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DNA AND RNA CLEAVAGE BY METAL COMPLEXES

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I. Introduction

The reason why Nature chose phosphate esters to serve as nucleotide linkages in the genetic material DNA may relate to the high stability of the phosphate ester with respect to hydrolysis and to its negative charge, which reduces the rate of nucleophilic attack on the DNA backbone (*1*). Because efficient hydrolysis of these phosphodiester linkers is necessary in DNA repair, and at many different stages of transcription and translation, enzymes are involved in catalyzing the hydrolysis of P–O bonds of the DNA phosphate backbone. These hydrolytic enzymes are in fact metalloenzymes with metal ions present at the active site, as will be discussed in Section III. Published structures of nucleases have allowed the positions of these ions (cal-

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cium, zinc, or magnesium) to be determined with respect to the phosphate residue, in order to better understand their role. It will be seen in Section III that modeling of these hydrolytic enzymes is very challenging for synthetic chemists and that considerable progress will have to be made before it is possible to create artificial nucleases capable of competing with natural ones. The time scale of evolution allows a continuous recycling process to improve the design of enzymes able to catalyze the hydrolysis of the P–O bonds of DNA phosphodiesters. Chemists have been working in this area for only 20 years! Despite the fact that more than 2400 site-specific restriction enzymes representing 188 different sequence specificities have been discovered (*2*), there is still a need for artificial endonucleases that are able to cleave DNA and RNA at specific sites.

On the other hand, as will be described in Section II, bio(in)organic chemists and biochemists have been more successful in understanding the mechanism of action of bleomycin, an antitumoral antibiotic able to cleave DNA by using three cofactors: iron, dioxygen, and an electron source (for early reviews, see Refs. *3–7*). This naturally occurring molecule is the paradigm of many metal-containing cleaving reagents synthesized during the past 15 years, whereby oxidative degradation of DNA and RNA is due to high-valent metal species or reactive oxygen entities produced at the metal center. These new DNA and RNA cleavers might have a future as antitumoral or antiviral agents if they can be used to create irreversible damage to the DNA present in cancer cells or to retroviral DNA integrated within infected cells.

II. Oxidative DNA and RNA Cleavage Mediated by Transition Metal Complexes

A. Bleomycin

Bleomycins (BLMs) are a family of glycopeptide antitumoral antibiotics produced by *Streptomyces verticillus*. The structure of the two major components (BLMs A_2 and B_2) is depicted in Fig. 1. The five nitrogen atoms of BLM indicated in Fig. 1 are able to chelate strongly several metal cations, e.g., iron, manganese, cobalt, copper, or zinc. Many gram-negative and gram-positive bacteria are resistant to BLM because of their capacity to produce a protein able to sequester this antibiotic. The crystal structure of a BLM-resistant protein has been determined, and a BLM–protein binding model proposed (*8*). The metal center of bound metallo-BLM is buried within the protein

