d(pGpG)- and d(pApG)-containing sequences by cisplatin.

c. Postscript: A Comment on Methodologies With few exceptions, none of the experimental studies described in this section could have been carried out in 1969, when Rosenberg first demonstrated the anticancer activity of *cis*-DDP. The techniques of DNA sequencing, monoclonal antibody formation, oligonucleotide synthesis, HPLC, FPLC, and many of the higher resolution gel electrophoresis methodologies employed were the result of later developments driven by rapid advances in the fields of molecular biology and immunology. Future progress in elucidating the molecular mechanisms of action of cisplatin and other inorganic pharmaceuticals will no doubt benefit from new technological discoveries and inventions of this kind yet to come.

5. Structure of platinum-DNA complexes

a. NMR Studies of Platinated Oligonucleotides Once the major spectrum of adducts formed by *cis*- and *trans*-DDP with DNA began to emerge, it was of immediate interest to learn to what positions on the nucleobases the platinum atom was coordinated. Proton NMR spectroscopy soon proved to be an invalu-

able tool for obtaining this information.[71,125,126] Several ribo- and deoxyribooligonucleotides containing GG, AG, or GNG sequences were synthesized, and allowed to react with *cis*-DDP or its hydrolysis products, and the resulting complexes were purified by chromatography. All GG-containing oligomers formed intrastrand crosslinks with the $\{Pt(NH_3)_2\}^{2+}$ moiety coordinated to the N7 atoms. This structure was deduced from several criteria. Most frequently studied were the nonexchangeable base protons H8 and H2 of adenine, H6 of thymine, H8 of guanine, and H5 and H6 of cytosine (Figure 9.9). Coordination of platinum to N7 of guanine causes a downfield shift of the H8 proton resonance. More importantly, however, it also lowers the pK_a of the N1 proton by ~ 2 units, because platination adds positive charge to the base. Thus, titration of the platinated oligonucleotide over a pH range, and comparison of the results to those obtained for the unplatinated oligomer, reveals a difference in the midpoint of the transition in chemical shift of the H8 proton by ~ 2 pH units if coordination occurs at N7. This effect is illustrated in Figure 9.19 for the adduct *cis*-$[Pt(NH_3)_2\{d(ApGpGpCpCpT)\}N7\text{-}G(2),N7\text{-}G(3)]$, where the pK_a of N1 is seen to shift from ~ 10 to ~ 8 upon platination.[71] The pH titration in this example also reveals the pH-dependent chemical shift of the cytosine ^1H resonances at a pH of ~ 4.5, corresponding to protonation of the N3 atoms. The protonation of adenine N7 ($pK_a \sim 4$) is also frequently observed in these studies. These results conclusively demonstrate platinum coordination at N7 of the two guanosine nucleosides.

Although several of the oligonucleotides studied have self-complementary sequences, such that they can form a double helix when unplatinated, in no such case was a duplex observed for their platinated forms. The presence of the platinum-induced crosslink presumably decreases the stability of the double-stranded form of the oligonucleotide. Another interesting result is that all intrastrand $\{Pt(NH_3)_2\}^{2+}$ adducts of d(GpG) or d(ApG) have an altered deoxyribose-sugar ring conformation. In normal, unplatinated form, these single-stranded or duplex oligonucleotides have a C2′-endo sugar pucker (Figure 9.9). Upon platination, the 5′-nucleotide switches to C3′-endo. This change is readily monitored by the ring proton-coupling constants $J_{H1'\text{-}H2'}$ and $J_{H1'\text{-}H2''}$. These protons constitute an ABX spin system such that the sum, $\Sigma^3J = {}^3J_{1'2'} + {}^3J_{1'2''}$, is most easily measured as the separation between the outermost peaks in the multiplet. For the C2'-endo conformation, a pseudotriplet occurs with $\Sigma^3J = 13.6$ Hz, and for C3′-endo, $\Sigma^3J = 7.5$ Hz. The 3′-guanosines in the adducts show greater conformational flexibility, having ~ 70 to 80 percent C2′-endo sugar puckers, depending upon the temperature.

Another conformational feature that could be deduced from ^1H NMR studies of all *cis*-DDP-platinated oligonucleotides containing an embedded d(GpG) sequence is that both guanosine nucleosides retain the anti orientation of the base around the C1′-N9 glycosidic linkage (Figure 9.9). This result was deduced from the lack of a pronounced nuclear Overhauser effect (NOE) between H8 and the H1′ protons, such as would occur in the syn conformation. An NOE between H8 resonances on the two coordinated nucleosides was observed for

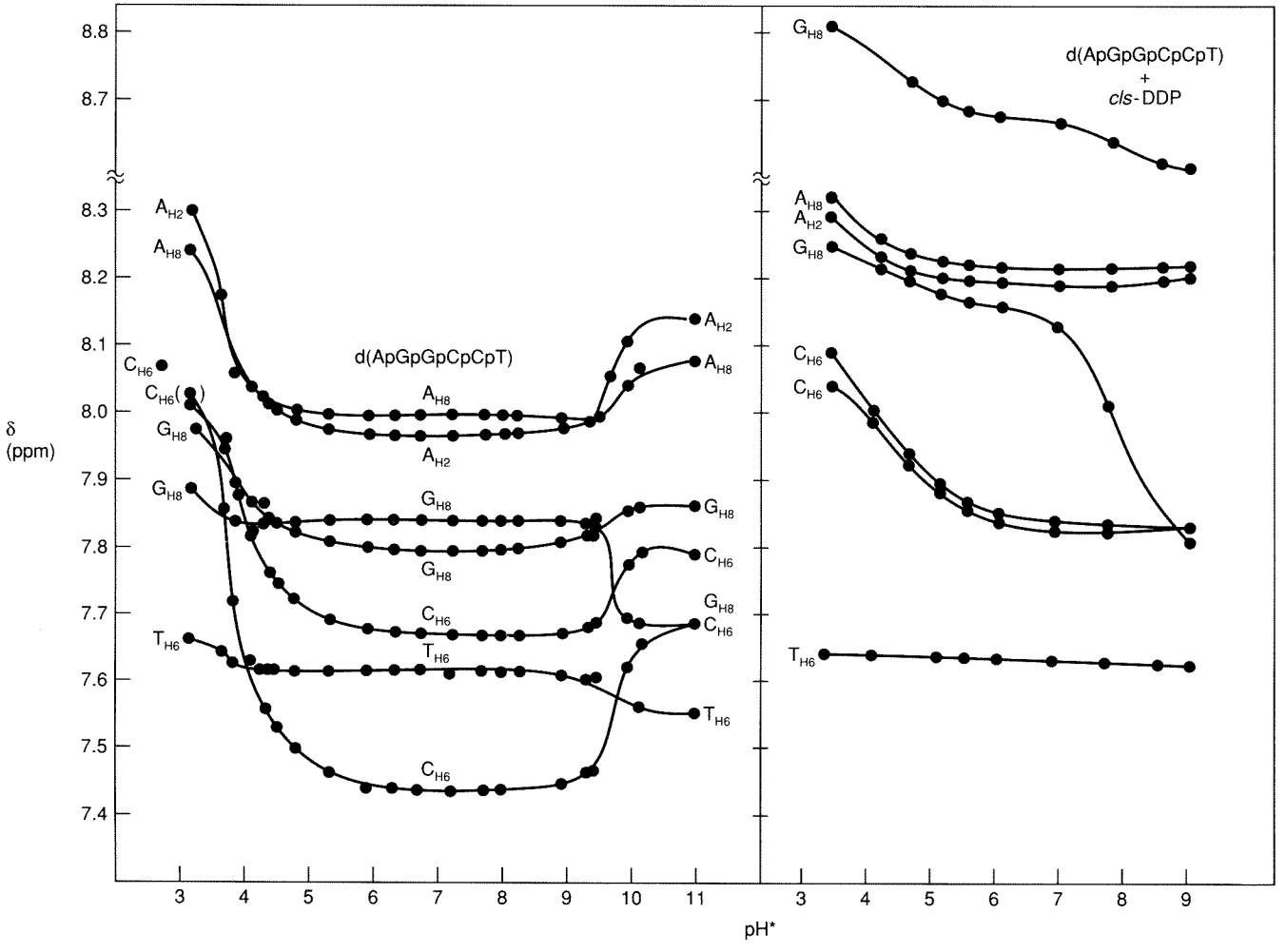


Figure 9.19
Chemical shift (δ) vs. pH* of the nonexchangeable base protons of D_2O solutions of $[d(ApGpGpCpCpT)]_2$ (3.5 mM, 35°C) and its *cis*-DDP adduct (2.5 mM, 70°C). The pyrimidine resonances of the latter sample show no chemical shift changes with temperature over the range $35 < T < 70$°C while the purine resonances show a slight temperature-dependent chemical shift change of up to 0.1 ppm. Tetramethylammonium chloride was used as the internal standard (δ 3.180). Reproduced by permission from J. C. Caradonna, S. J. Lippard, M. J. Gait, and M. Singh, *J. Am. Chem. Soc.* **104** (1982), 5793.

adducts of d(ApTpGpG) and d(CpGpG), indicating that the two bases are in a head-to-head orientation with respect to the platinum coordination plane. In other words, both O6 atoms lie on the same side of that plane. Two oligonucleotides containing *cis*-[$Pt(NH_3)_2$(ApG)] adducts have been examined; their structural properties closely resemble those of the (GpG) adducts, with platinum coordinated to N7 of both purine bases.