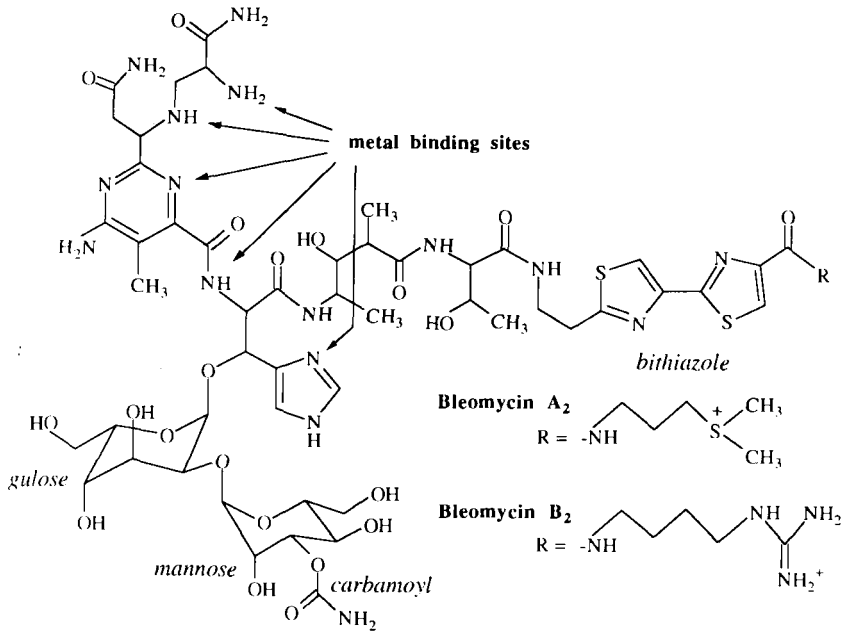


FIG. 1. Structures of bleomycin A_2 and B_2.

dimer, making unaccessible the sixth coordination site for oxygen activation (see below for the mechanism of action of bleomycin).

This potent anticancer drug is now widely used in chemotherapy in association with several other drugs (e.g., vinblastine, methotrexate, carboplatine, ifosfamide, or etoposide) in the treatment of Hodgkin's disease (*9*), of head and neck cancers (*10*, *11*), of disseminated germ cell tumors (*12*), and of poor-prognosis epidemic Kaposi's sarcoma (*13*) (for previous articles on the clinical use of BLM, see references listed in Refs. *9–13*).

One of the unique features of BLM is its lack of significant hepatic, renal, and bone marrow toxicities, undesired effects of many anticancer drugs. Two drawbacks are the tumor resistance and the BLM-induced pulmonary toxicity. Both effects are related to the level of BLM hydrolase, a protease that binds to DNA and inactivates BLM by hydrolyzing the β-alanine carboxamide moiety (*14*, *15*). Cells with high levels of BLM hydrolase are resistant to bleomycin, whereas lungs are sensitive to BLM-induced tissue injuries because of low levels of this enzyme. The structure of this protease, which binds DNA and is conserved from bacteria to humans, has been solved (*16*).

The cytoxicity of BLM toward cancer cells is oxygen dependent and

related to its ability to induce single- and double-stranded DNA breaks. Cell penetration limits the cytotoxicity of this DNA cleaver (*17*). By electropermeabilization, it has been shown that BLM is highly cytotoxic. After BLM internalization, cell apoptosis is observed and is probably due to the direct internucleosomal cleavage of chromatin (*18*).

Early studies on the mechanism of DNA cleavage by metallobleomycins have been reported in well-organized review articles (*3–7*), so here the focus is on advances in (1) the syntheses of BLM and structures of BLM complexes, (2) the interactions of BLM with DNA, (3) the nature of "activated" BLM, (4) the mechanism of DNA (and RNA) cleavage, and (5) BLM models.

1. Structures and Syntheses of Bleomycin Derivatives

As depicted in Fig. 1, the structure of BLM can be regarded as the association of three different domains: (1) a bithiazole unit with a positively charged lateral chain mainly responsible for DNA binding, (2) a metal-chelating system involving the pyrimidine, β-aminoalanine, and β-hydroxyimidazole residues [this proposal is confirmed by the determination of the three-dimensional structure of a Cu^{II}–BLM analogue (*19*); a sixth coordination site is vacant and available for the interaction with molecular oxygen in the case of the Fe^{II}–BLM complex], and (3) a disaccharide moiety containing a gulose and a carbamoylated mannose.

The total synthesis of BLM A_2 and related compounds has been achieved by Boger and co-workers (*20*). The approach developed by these authors also included the synthesis of 2-*O*-(3-*O*-carbamoyl-α-D-mannopyranosyl)-L-gulopyranose and its incorporation into a total synthesis of BLM structural analogues (*21*). The chemical synthesis of this disaccharide was also achieved by Kobayashi and Oshitari (*22*).

2. Interactions of Bleomycin with Metal Ions and DNA

The common factor of all bleomycins is their high capacity to bind metal ions strongly. The Cu^{II}–BLM complex is slightly more stable than the corresponding Fe^{III} analogue, Log K_{aff} being 18.1 and 16.0, respectively (*6*). In blood, BLM rapidly forms a copper complex that is thought to be involved in cellular uptake. ^{14}C-Labeled copper–BLM–A_2 is poorly internalized within KB_3 cells, but, surprisingly, the accumulation of the deglyco derivative within cell nuclei was higher than for BLM (*23*). This result is controversial with the classical idea that sugar residues of antitumoral agents are present to facilitate cell penetration.

Several recent investigations indicate that BLM binds to DNA not only by the bithiazole moiety but also by the metal binding domain and the disaccharide entity, which are also involved in the overall DNA binding and recognition process. Without bithiazole, BLM is unable to bind to DNA (*24*) and it is reasonable to assume that the two other structural factors (the metal-binding domain and the disaccharide) are involved in the fine tuning of sequence-specific BLM–DNA interactions. The important role of the bithiazole in DNA binding and cleavage efficiency has been confirmed by using analogues containing modified bithiazoles (*25*). BLM-bithiazole behaves as a (partial) intercalating agent, because Cu^{II}–BLM unwinds DNA. Bailly and Waring found that the 2-amino group of guanine plays an important role in the recognition of DNA by BLM (*26*), in agreement with nuclear magnetic resonance (NMR) studies on Zn–BLM and Co–BLM incubated with the oligodeoxyribonucleotides (ODNs) $d(CGCTAGCG)_2$ and $d(CCAGGCCTGG)_2$, respectively (*27*, *28*). These two latter studies confirmed that the metal-binding domain of BLM is folded and interacts with the minor groove of double-stranded DNA. In the Zn–BLM–oligonucleotide complex, the metal center is only 3.3 Å away from the closest deoxyribose C-4′–H. If we assume a 1.7 ± 0.1 Å metal–oxygen distance for a putative metal–oxo species, the oxygen atom of the oxo group will be 1.6 ± 0.3 Å from the abstracted hydrogen atom, depending on the metal–oxygen–hydrogen angle, very close to the expected oxygen atom distance in the transition state of the removal of H · at a 4′ position of a deoxyribose unit.

The recognition of B-DNA by BLM is enantiospecific because BLM is unable to cleave the nonnatural L-$d(CGCGCG)_2$ duplex, whereas the nature D oligomer is efficiently cleaved (*29*).

3. *Nature of "Activated Bleomycin"*

The oxidative degradation of DNA by BLM involves abstraction of the hydrogen atom at the 4′ positions of deoxyribose units (see Section II,A,4 for details on DNA cleavage) by a transient species, the so-called activated BLM resulting from activation of the Fe–BLM by two reducing equivalents in the presence of molecular oxygen (*3*, *4*, *30*). This drug–iron–oxygen complex is kinetically competent to initiate the attack on DNA (*31*). The DNA cleavage pattern is not in favor of diffusible HO · species as H atom abstracting agents, so "activated bleomycin" is a metal-centered oxygen-containing complex.

It has been hypothesized that activated BLM is a high-valent iron–oxo complex generated by a reductive activation of molecular oxygen reminiscent to that observed in cytochrome *P*-450 monooxygenases

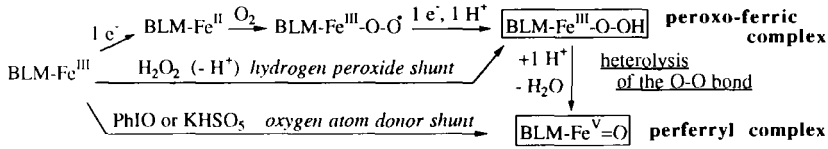


FIG. 2. Different routes for the generation of "activated bleomycin." The formal oxidation state (V) of the bleomycin–iron–oxo species (perferryl complex) is two oxidant equivalents above BLM–Fe^{III}, but one oxidant equivalent might be located on the ligand, as in Compound I of peroxidases, with an $iron^{IV}$–oxo–ligand radical cation structure.