In order to study double-stranded DNAs platinated on one strand, it was necessary to adopt a special strategy. First, the desired oligonucleotide is synthesized. It is preferable that the DNA strands not be self-complementary, since the affinity of such an oligomer for itself is so much greater than that for its platinated form that the desired, singly platinated duplex will not form. After the platinated single strand is synthesized and purified, the complementary strand is added. Several duplex oligonucleotide-containing *cis*-[$Pt(NH_3)_2${d(pGpG)}]-embedded adducts prepared in this manner have been studied by ^{1}H NMR spectroscopy. With the use of two-dimensional and temperature-dependent techniques, both the nonexchangeable base and sugar protons as well as the exchangeable (guanine N1 and thymine N3) N-H (imino) proton resonances were examined. The last are useful, since they give some measure of the extent to which the double helix remains intact. When not base-paired to their complements in the other strand, these protons exchange more rapidly with solvent (water) protons, leading at moderate exchange rates to broadening of the resonances and, at high exchange rates ($>10^7$ s^{-1}), disappearance of the signals. Several interesting results were obtained in these studies. In all of them, platination of the d(GpG) sequence brought about the same C2′-endo → C3′-endo sugar-ring pucker switch for the 5′-guanosine as seen in the single-stranded adducts. Head-to-head, anti conformations were also observed. At low temperatures, below the melting transition temperature, above which the duplex becomes single-stranded, the imino proton resonances were observed. This result was interpreted to mean that normal, Watson-Crick base pairs can still exist between the *cis*-DDP d(GpG) adduct and the d(CpC) sequence on the complementary strand. In the case of [d(TpCpTpCpG*pG*pTpCpTpC)]·[d(GpApGpApCpCpGpApGpA)], where the asterisks refer to the sites of platination, the imino proton resonances were assigned with the assistance of NOE experiments.[125] Temperature-dependent studies showed that, in the range $-4° < T < 46°C$, the imino resonances of the coordinated guanosine nucleosides broadened first with increasing temperature. Apparently the base pairs of the intrastrand crosslinked, platinated duplex DNA are disrupted, or "melted," outward from the point of platination as well as from the ends. Since the amino hydrogen atoms involved in base pairing were not observed in this study, a completely definitive structural analysis was not possible. Nevertheless, the authors proposed that the duplex would be kinked by an angle of $\sim 60°$ at the *cis*-DDP binding site in order to preserve full duplex character.

Another useful NMR nucleus for monitoring cis-DDP-DNA interactions is ^{195}Pt, which is 34 percent abundant with $I = \frac{1}{2}$. When used in conjunction with ^{15}N ($I = \frac{1}{2}$) enriched NH_3 ligands, ^{195}Pt NMR resonances provide a powerful

means for characterizing complexes in solution. The ^{195}Pt and ^{15}N chemical shifts are both sensitive to the ligand trans to the NH_3 group, as is the ^{195}Pt-^{15}N coupling constant.[127] ^{195}Pt NMR studies of *cis*-DDP binding have been carried out using nucleobases, small oligonucleotides, and even double-stranded fragments of 20 to 40 bp in length, as previously described (Section V.D.1). The major contribution of this method is to show whether platinum coordinates to a nitrogen or an oxygen donor atom on the DNA, since the ^{195}Pt chemical shift is sensitive to this difference in ligation.

b. X-ray Structural Studies In recent years several oligonucleotide duplexes have been crystallized and characterized by x-ray diffraction methods. The probability of forming suitable single crystals of DNA fragments is disappointingly low, however, with only 1 in 10 such attempts being successful. Correspondingly, it has been difficult to crystallize platinated oligonucleotides. An alternative approach has been to soak nucleic-acid crystals of known structure with the platinum reagent in the hope of forming an isomorphous derivative, the structure of which could be obtained by using the changes in phases from the native material. In attempts to characterize a *cis*-DDP nucleic acid adduct, crystals of $tRNA^{Phe}$ and the self-complementary dodecamer d(CpGpCpGpApApTpTpCpGpCpG) were soaked with cisplatin solutions in the hope of obtaining useful metric information.[123,128,129] These efforts have thus far failed to produce a high-resolution structure, although they confirm the predilection for platinum to coordinate to the N7 position of purine rings. Addition of *cis*-DDP tends to disorder the crystal, with platinum going to several sites of partial occupancy.

A more fruitful approach has been to crystallize a purified oligonucleotide containing the coordinated *cis*-$\{Pt(NH_3)_2\}^{2+}$ moiety. The first x-ray structure to be deciphered through such a strategy was that of *cis*-$[Pt(NH_3)_2\{d(pGpG)\}]$.[130] This compound crystallizes with water solvent and glycine buffer molecules in the lattice. The crystals were grown at pH 3.8, where the terminal phosphate is monoprotonated in order to provide a neutral complex of diminished solubility. Two crystalline forms have been obtained, and both structures solved, one to 0.94 Å resolution. The latter contains four crystallographically independent molecules, which, although complicating the structure solution, afforded four independent views of the major adduct formed by *cis*-DDP with DNA. The four molecules form an aggregate, held together by hydrogen bonding and intermolecular base-base stacking interactions (Figure 9.20). There are two conformationally distinct classes that comprise molecules 1 and 2, and molecules 3 and 4; within each class, the molecules are related by an approximate C_2 symmetry axis.

The molecular structure of molecule 1 is displayed in Figure 9.21; geometric information about all four molecules is contained in Table 9.4. As expected from the NMR studies, platinum coordinates to N7 atoms of the guanine bases, which are completely destacked (dihedral angles range from 76.2 to 86.7°), to form a square-planar geometry. The bases have a head-to-head configuration

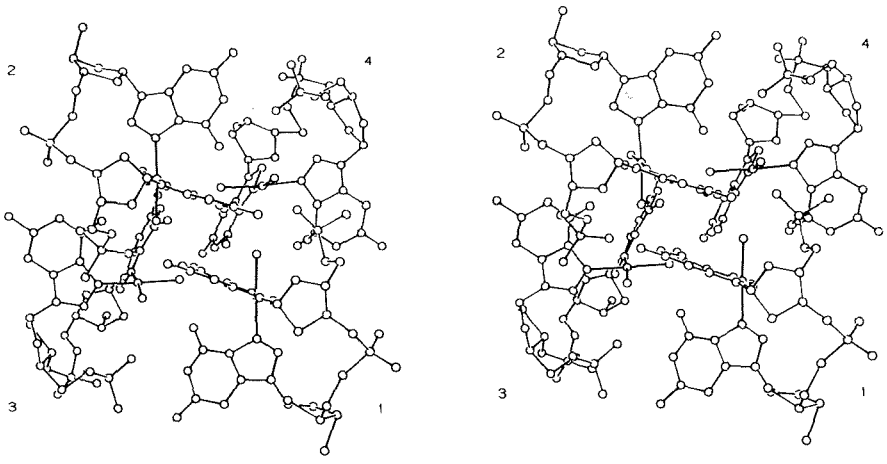


Figure 9.20
Stereoview of aggregate of four *cis*-[$Pt(NH_3)_2${d(pGpG)}] molecules (reproduced by permission from Reference 130).

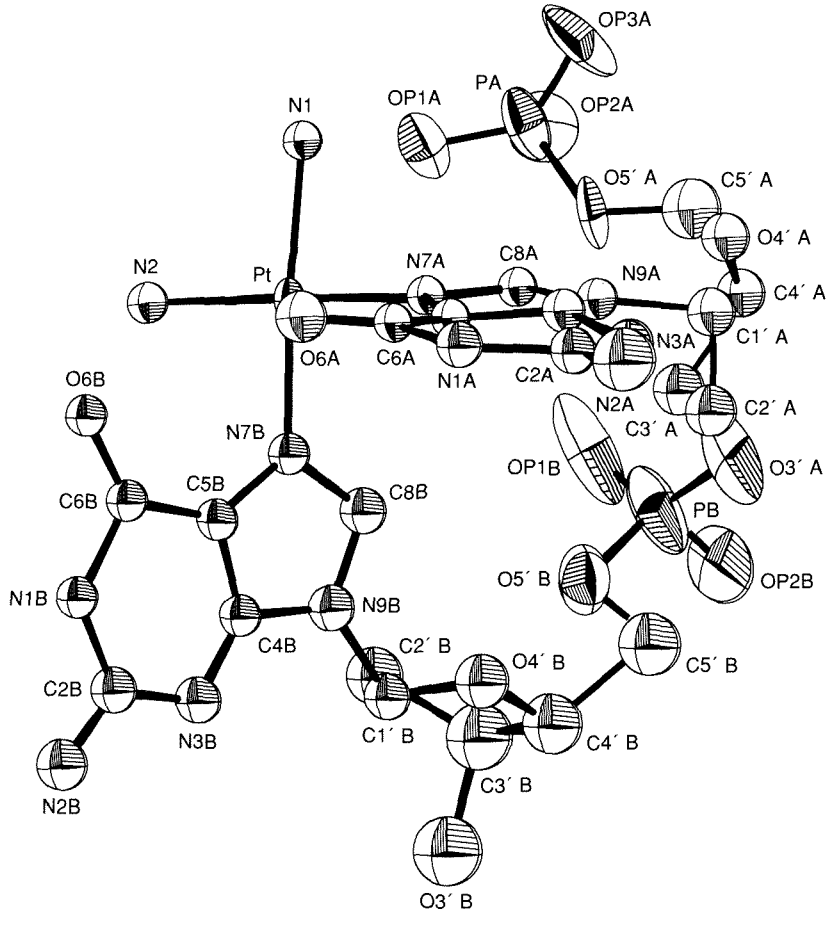


Figure 9.21
Molecular structure of *cis*-[$Pt(NH_3)_2${d(pGpG)}].

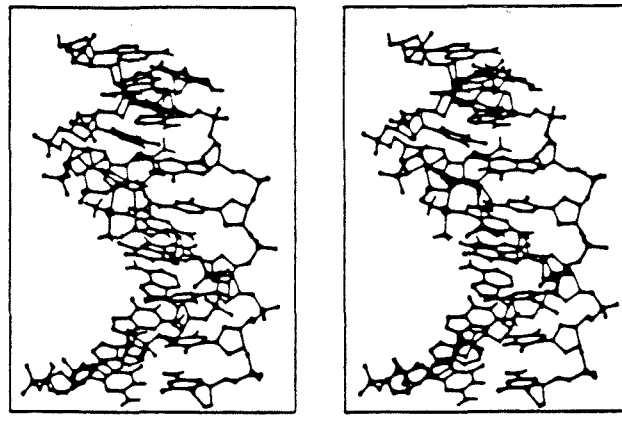
and conformational angles χ (Table 9.4 and Figure 9.9) that fall in the anti range. The sugar puckers of the 5′-deoxyribose rings for all four molecules have a C3′-endo conformation, and some of the 3′-sugar carbon atoms exhibit large thermal parameters suggestive of a less well-ordered structure. These results further demonstrate the similarity of the structure as detected in the solid state by x-ray diffraction and in the solution state by NMR spectroscopy.

An interesting additional feature of the *cis*-[$Pt(NH_3)_2${d(pGpG)}] crystal structure is a hydrogen bonding interaction between an ammine ligand and the oxygen atom of the terminal phosphate group (OP1A$\cdots$N1, Figure 9.21). This intramolecular hydrogen bond is prominent in three of the four molecules in the asymmetric unit. Although the relevance of this hydrogen bonding interaction to the solution structure and molecular mechanism of cisplatin is presently unknown, it is interesting to note that the antitumor activity of platinum amine halide complexes is reduced when protons on coordinated NH_3 are replaced by alkyl groups.[34]

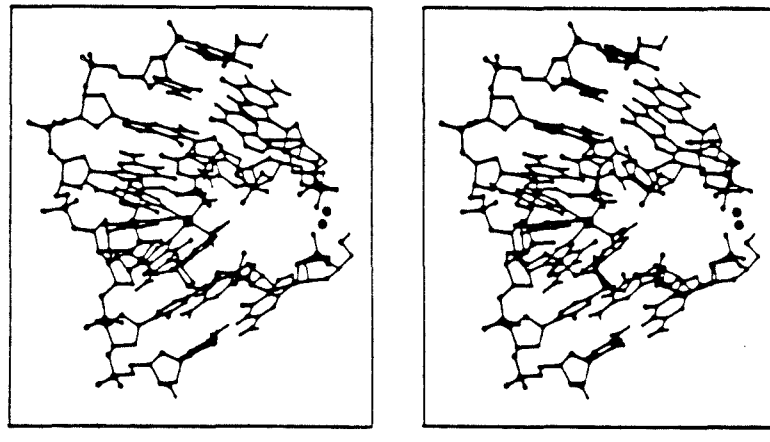
A second *cis*-DDP-oligonucleotide adduct characterized by x-ray crystallography is the neutral molecule *cis*-[$Pt(NH_3)_2${d(CpGpG)}].[131] Here again, there are several (three) molecules in the asymmetric unit. Although determined at lower resolution, the structure is similar in most respects to that of *cis*-[$Pt(NH_3)_2${d(pGpG)}] except for the presence of some weak $NH_3\cdots$O6(guanosine) intramolecular hydrogen bonding interactions and a few unusual sugar-phosphate backbone torsional angles. Also, no NH_3(H)$\cdots$phosphate(O) hydrogen bonds were observed.

From the foregoing discussion, it is apparent that adequate x-ray structure information is available for the *cis*-$\{Pt(NH_3)_2\}^{2+}$/d(pGpG) intrastrand crosslink. What is needed now are structures of the minor adducts and, most importantly, of adducts in double-stranded DNA. Very recently, dodecanucleotide duplexes containing *cis*-$\{Pt(NH_3)_2\}^{2+}$/d(pGpG) adducts have been crystallized, the structures of which are currently being investigated.[132]

c. Molecular Mechanics Calculations on Platinated Duplexes As a supplement to x-ray structural information on double-stranded oligonucleotides containing an embedded *cis*-[$Pt(NH_3)_2${d(pGpG)}] adduct, several models have been constructed by using a molecular mechanics approach.[133] In this work, a set of coordinates was first obtained by amalgamation of structural information about standard double-helical DNAs and the platinated d(pGpG) fragment. Various starting structures were assumed, both linear and bent. The models were then refined according to various charge and stereochemical constraints built into the calculation. The results, which can reveal only what is feasible and not necessarily what actually happens, for both linear and bent structures are depicted in Figure 9.22 for two of the duplex sequences studied. In the linear model, the 5′-coordinated guanosine is rotated out of the stack, and its hydrogen bonding to the cytosine on the complementary strand is seriously disrupted. The imino N-H group is still involved in H-bonding, however; so this structure is not inconsistent with the NMR results. Two classes of kinked platinated duplex structures were encountered, with bending angles of 61 and 50°. In one of these, all



(A)



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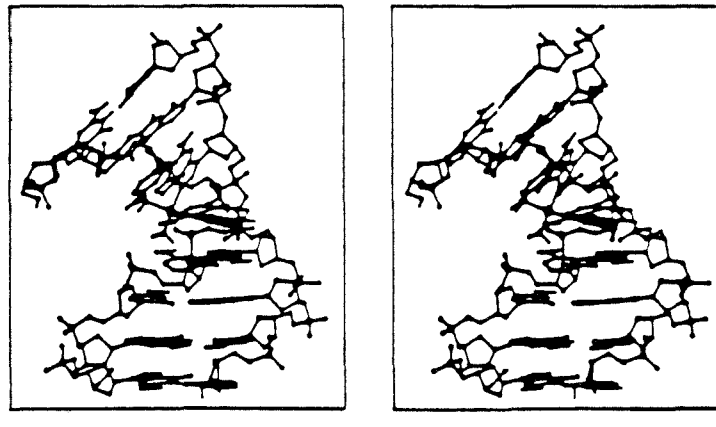

(C)

Watson-Crick hydrogen bonds remain intact. These kinked structures are supported most strongly by the gel-electrophoresis experiments discussed in Section V.D.3.b.v.

Molecular mechanics and the related molecular dynamics calculations are a potentially valuable tool for the bioinorganic chemist interested in how metal complexes might perturb the structures of biopolymers. Analysis of the results for cisplatin-DNA binding reveals that, compared with the sum of all contributions from the biopolymer, the Pt-DNA interactions constitute a small part of the overall energy. For the most accurate results, it is important to know the charge distributions on the metal and its ligands as well as the effects of solvent interactions. Much work needs to be done in these areas before the results of molecular mechanics and dynamics calculations can be used reliably to predict or analyze structures. At present, however, they are far superior to examination of space-filling molecular models, for example, and produce quantitatively revealing structural diagrams.

d. Platinum-Nucleobase Model Complexes Several studies have been carried out of the *cis*-diammineplatinum(II) moiety coordinated to nucleobases in which the N9 (purine) or N3 (pyrimidine) positions either have been alkylated, to simulate the glycosidic linkages, or in which the actual nucleotide (AMP, dGMP, etc.) is employed.[134] These investigations are in many respects analogous to the synthesis and characterization by bioinorganic chemists of model complexes for the active site of a metalloenzyme. Their purpose is to simplify the problem, revealing kinetic, thermodynamic, and structural preferences of the primary building blocks involved in the metallodrug-biopolymer interaction, without the profound stereochemical constraints of the latter. Early studies of *cis*- and *trans*-DDP adducts with nucleobases (i) revealed the kinetic preferences for platinum binding to GMP and AMP, (ii) mapped out the preferred sites of platination (N7 of A and G; N1 of A; N3 of C; no N7-O6 chelate; no ribose or deoxyribose binding; only rare binding to phosphate oxygen atoms), (iii) demonstrated that Pt-N7 binding to G lowered the pK_a of N1-H by ~ 2 units, and (iv) led to the discovery of interesting new classes of coordination complexes such as the *cis*-diammineplatinum pyrimidine blues and metal-metal bonded diplatinum(III) complexes.

Figure 9.22 *(facing page)*
(A) Stereoscopic view of the unkinked, platinated model of duplex d(TpCpTpCpG*pG*pTpCpTpC) from molecular-mechanics calculations. Counter ions used to stabilize the negative charge of the phosphates are not shown. (B) Stereoscopic view of the "high-salt" kinked, platinated model of duplex d(GpGpCpCpG*pG*pCpC) from molecular-mechanics calculations. Counter-ions are not depicted, with the exception of the bridging ion. (C) Stereoscopic view of the "low-salt" kinked, platinated model of duplex d(GpGpCpCpG*pG*pCpC). Counter-ions are not depicted. Reproduced with permission from Reference 133.