(*30*, *32*) (see Fig. 2). Such a hypothesis (see the peroxide shunt in Fig. 2) has been supported by using single-oxygen atom donors, iodosylbenzene or potassium monopersulfate, as cofactors in DNA cleavage or olefin epoxidations catalyzed by iron– or manganese–bleomycin (*33–35*; and see Ref. *36* for a review of oxygen donors in metalloporphyrin-catalyzed oxygenations). The precursor of such high-valent iron–oxo species is the hydroperoxo complex BLM–Fe^{III}–OOH as depicted in Fig. 2. This intermediate, prepared from Fe^{III}–BLM and hydrogen peroxide, has been recently characterized by electrospray mass spectrometry (*37*). The d^5 BLM–Fe^{III}–OOH, prepared by reacting H_2O_2 with a dual syringe pump interfaced with the electrospray source, exhibits a peak at $m/z = 751$ (with $z = 2^+$). This peak shifts at 753 with $H_2{}^{18}O_2$. The electron spin resonance (ESR) spectrum of activated BLM with characteristic g values at 2.26, 2.17, and 1.94 was assigned to the low-spin d^5 BLM–Fe^{III}–OOH complex (*30*, *38*). The stability of BLM–Fe^{III}–OOH, and the slow scission of the peroxidic O–O bond to generate a perferryl species as in cytochrome *P*-450 or metalloporphyrin models, might be due to the weak electron transfer from the metal and its ligands to the antibonding orbital of the O–O bond to promote the heterolytic cleavage (porphyrin ligands and proximal ligands of heme proteins are better electron reservoirs than are BLM ligands), and not the reverse as stated by Mascharak *et al.* (*38*) (see Fig. 3).

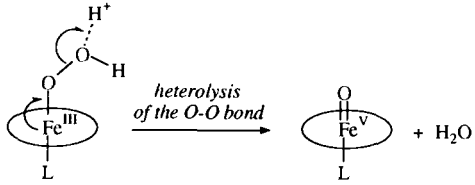


FIG. 3. Push–pull mechanism for the heterolysis of the O–O bond of an iron–peroxo complex. The oval indicates an electron-rich ligand, such as a porphyrin; L is a heme-proximal ligand (cysteine or histidine).

From oxygen isotope studies, Burger *et al.* reported that activated BLM formation is at least 10-fold faster than its decay (*39*). No significant amount of superoxide or peroxide is released during the decomposition of activated BLM. The decay of BLM–Fe^{III}–OOH to a highly reactive iron–oxo as the proximate DNA-attacking species cannot be excluded (*39*).

4. Mechanism of DNA (and RNA) Cleavage

Recent studies with *Euglena gracilis* and human leukemia HL-60 cells have assessed the need for intracellular iron for cellular DNA damage and cell growth inhibition by BLM (*40*). Growth inhibition and both single- and double-strand DNA breaks were substantially reduced in iron-deficient cells. In iron-deficient HL-60 cells, metal-free BLM and Cu–BLM are mainly inactive as cellular DNA cleavers. The earlier findings that free BLM, Zn–BLM, or Cu–BLM had the same cytotoxic activity as Fe–BLM can now be explained by their conversion into Fe–BLM inside host cells (*40*).