Attempts to model the intrastrand d(GpG) crosslink with nucleobases have met with only moderate success. Usually the O6 atoms of the two guanosine rings are on opposite sides of the platinum coordination plane (''head-to-tail'' isomer). Only for *cis*-$[Pt(NH_3)_2(9\text{-}EtG)_2]^{2+}$ was the correct isomer obtained. Nucleobase complexes of the *cis*-diammineplatinum(II) moiety have been valuable for testing the controversial proposal of N7,O6 chelate formation, which to date has not been observed. Several interesting discoveries of metal-nucleobase chemistry are that metal binding can stabilize rare tautomers, for example, the 4-imino, 2-oxo form of cytosine, through N4 binding, that coordination of platinum often produces unusual base pairing, and that metal migration from one donor site to another on an isolated nucleobase can occur. These model studies will continue to provide valuable insights into the possible chemistry of platinum antitumor drugs with DNA.

e. trans-DDP-DNA Adducts Because *trans*-DDP is biologically inactive, it has received less attention than the *cis* isomer. Nevertheless, knowledge of its binding to DNA is important to have as a reference point for mechanistic comparison with the active compounds. Shortly after replication mapping experiments established that *trans*-DDP binds preferentially to d(GpNpG) and d(ApNpG) sequences,[135] several synthetic oligonucleotides containing such sequences were prepared and used to investigate reactions with the *trans* isomer.[136–138] Kinetic studies of *trans*-DDP with d(GpCpG) and d(ApGpGpCpCpT) revealed the presence of, presumably monofunctional, intermediates that closed to form both intra- and interstrand products. In the reaction with d(GpCpG), the 1,3-intrastrand G-G chelate accounted for 70 percent of the product, and 21 percent of the remaining material was unreacted oligonucleotide. Proton NMR studies of purified *trans*-$[Pt(NH_3)_2\{d(GpCpG)\}]$ as well as the d(GpTpG) analog established platinum binding to N7 positions of the two *trans* guanosine nucleosides. As with the *cis*-$[Pt(NH_3)_2\{d(pGpG)\}]$ adducts, the 5′-guanosine residue no longer retained the normal B-DNA type conformation; instead, the sugar ring pucker switched to C3′-endo. A fairly detailed ^1H NMR characterization of *trans*-$[Pt(NH_3)_2\{d(ApGpGpCpCpT)\text{-}N7\text{-}A(1),N7\text{-}G(3)\}]$ revealed very similar features. This example nicely illustrates the different stereoselectivity of *cis*- and *trans*-DDP binding to DNA. The *cis* isomer forms exclusively an intrastrand d(GpG) crosslink, whereas the *trans* isomer makes a 1,3-d(A*pGpG*) adduct. A schematic depiction of the *trans*-$\{Pt(NH_3)_2\}^{2+}$ adduct is shown in Figure 9.23. As can be seen, the two purine rings enclose a large, 23-membered ring, the central guanosine residue is ''bulged out,'' and the 5′-residue has a C3′-endo sugar pucker. This structure may be compared with that of *cis*-$[Pt(NH_3)_2\{d(pGpG)\}]$ (Figure 9.21), where the platinum is part of a smaller, 17-membered ring. Both space-filling model building studies and molecular mechanics calculations reveal that it would be stereochemically very unfavorable for the *trans*-$\{Pt(NH_3)_2\}^{2+}$ fragment to replace the *cis* analogue in an intrastrand crosslinked d(GpG) structure of the kind shown in Figure 9.21. Thus, for bidentate adducts, it seems clear that the important difference between *cis*- and *trans*-DDP binding to sin-

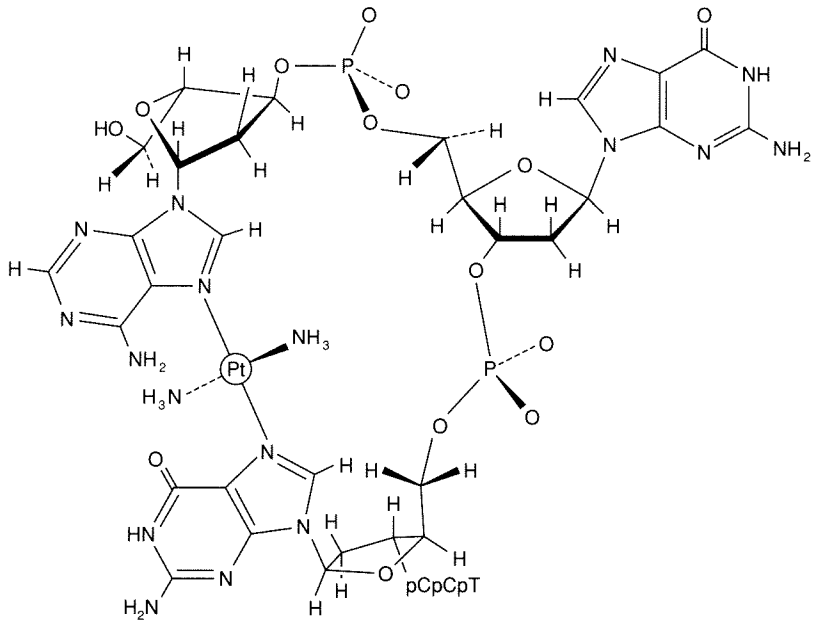

Figure 9.23
Structure of the intrastrand 1,3-d(A*pGpG*) crosslink formed in the reaction of *trans*-DDP with d(ApGpGpCpCpT). Reproduced by permission from Reference 136.

gle-stranded DNA is revealed by the structures shown in Figures 9.21 and 9.23, respectively.

Information about *trans*-DDP binding to double-stranded DNA is scanty, but very recent studies indicate that the *trans*-[$Pt(NH_3)_2${d(GpApG)-N7-G(1),N7-G(3)}] intrastrand crosslinked fragment can be embedded in duplex dodecamers.[139] Interestingly, for one sequence the melting temperature (T_M) of this duplex is not reduced over that of the unplatinated DNA fragment, in contrast to results for *cis*-DDP intrastrand d(GpG) adducts. This intriguing result, which agrees with earlier T_M studies of DNA platinated by *trans*-DDP, does not yet have a structural rationale. It is possibly relevant to the processing of bifunctional *trans*-DDP-DNA adducts *in vivo*.

f. Effects of Platination on DNA Structure It is valuable to summarize at this stage all that has been learned concerning the changes in DNA structure that occur upon *cis*- or *trans*-DDP binding. *cis*-DDP intrastrand crosslinks result in unstacking of neighboring bases and a switch in the sugar pucker of the 5′-nucleoside from C2′-endo, the standard B-DNA conformation, to C3′-endo, a conformation encountered in A-DNA. These various forms of DNA have already been introduced in the previous chapter. Watson-Crick base pairing, although weakened, is probably maintained. Evidence that base pairing is altered comes from studies with antinucleoside antibodies that bind appreciably better to DNA platinated with *cis*-DDP than to unmodified DNA. These antibodies

recognize the nucleobases much better in platinated than in unplatinated DNA, presumably because platination disrupts the double helix. Additional support for base-pair disruption comes from gradient gel-denaturation experiments using site-specifically platinated DNA (see Section V.D.8.b). Intrastrand crosslinking by *cis*-DDP also bends the helix by about 34° and unwinds the duplex by 13°. When *trans*-DDP forms 1,3-intrastrand crosslinks, the nucleotides situated between the platinated residues may be bulged out; consistent with this picture is the fact that they present an especially good target for antinucleoside antibodies. In 1,3-intrastrand d(GpNpG) or d(ApNpG) adducts, the 5′-nucleoside sugar pucker is altered to C3′-endo. Intrastrand crosslink formation by *trans*-DDP also leads to DNA bending, but the platinum serves as the locus for a hinge joint and not for cooperative bending. These different effects of platination on DNA structure brought about by the two isomers are likely to be related to their different biological activities.

6. Effects of DNA structure on platinum binding

a. A-, B-, and Z-DNA[140] As discussed in more detail in Chapter 8, double-helical DNA can adopt different polymorphic forms depending on the conditions in solution or polycrystalline fiber. Even within a given DNA molecule, there can be sequence-dependent local secondary and tertiary structural differences that constitute important signals for cellular DNA binding and processing molecules. An example already discussed is the recognition of palindromic sequences by type II restriction endonucleases. As shown in Figure 9.24, three such DNA polymorphs are the right-handed A- and B- and the left-handed Z-forms. Most commonly encountered in solution is B-DNA, characterized by well-classified major and minor grooves designated by arrows in Figure 9.24. The targets of platinum binding, guanine N7 atoms, are situated in the major groove.

To what extent do sequence-dependent local structural modulations affect platinum binding? Although no general answer to this question can be given, there are several interesting anecdotal pieces of information worth mentioning. Z-DNA, a form favored by alternating purine-pyrimidine sequences such as in poly d(GC), does not constitute a particularly good target for *cis*-DDP binding. For one thing, it lacks the preferred d(GpG) or d(ApG) sequences. The monofunctional $[Pt(dien)Cl]^+$ complex, however, facilitates the B-DNA → Z-DNA conformational transition, as demonstrated by circular dichroism and ^{31}P NMR spectroscopic data.[141] In Z-DNA, the guanosine nucleoside adopts the syn conformation (Figure 9.9), which is presumably favored by placing a bulky $\{Pt(dien)\}^{2+}$ moiety on N7. Moreover, the local charge density on DNA is greater in Z- than B-DNA, owing to the closer proximity of the phosphate groups, and the former is presumably stabilized by the +2 charge on the platinum complex.

b. Effects of Local Sequence and of Free and Linked Intercalators on Platinum Binding Of more interest perhaps to anticancer drug-DNA interactions is the fact that some d(GpG), d(ApG), and even d(GpA) targets for *cis*-DDP bind-