Several studies with DNA fragments have shown that the most frequent cleavage sites are at G-pyrimidine sequences. Frequency of cleavage decreases as follows: 5′-GC ~ GT > GA ≫ GG (*41*, *42*). Breaks are also observed at 5′-AT sites in DNA fragments having AT-rich sequences. DNA cleavage is efficiently performed by BLM in the presence of iron(III) ions and hydrogen peroxide or in the presence of iron(III) ions, molecular oxygen, and a reducing agent. DNA strand scission is accompanied by the simultaneous release of free bases, base propenal derivatives **A**, 3′-phosphoglycolates **B**, 5′-phosphate ends **C**, and alkali-labile 4′-hydroxylated abasic sites **D** revealed by hydrazine (Fig. 4) (*3*, *4*, *43*, *44*). As demonstrated by deuterium isotope effects H-4′ abstraction is the rate-determining step (*45*). The k_H/k_D value ranges from 2 to 7 depending on structural changes on BLM derivatives (*46*). Then the radical at 4′ can react according to three different pathways: (1) with molecular oxygen (the so-called oxygen-dependent route) to generate an unstable hydroperoxide at 4′, which breaks down via a Criegee-type rearrangement to the corresponding base propenal, 3′-phosphoglycolate, and 5′-phosphate derivatives, (2) with the coordinated OH group of BLM–Fe^{IV}–OH via an oxygen rebound mechanism to give the 4′-hydroxylated site **E**, or (3) with BLM–Fe^{IV}–OH via an electron transfer to produce an intermediate cation **F**, which reacts with water to give **E**. Experiments with ^{18}O-labeled water indicate that this latter pathway is the main route to produce **E** (*47a*).

Recent kinetic data on DNA strand scission initiated by BLM indicate that strand cleavage precedes deoxyribose 3′-phosphate bond

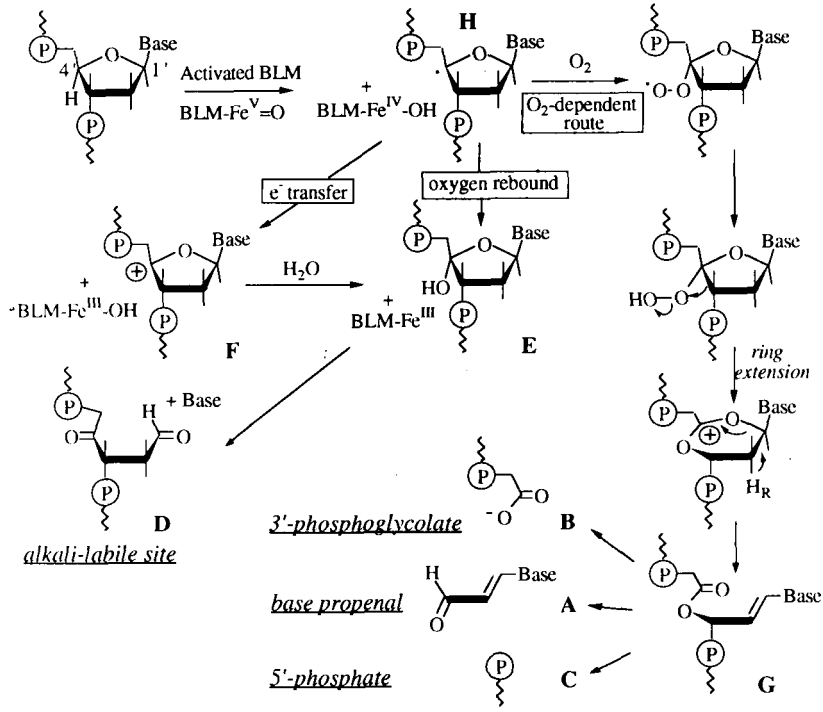


FIG. 4. Mechanism of the oxidative DNA cleavage by activated iron–bleomycin.

cleavage (*47b*). Strand scission occurs with a $t_{1/2}$ = 4.1 ± 0.5 min at 4°C, faster than base propenal ($t_{1/2}$ = 6.7 ± 0.3 min) or the 5′-phosphate end ($t_{1/2}$ = 7.4 ± 0.8 min) formation. So the common precursor **G** of products **A**, **B**, and **C** is decomposed via the cleavage of the C-3′–O bond to generate first the 3′-phosphoglycolate and consequently the DNA strand scission. The intermediate radical **H** resulting from H atom abstraction by activated BLM has been generated by photolysis of a single-stranded oligonucleotide modified with a photoactivatable substituent at 4′ (*48*, *49*). Giese *et al.* (*48*, *49*) proposed a mechanism involving the release of the 5′-phosphate directly from intermediate **H**. This proposal seems to be in contradiction with the kinetic data obtained by Burger *et al.* (*39*) (see above).

H-1′ abstraction by Fe–BLM from ODN duplexes containing a ribonucleotide unit was evidenced (*50*), but degradation of DNA–RNA hybrids by BLM involves C-4′ rather than C-1′ chemistry (*51*); the DNA strand of this heteroduplex is preferentially cleaved (*52*).

Double-strand breaks induced by BLM result from two single-

strand breaks generated on each strand by the same bleomycin molecule acting as catalyst (*53*, *54a*). The essential factor establishing the ratio of single-strand to double-strand cleavage at a specific site is related to the efficiency by which Fe–BLM can be reactivated. The specific cleavage of a DNA triple helix by iron–bleomycin has also been observed (*54b*). The strongest damage is located at the 5′ duplex–triplex junction.

It can also be noted that BLM-dependent damage to bases is a minor side reaction (*55*). Thus 7,8-dihydro-8-oxodeoxyguanosine, the predominant tautomer of 8-OH-dG, is formed in calf thymus DNA treated with BLM (*56*).

In spite of early reports that RNA is not degraded by BLM (*57*), additional studies indicate Fe–BLM-mediated degradation of certain RNA substrates, notably transfer RNAs, tRNA precursor transcripts, and HIV-1 reverse transcriptase mRNA (*6*, *58*). The RNA cleavage is much more selective than DNA cleavage. *Bacillus subtilis* $tRNA^{His}$ precursor was cleaved at U35 and the corresponding "tDNA" was also cleaved at the corresponding T35 site by BLM at low concentration (*59*). Keck and Hecht have found that metal-free BLM is able to mediate the sequence-specific hydrolysis of yeast $tRNA^{Phe}$ at Py–Pu sites not involving modified bases (*60*). The fact that RNA might constitute a therapeutically relevant target for BLM is now an open question.

5. *Bleomycin Models and Conjugates*

Sawai *et al.* (*61*) reported that enantiomeric models containing a D- or L-histidine entity exhibited a DNA cleaving activity in a chiral discrimination manner. Using a 4-substituted pyridine and two histidine residues, Sugiura *et al.* (*62*) have shown that the oxygen activation efficiency of the corresponding iron complexes increased with electron-donating substituents (methoxy and dimethylamino groups).

Mascharak *et al.* (*63*) have prepared a structural BLM model PMAH containing pyrimidine, imidazole, and primary and secondary amine building blocks. The $iron^{II}$–PMAH complex was studied by X-ray absorption, magnetic circular dichroism, and resonance Raman spectroscopy (*63*). This Fe–BLM model exhibits a five-coordinate, square pyramidal geometry in the solid state and a distorted octahedral geometry in solution with a solvent molecule at the sixth position. Similar spectral features have been found for Fe^{II}–BLM. This PMAH–Fe^{II} complex binds O_2 to generate PMAH–Fe^{III}–OOH, a low-spin hydroperoxo–iron(III) complex (*64*) able to promote the lipid peroxidation as BLM (*65*).

Lown *et al.* (*66*) have prepared functional models of bleomycin com-

posed of a metal-complexing motif similar to PMAH (with a pyridine instead of a pyrimidine) and an oligo-*N*-methylpyrrole peptide as DNA-binding entity. The DNA cleavage activity increases with the number of *N*-methylpyrrole units, but sequence selectivity studies on restriction fragments indicate that the models with lexitropsin-type vectors cleave DNA at AT-rich DNA sequences, as expected for minor-groove binders.