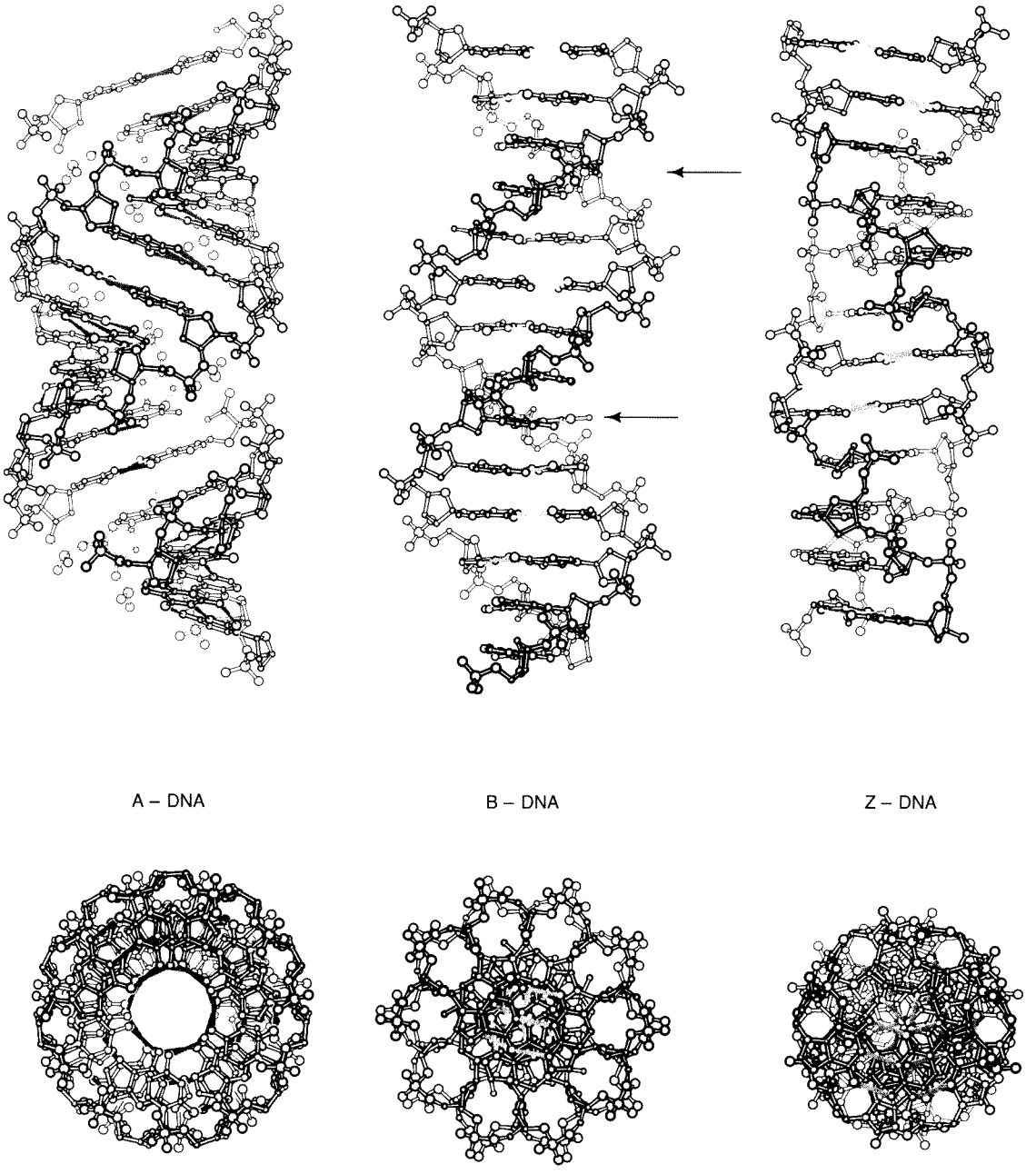

Figure 9.24
Representations of side (above) and top (below) views of three major classes of double-stranded DNA. For B-DNA, arrows near the top and bottom of the helix designate the minor and major grooves, respectively. Reproduced with permission from Reference 140.

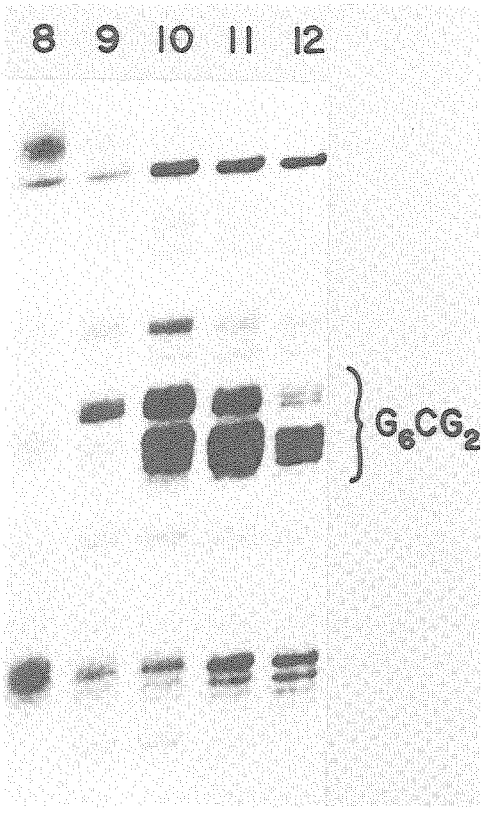

Figure 9.25
Autoradiograph of Exo III mapping results for *cis*-DDP binding to a 165-bp DNA restriction fragment. The Pt/nucleotide ratio is 0.05. Lanes 8—12 contain DNA platinated in the presence of 0, 0.012, 0.057, 0.12, and 0.23 Etd/nucleotide, respectively. For more details, see Reference 142.

ing are very sensitive to the sequences in which they are embedded. This phenomenon was first discovered during exonuclease III mapping studies of cisplatin binding to a 165-bp restriction fragment from pBR322 DNA.[142] Although *cis*-DDP binding stops the enzyme at G_3, G_5, and GAGGGAG sequences, at a $(D/N)_b$ ratio of 0.05 there is little evidence for coordination to an apparently favored G_6CG_2 sequence (Figure. 9.25, lane 8). When platination was carried out in the presence of the DNA intercalator EtdBr (Figure 9.11), however, the G_6CG_2 sequence became a Pt binding site (Figure 9.25, lanes 9-12). A more extensive exonuclease III mapping study of this phenomenon suggested that d(CGG)-containing sequences in general are less well platinated by *cis*-DDP.[143,144] Moreover, only EtdBr, and not other acridine or phenanthridinium type intercalators, was able to promote an enzyme-detectable *cis*-DDP binding to these sequences. A suggested explanation for these results is that local d(purCGG) sequences might have an A-DNA-type structure (Figure 9.24) in which the major groove is narrow, inhibiting access of platinum to N7 of guanosine nucleosides. In the presence of the intercalator EtdBr, the local DNA structure might be altered in such a manner as to permit platinum binding.[143,144]

In accord with this interpretation, and further to delineate a possible reason why acridines and deaminated ethidium cations do not promote cisplatin binding to d(purCGG) sequences, NMR studies were performed that revealed the mean

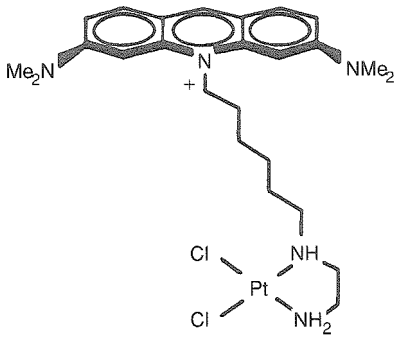

Figure 9.26
Structure of AO-Pt in which the $\{Pt(en)Cl_2\}$ moiety is linked by a hexamethylene chain to acridine orange.

residence time of EtdBr on DNA to be 6 to 21 times longer than that of any of the other intercalators examined.[144] Thus, for these latter intercalators, the local DNA structure presumably can relax back to one unfavored for cisplatin binding before it can diffuse to the site. Moreover, when acridine orange (AO), one of the five intercalators studied that does not promote cisplatin binding to excluded sites, was covalently attached to dichloroethylenediamineplatinum(II) (Figure 9.26) via a hexamethylene linker chain, the resulting AO-Pt molecule was able to bind to all d(CpGpG) sites, as determined by exonuclease III mapping. In the tethered molecule, the high local platinum concentration near the intercalator binding site facilitates attachment of the $\{Pt(en)\}^{2+}$ moiety to DNA before the acridine orange fragment can diffuse away and the structure can relax to reform the excluded site.

Subsequently, the excluded site phenomenon was found for *cis*-DDP binding, as assayed by the 3′-5′-exonuclease activity of T4 DNA polymerase.[145] Enzyme stopping sites were observed at all platinated d(GpG) sequences, but only weakly when at a d(GTGGTC) site. Similarly, d(ApG) was not modified when embedded in pyGAGCpy and pyGAGCA sequences. Although most d(GpA) sequences were not platinated, as detected by T4 mapping, a few were. These results further underscore the importance of local sequence modulation of cisplatin binding to DNA.

c. DNA-Promoted Reaction Chemistry In the EtdBr-enhanced binding of *cis*-DDP to DNA, a small fraction (<5 percent) of the intercalator is strongly bound and can be dialyzed out only very slowly.[145] The detailed structure of this DNA-cisplatin-EtdBr ternary complex has been established, and involves *cis*-$\{Pt(NH_3)_2\}^{2+}$ binding to the exocyclic amino groups of ethidium as well as to donor sites on DNA.[146] This assignment was proved by synthesizing *cis*-$[Pt(NH_3)_2(Etd)Cl]^{2+}$ complexes in dimethylformamide solution and then allowing them to react with DNA. The optical spectra of the resulting adducts were identical to that of the ternary complex. The reaction of *cis*-DDP, EtdBr, and DNA to form the ternary complex is promoted by the favorable orientation of the exocyclic amino group of intercalated Etd with respect to the coordination plane of platinum bound to the double helix. The N-8 exocyclic amino group of ethidium, bound intercalatively at a site adjacent to a purine N-7 coordinated *cis*-$\{Pt(NH_3)_2Cl\}^{+}$ moiety, is positioned above the platinum atom in a structure

resembling the transition state for a square-planar substitution reaction. The structure of this transition state has been modeled in a molecular mechanics calculation (Figure 9.27 *See color plate section, page C-16.*),[147] and evidence has been obtained that indicates selective binding of platinum to the Etd N-8 amino position.[148]

Since cisplatin is usually administered in combination chemotherapy with other drugs, many of which contain intercalating functionalities, strong covalent, DNA-promoted interactions between drug molecules at a target site must be considered as possibly relevant to the molecular mechanism of action. In such a situation, there must be a strong binding preference for both drug molecules for the same target sequences, since on probability grounds alone it is unlikely that both would migrate to the same site by random diffusion at the low concentrations found *in vivo*.

d. Effects of DNA Function on Platinum Binding Although there is yet little known about this topic for cisplatin, it is worth pointing out that other DNA-targeted drugs, such as bifunctional alkylating agents, bind preferentially to actively transcribed genes. It is therefore possible that platinum exhibits such preferences, for example, to single-stranded DNA at or beyond the transcription fork, compared to duplex DNA in chromatin structures. Or, perhaps, it too binds selectively to actively transcribed DNA. Investigation of these possibilities seems worthwhile.

7. Speculations about the molecular mechanism

a. Is There a Single Mechanism? Most investigators now agree that DNA is the cytotoxic target of cisplatin. We have seen that the drug inhibits DNA replication by binding to the template and halting the processive action of DNA polymerase. Less well-studied is the inhibition of transcription by platinum-DNA adducts, but recent evidence clearly indicates that they can do so. Studies of the effects of platinum on cells growing in culture reveal that DNA replication and cell growth can continue without cell division in the presence of low levels (1 μg/mL) of *cis*-DDP; cells are arrested at the G2 phase, the stage of cell growth just preceding division.[105] The G2 arrest was reversible, but at higher cisplatin levels (8 μg/mL), cell death occurred. These observations led to the speculation that, perhaps, post-replication DNA repair can handle the toxicity associated with a platinum-damaged template, at least for DNA synthesis, but that there is no known pathway by which transcription can circumvent Pt-DNA lesions. Possibly, inhibition of transcription is ultimately a more lethal event than inhibition of replication. This idea is inconsistent with the well-established fact that thymidine incorporation into DNA is more affected by low levels of cisplatin than is uridine incorporation into RNA. Might there be more than one biochemical pathway by which cisplatin manifests its anticancer activity? Further work is necessary to address this intriguing question.

b. Is There a "Critical Lesion"? We now have an excellent understanding of the major DNA adducts made by *cis*-DDP, their structures, and the corresponding DNA distortions. Information about adducts made by the inactive *trans* isomer, though not as complete, is also substantial. During the period when this knowledge was being accumulated, it was of interest to learn whether a "critical lesion," a specific DNA adduct with a unique molecular structure, might be responsible for the antitumor activity of the drug. At present, it appears that all bidentate adducts made by *cis*- and *trans*-DDP can inhibit replication, although they may not be equally efficient at doing so.[149] Even monofunctional adducts of the kind formed by *cis*-$[Pt(NH_3)_2(4\text{-Br-py})Cl]^+$ can block replication.[96] Thus, it might be better to think about the concept of "critical lesion" in a functional sense, where the rates of adduct formation, removal, and enzyme inhibition together determine which family of adducts will exhibit antitumor activity and which will not. Here the biochemistry of the host cell will also be an important determinant. Clearly, more studies are required to delineate these possibilities.

c. Replication and Repair in the Tumor Cell[150] If the anticancer activity of cisplatin arises from damaged DNA templates, then the drug could be selectively toxic to cancer versus normal cells of the same tissue if repair of DNA damage occurred more efficiently in the latter. The best way to study this phenomenon would be to measure the platinum-DNA levels and list the spectrum of adducts formed in tumor versus normal biopsy tissue obtained from patients undergoing cisplatin chemotherapy. As described previously, methodologies are now reaching the point where such experiments can be carried out in order to test the key hypotheses about the mechanism of action of cisplatin. In addition, powerful new methods have recently been developed to screen for DNA binding proteins. If one could identify proteins that bind selectively to *cis*-DDP-platinated DNA and determine their function, further insights into cellular replication and repair phenomena would be forthcoming. Such cellular factors that bind selectively to DNA containing cisplatin adducts have, in fact, recently been discovered.[107] The experiments that led to this finding and their possible implications for the molecular mechanism of cisplatin are described in the next section.

d. Structure-Specific (or Damage) Recognition Proteins[150] If selective repair of platinum-DNA adducts in cells of different origin is an integral part of the anticancer mechanism of *cis*-DDP, then it is important to identify the cellular factors associated with this phenomenon. In bacteria, *cis*-DDP adducts on DNA are removed by excision repair, a process in which the lesion is first identified and then excised by the uvrABC excinuclease system.[151] In this process, the uvrA protein first binds to the adducted DNA. Subsequently, the uvrB and C proteins excise the damaged strand, which additional cellular proteins rebuild by copying the genetic information from the remaining strand.

The repair of *cis*-DDP intrastrand crosslinks in mammalian cells is much less well understood. Under the assumption that an analogue of the uvrA protein might exist in such cells, experiments were carried out to try to isolate and clone the gene for such a protein. In particular, the mobility of platinated DNA restriction fragments of defined length was found to be substantially retarded in electrophoresis gels following incubation in extracts from human HeLa cells.[107] This gel-mobility shift was attributed to the binding of factors termed "damage recognition proteins" (or DRPs). Subsequent studies with site-specifically platinated oligonucleotides (see V.D.8) revealed that the cisplatin DRP binds specifically to DNA containing the intrastrand *cis*-$[Pt(NH_3)_2\{d(pGpG)\}]$ or *cis*-$[Pt(NH_3)_2\{d(pApG)\}]$ crosslink. In parallel work, the gene encoding for a DRP was cloned[152] and used to demonstrate the occurrence of such a protein in nearly all eukaryotic cells. Since binding of the DRP to platinated DNA is not specific for the ammine ligands opposite the crosslinked nucleobases, the interaction is thought to involve recognition of local changes in the twist and bending of the double helix. Figure 9.28 depicts one possible structure for the complex formed between *cis*-DDP platinated DNA and a DRP. More recently, the cloned proteins were found to contain a high mobility group (HMG) protein box, and even HMG1 itself binds to cisplatin-modified DNA.[152] The class of proteins was renamed "structure-specific recognition proteins" (SSRPs).

The discovery of SSRPs that bind specifically to cisplatin-modified DNA raises several questions that are the subjects of current study. The first is to determine whether the proteins are an integral component in the mechanism of action of the drug. Although it has not yet been possible to induce the proteins by treating cells with cisplatin, nor have elevated or suppressed levels been found in platinum-resistant cells, deletion of an SSRP gene in yeast has recently afforded a mutant strain less sensitive to cisplatin than wild-type cells.[153] This result links a yeast SSRP with cellular sensitization to the drug. Such a protein could contribute to the molecular mechanism in one of several ways (Figure 9.28). It might be the analogue of uvrA, which, as mentioned above, recognizes damage and signals the cell to perform excision repair. If so, then one would

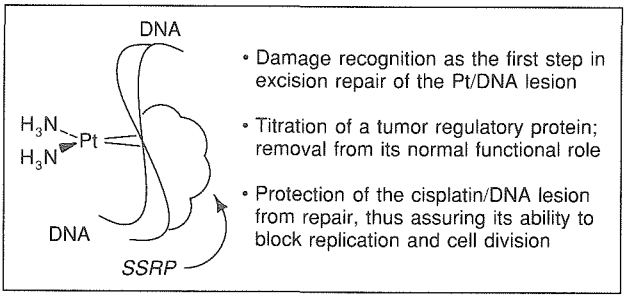

Figure 9.28
Model depicting the binding of an SSRP to cisplatin-damaged DNA and several hypotheses for its role in the molecular mechanism.

like to depress the levels of the protein in cancer cells to make them more sensitive to the drug. A second possibility is that the true role of the SSRP is to serve as a tumor-cell activator, and that cisplatin lesions titrate it away from its functionally active sites on the DNA. Alternatively, binding of the protein could protect cisplatin adducts from repair, preserving their lethality at the time of cell division and leading to the arrest of tumor growth. This last hypothesis would require more of the SSRP in cells sensitive to the drug. Studies are currently in progress to delineate these three and other hypotheses, and to learn whether the discovery of the SSRPs has heralded the final chapter in the quest for the molecular mechanism of cisplatin or merely been an entertaining sidelight.

e. Drug Resistance: What Do We Know?[150] Perhaps the most serious problem for successful chemotherapy of tumors is drug resistance.[102] In most tumors there exists a subpopulation of cells that are naturally resistant to a given drug; as the sensitive cells are killed, these refractory clones take over. In addition, resistance can be acquired by tumor cells following repeated application of the drug. Attempts to identify mechanisms responsible for cisplatin resistance have therefore been the subjects of considerable research activity. Other DNA-damaging agents sometimes amplify genes as a mechanism of drug resistance. An example is the multidrug resistance phenomenon, in which a gene encoded for a P-glycoprotein is amplified in cells resistant to agents such as daunomycin. This protein is believed to increase efflux of the drug through the cell membrane by an ATP-dependent, energy-driven pump. There is currently an intensive search underway to see whether the cisplatin resistance phenomenon has a genetic origin. If a cisplatin resistance gene could be cloned and its phenotype identified, a powerful new avenue would be opened to overcome drug resistance.

8. Site-specifically platinated DNA[154]

a. The Problem Much of the information obtained about the mechanism of action of cisplatin has been derived from experiments where Pt-DNA binding has occurred *in vivo* or *in vitro*, with the use of random-sequence DNA having all available targets for the drug. In these studies, platination is controlled by the inorganic chemistry of *cis*-DDP in the medium and the accessibility of target sites on the DNA, as already discussed in considerable detail. As such, this situation best represents drug action as it actually occurs in the tumor cell. On the other hand, the resultant spectrum of DNA adducts makes it difficult, if not impossible, to understand the structural and functional consequences of any specific adduct. In order to address this problem, a methodology has been developed in which a single platinum adduct is built into a unique position in the genome. This approach is powerful and has the potential to be extended to the study of many other metal-based drugs. In this section, we discuss the strategy used to construct such site-specifically platinated DNA molecules and the information obtained thus far from their study. Some uses have already been discussed.

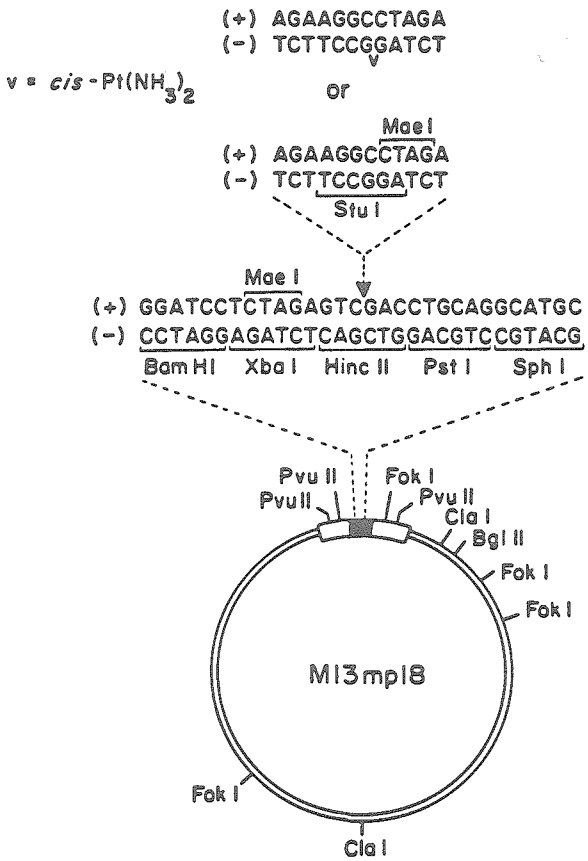

Figure 9.29
Map of the genome created by insertion of *cis*-DDP platinated or unplatinated d(TpCpTpApGpGpCpCpTpTpCpT)-d(ApGpApApGpGpCpCpTpApGpA) into the Hinc II restriction site of bacteriophage M13mp18 DNA.

b. Synthesis and Characterization Figure 9.29 displays the map of a genome constructed by insertion of platinated or unplatinated dodecanucleotide duplexes d(pTpCpTpApGpGpCpCpTpTpCpT)·d(pApGpApApGpGpCpCpTpApGpA) into DNA from bacteriophage M13mp18. This genome was constructed in the following manner.[154] Double-stranded DNA from M13mp18 was first digested with Hinc II, a restriction enzyme that recognizes a unique six-base-pair sequence in the DNA and cleaves the double helix there, leaving a blunt-ended (no overhanging bases) cleavage site. The unplatinated dodecamer duplex was next ligated into the Hinc II site, and the DNA amplified *in vivo*. The dodecamer can insert into the genome in two different orientations, the desired one of which, termed M13-12A-Stu I, was identified by DNA sequencing. The presence of the insert in the new DNA was checked by its sensitivity to the restriction enzyme Stu I, which cleaves at the d(AGGCCT) sequence uniquely situated in the dodecamer insert, and the absence of cleavage by Hinc II, the site for which was destroyed. Next, Hinc II-linearized M13mp18 replicative form (RF)

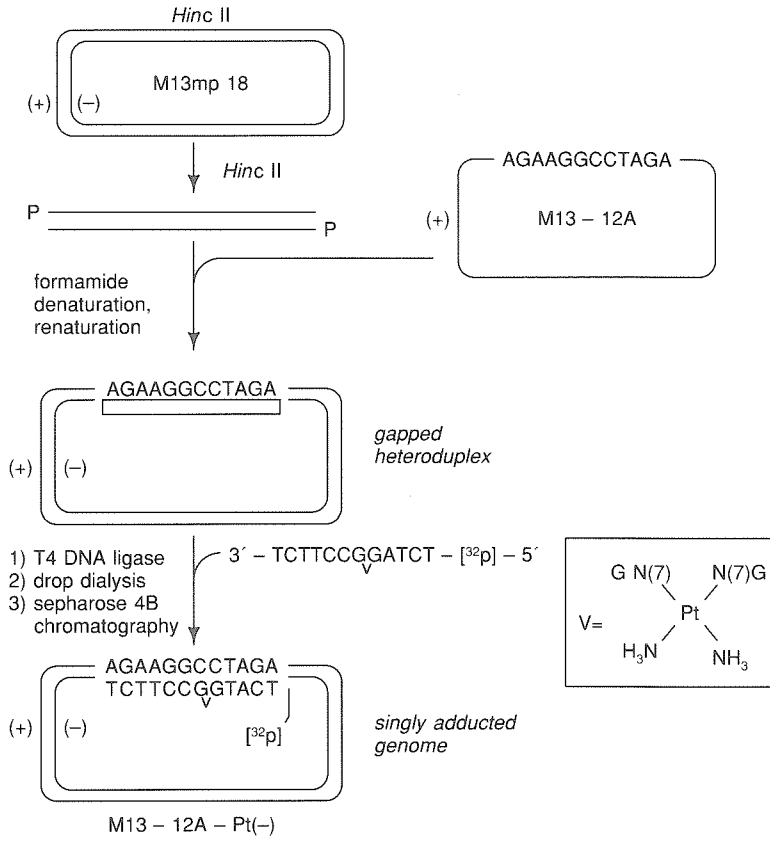

Figure 9.30
Scheme for constructing site-specifically platinated genomes via gapped heteroduplex synthesis (reproduced by permission from Reference 154).

DNA was allowed to form a heteroduplex with excess viral DNA (which has only the + strand) in the presence of the denaturant formamide, which was dialyzed away during the experiment. The resulting circular DNA has a gap in the minus strand into which the platinated d(TCTAG*G*CCTTCT) was ligated (Figure 9.30). The latter material was prepared by the methods described in Section V.D.5.a and characterized by ^{1}H NMR spectroscopy. The resulting site-specifically platinated DNA contains a single *cis*-[Pt(NH$_3$)$_2${d(pGpG)}] intrastrand crosslink built into a unique position. The methodology is general, and has been used to create other known platinum-DNA adducts site-specifically in M13mp18.

The chemical properties of the platinated DNA, termed M13-12A-Pt(-)-Stu I, were investigated by enzymatic, digestion and gel electrophoresis experiments. Platinum completely inhibits cleavage of the DNA by Stu I, as expected from the earlier restriction enzyme mapping studies. In addition, the *cis*-[Pt(NH$_3$)$_2${d(pGpG)}] and *cis*-[Pt(NH$_3$)$_2${d(pApG)}] intrastrand crosslinks were

found to inhibit a variety of DNA polymerases, with only a small amount of bypass of the platinum lesion.[149] These results indicate that the most abundant adducts of cisplatin on DNA are able to block replication efficiently.

c. Biological Properties When M13-12A-Pt(-)-Stu I DNA was introduced into *E. coli* cells by transformation, DNA synthesis was uninterrupted, because the cell can both repair the damage and use the unmodified (+) strand for synthesis. Consequently, a slightly different strategy was used to construct single-stranded M13-12A-Pt(+)-Stu I DNA, the details of which are available elsewhere.[154] This platinated template, in which the damage can neither be repaired nor bypassed by known mechanisms *in vivo*, was then transformed into *E. coli* cells co-plated with GW5100 cells. Under these conditions, viral DNA replication is detected by the expression of the β-galactosidase gene, which, in the presence of appropriate reagents in the medium, leads to formation of blue plaques on a clear background. The results clearly indicate that many fewer plaques appear when M13-12A-Pt(+) is introduced into the cells than when M13-12A-u(+) was employed, where u stands for unmodified DNA. In three repeats of this experiment, survival of DNA containing only a single *cis*-[$Pt(NH_3)_2${d(pGpG)}] crosslink was only 11 $\pm$ 1 percent.

These data provide unambiguous proof that the most frequent DNA adduct formed by cisplatin is toxic, capable of inhibiting replication when only a single such lesion is present on a natural DNA template of 7,167 nucleotides. The fact that as many as 10 percent of the transformed cells can bypass or repair the lesion is also of interest, and parallels the results found *in vitro*. In related work, it was found that the *cis*-[$Pt(NH_3)_2${d(pGpG)}] intrastrand crosslink is not very mutagenic, but that *cis*-[$Pt(NH_3)_2${d(pApG)}] intrastrand adducts are considerably more so. This finding is important, since mutations could lead to long-term secondary tumor production in patients treated with cisplatin. The methodology affords a way to screen new compounds that one would like to be equally effective at inhibiting replication but less mutagenic. In addition, by using repair-deficient mutant cell lines, as well as cisplatin resistant cells, one can study the effects of varying the properties of the host cells. Incorporation of site-specifically platinated DNA sequences into appropriate shuttle vectors will also facilitate investigation of toxicity, repair, and resistance in mammalian cells.

d. Prospectus The foregoing discussion illustrates the power of site-specifically platinated DNAs as a probe of the molecular mechanism of the drug. We recall that similar strategies were employed to obtain uniquely modified DNA in the bending[92,93] and unwinding[95] experiments discussed previously. In principle, this technique can be applied to examine other aspects of the molecular mechanism of other metallochemotherapeutic agents. The requirements are a synthetic route to the uniquely modified genome, for which both the inorganic coordination chemistry and molecular biology must be amenable, an adduct stable to the biological conditions for DNA synthesis, and a method (usually genetic) for scoring the biological effects being investigated. Site-specifically platinated

DNAs allow the bioinorganic chemist to have maximal control over the genetics and should continue to provide valuable information about the molecular mechanism of action of cisplatin.

E. Design of New Inorganic Anticancer Drugs

1. Objectives

Although chemotherapy has made significant contributions to cancer treatment, the effect of cisplatin on testicular cancer being a showcase example, early detection and surgical removal of all neoplastic tissue still remain the preferred means of combating most forms of the disease. What steps need to be taken to devise better chemotherapeutic agents? One answer is to understand the biochemical mechanisms that underlie the transformation of normal into neoplastic cells and to attack the disease on the basis of that knowledge. The value of this approach is indisputable, but it need not be the only one. We have seen that *cis*-$[Pt(NH_3)_2Cl_2]$, a simple third-row transition-metal complex containing no carbon atoms, can contribute significantly to cancer chemotherapy. This example alone should lead us to search for improved inorganic drugs based on the evolving knowledge of the mechanism of action of *cis*-DDP. What then should our objectives be? Three answers are immediately apparent. First, we need to find compounds that are active against resistant cells. Such compounds are termed "second-generation" platinum drugs, and are the focus of much activity in the pharmaceutical industry. Their development will be facilitated by understanding the fundamental biochemistry of cisplatin drug resistance, designing complexes to circumvent the cellular resistance mechanisms. Second, there needs to be an improved spectrum of activity, to be provided by the so-called "third-generation" compounds. The major cancers of the colon, breast, and lung are not effectively diminished by cisplatin chemotherapy. Finally, cisplatin toxicity is often dose-limiting, and there is a need for agents with a greater chemotherapeutic index-to-toxicity ratio. Some of these objectives may ultimately be met by modifying the mode of delivery of cisplatin, for example, by encapsulating the drug in a tumor-seeking liposome or attaching it to a tissue-specific monoclonal antibody. A major step in alternative delivery has recently been taken with the development of a class of oral platinum complexes that have just entered clinical trials.[54] These complexes are platinum(IV) cycloalkylamine species of the kind *cis, trans, cis*-$[Pt(NH_3)(C_6H_5NH_2)(O_2CCH_3)_2Cl_2]$. The prospects are reasonably good that new platinum and other metal anticancer drugs can be designed in a bioinorganic chemical approach to the problem.

2. Strategies for drug development

a. Can We Build on Our Knowledge About Cisplatin? If we consider what is known about the molecular mechanism of cisplatin, what properties are desirable in the design of new metal complexes for testing? The molecules should

be reasonably stable kinetically and soluble in biological fluids, cross the cell membrane, bind covalently to DNA, and inhibit gene function. As described previously in this section of the chapter, powerful methods are now available to screen compounds for these properties in a relatively short time. But there are additional factors required for metallodrug anticancer activity, above and beyond these criteria; *trans*-DDP, after all, has all five of the above properties and is not active. Probably one should add to the list the requirement that the complex have two substitutionally labile *cis* sites for intrastrand crosslinking of adjacent DNA nucleotides; such a criterion would, of course, rule out molecules like *trans*-DDP. Recall, however, that *cis*-$[Pt(NH_3)_2(4\text{-X-py})Cl]^+$ complexes (X = Br, Me) are active. These cations have the five properties listed above, but, as far as is currently known, bind only monofunctionally to DNA. The pyridine ring moiety of a covalently attached platinum atom could possibly intercalate into a neighboring interbase pair site on the DNA, making a pseudo-intrastrand crosslinked adduct structurally similar to the *cis*-DDP-d(pGpG) structure. Further information is required about these active, monofunctional cations before any firm conclusions can be drawn. Nevertheless, it is useful to remember that, if the requirement of two substitutionally labile *cis* ligands had been rigorously followed, this new class of monofunctional platinum complexes would not have been discovered.

Another rationale for designing new platinum or other metal antitumor drugs could emerge with a better understanding of the SSRPs in the mechanism of action of cisplatin. For example, if they serve to protect cisplatin lesions on DNA from repair, one would want to design complexes that form adducts that bind even more strongly to the purified protein. The strength of this binding interaction, having been a serendipitous discovery, surely cannot have been maximized. A tighter SSRP-platinated DNA complex would require the use of less platinum, and thus afford lower toxicities.

b. Is Platinum Uniquely Suited? Given the above criteria, is platinum the only metal to be chosen for further drug development? The answer to this question is "probably not," but a few points need to be kept in mind. Given the assumption that the geometry of the *cis*-$[Pt(NH_3)_2\{d(GpG)\}]$ intrastrand crosslink was important for the antitumor activity of cisplatin, computer graphics methods were employed to probe the stereochemical consequences of modifying this structure.[155] Addition of axial chloride or water ligands in fifth and sixth coordination positions to form pseudo-octahedral adducts, for example, introduces several steric clashes with the guanosine O6 atoms. An octahedral complex, for example *cis,cis,cis*-$[Pt(NH_3)_2Cl_2(OH)_2]$, bifunctionally coordinated to DNA either would not form an intrastrand d(GpG) crosslink or would form an adduct structurally different from that made by *cis*-DDP. This octahedral Pt(IV) complex, known as "tetraplatin" in the pharmaceutical industry, is active, but is believed to be reduced *in vivo* to platinum(II) before coordinating to DNA.[156,157] These considerations might imply that the best strategy for inorganic drug development would be to employ square-planar d^8 complexes. Clearly there are as

yet no definitive answers. Nevertheless, the criteria derived from the mechanism of action studies of cisplatin represent an excellent starting point for designing new antitumor metallodrugs.

c. How Important Are Amine Ligands? Here again, the answer is not unequivocal, but amines (including NH_3) are probably ideally suited ligands for covalent DNA-binding metal complexes. Even completely inert complexes such as $[Co(NH_3)_6]^{3+}$ show sequence and DNA polymorph binding preferences,[158] suggesting that the N-H bonds orient toward the phosphate and heterocyclic nitrogen atoms in the major groove, forming hydrogen-bonding interactions. This chemistry is analogous to the binding and recognition of organic amines and polyamines, such as spermine and spermidine, by nucleic acids. Apart from amines, hydrophobic groove-binding and/or intercalating ligands such as o-phenanthroline and its derivatives should be considered. Molecules such as $[Rh(DIP)_3]^{3+}$, where DIP = 4,7-diphenyl-1,10-phenanthroline, bind to DNA and have proved to be useful structural probes (Chapter 8). Recent work has shown that $[Rh(DIP)_2Cl_2]^+$ binds preferentially to d(GpG) sequences, like cisplatin, although its antitumor properties have not yet been investigated.[159]

3. Second- and third-generation platinum anticancer drugs

Improvements over cisplatin have been made, most notably the molecule carboplatin (Figure 9.4), which is less nephrotoxic and has been reported to be effective in some patients where cisplatin chemotherapy has failed. These properties come solely from the dicarboxylate leaving group, which is kinetically more inert to substitution. Studies with monoclonal antibodies have shown the DNA adducts of carboplatin to be identical with those formed by *cis*-DDP.[117] Other platinum compounds that have undergone clinical trials are close analogues of cisplatin, *cis*-$[PtA_2X_2]$, or tetraplatin, *cis,cis,cis*-$[PtA_2X_2Y_2]$, that obey the classic structure-activity relationships. The activity of cationic triamines, *cis*-$[Pt(NH_3)_2LCl]Cl$, where L is pyridine, a substituted pyridine, pyrimidine, or purine, against S180 ascites and L1210 tumors in mice opens a new vista of possible structures to be tried. The intercalator-linked complex AO-Pt (Figure 9.26) has also been found to show activity in the S180 ascites system, suggesting a further class of complexes that could be studied. The oral compounds, *cis,trans,cis*-$[Pt(NH_3)(C_6H_5NH_2)(O_2CCH_3)_2Cl_2]$, survive the digestive tract, are taken across the gastrointestinal mucosa, and metabolize to *cis*-$[Pt(NH_3)(C_6H_5NH_2)Cl_2]$, a cisplatin analogue.[54] As such they are effective prodrugs that could become the major platinum agent in clinical use. Until these recent advances, there was a general impression that, by chance, the best compound discovered was the first one, cisplatin. There is now sufficient reason to expect that innovative experimentation will lead to improved drugs, bearing in mind the comment made earlier (Section IV.G.) that sustained individual effort for up to a decade can be required to move a compound from the laboratory bench into the clinic.

4. Nonplatinum antitumor metal complexes

a. Soft Metals As mentioned in Section IV.E., some compounds of Pd(II), Au(I), Rh(II), and Ru(II or III) have been screened for antitumor activity, but much more work needs to be done in this arena. The higher metal-ligand exchange rates of Pd(II), $\sim 10^5$ faster than those of Pt(II), make these complexes potentially more toxic, as some preliminary animal studies have shown. By use of chelating or organometallic complexes, however, this problem might be avoided. The properties of Ru, Rh, and to a lesser extent Au amine and polypyridine complexes would seem to make them attractive candidates, and indeed there appears to be renewed interest in these molecules.[160] Inorganic chemists interested in pursuing drug development with these metals need to forge alliances with biological colleagues equipped to do the necessary animal screening and to develop in-house expertise for cell culture and related biochemical work. The techniques are not all that difficult, and it is actually fun to undertake studies of the biological consequences of metallodrug chemistry.

b. Metallocenes and Metallocene Dihalides[36,37] Although complexes such as $[(C_5H_5)_2TiCl_2]$ are superficially analogous to *cis*-DDP, in being potentially bifunctional DNA crosslinking agents, their hydrolytic reactions are sufficiently different to cast doubt on the value of this comparison. The fact that antitumor activity has been found for this very different class of inorganic compound, however, suggests that perhaps bioinorganic chemists have explored only a very small sample of possible metallodrugs.

VI. RETROSPECTIVE

The topics discussed in this chapter are helping to expand bioinorganic chemistry from a subject that arose chiefly from spectroscopic analysis of metal centers in proteins, because they were uniquely convenient functional groups, to a discipline where fundamental knowledge about metal functions and the application of metals as diagnostic and chemotherapeutic agents are making important contributions to medicine. As the case study of cisplatin is intended to demonstrate, progress in understanding how metals function in chemotherapy can be made only by the combined efforts of many disciplines, including synthetic and physical inorganic and organic chemistry, molecular and cell biology, immunology, pharmacology, toxicology, and clinical medicine. Although we have not yet reached the day where chemotherapeutic agents can be rationally designed from knowledge of a molecular mechanism, such a concept does not seem that farfetched. If nothing else, knowledge of fundamental bioinorganic processes related to metal-macromolecule interactions will continue to grow enormously through efforts to achieve this ultimate goal.

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