Bleomycin can be covalently attached to oligonucleotides or DNA binding proteins (*67–69*). A BLM–oligonucleotide conjugate cleaves DNA at GT sequences located near the junction site of the conjugate with its target (*67*, *68a*). A catalytic site-specific cleavage of a DNA target by a short oligonucleotide linked to BLM has also been reported by the same research group (*68b*). Up to three catalytic cycles have been obtained. The iron-binding motif of BLM (pyrimidoblamic acid β-hydroxy-L-histidine) linked to the terminal amine of the DNA binding domain of Hin recombinase interacts with DNA at the expected Hin binding sites (*69*). The corresponding iron complex cleaves DNA in the presence of dithiothreitol. Analysis of the cleavage pattern suggests that this site-specific DNA is mediated by a localized diffusible species, in contrast with BLM, which proceeds via nondiffusible species.

B. Copper Complexes

1. Bis(1,10-phenanthroline)copper

The complex $Cu^{I}(phen)_2$ **1** [see Fig. 5; another commonly used abbreviation is $Cu^{I}(oP)_2$] was the first to be recognized as an efficient chemical nuclease by Sigman *et al.* (*70–72*). The oxygen-dependent DNA cleavage activity of $Cu^{I}(phen)_2$ was the cause of the inhibition of *Escherichia coli* DNA polymerase I activity in the presence of a chelating agent, a thiol, and traces of copper salts (*70*). To generate these single-strand DNA breaks, two modes of activation can be used: hydrogen peroxide or molecular oxygen in the presence of a reducing agent (a thiol or ascorbic acid) (*73–75*). The active species responsible for DNA cleavage is still a matter of debate. A popular view is to consider that $Cu^{I}(phen)_2$ [or $Cu^{II}(phen)_2$ before being reduced] freely diffuses along double-stranded DNA, binds to DNA (see below for details on nucleic acid interactions), and then reacts with hydrogen peroxide to cleave DNA. Some reports indicate that freely diffusible hydroxyl radicals are not responsible for DNA strand scission (*73*, *76*), but others mention that the DNA-damaging species can diffuse over

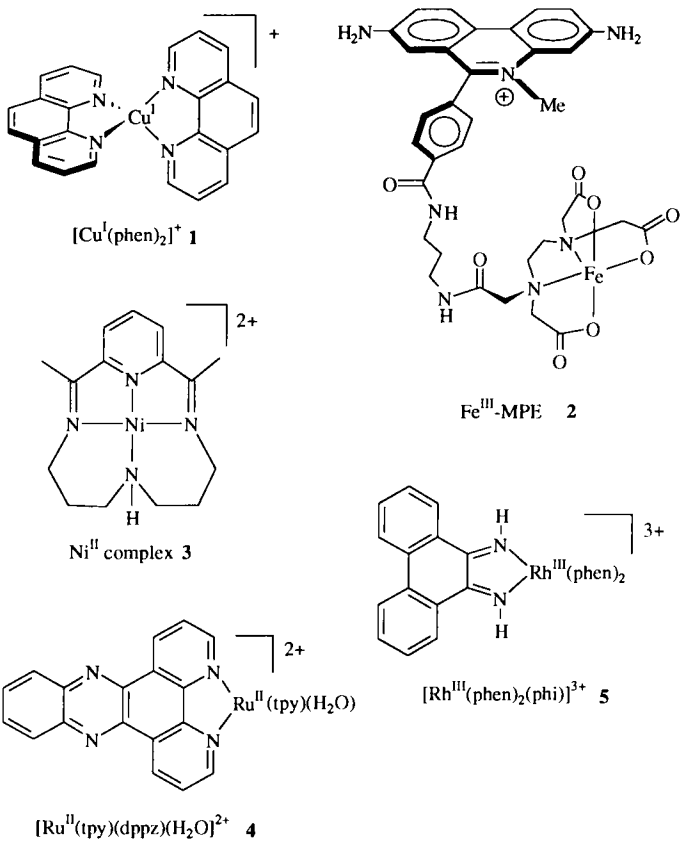


FIG. 5. Structures of the metal complexes **1**–**5** having an oxidative nuclease activity.

a limited range from the binding site of **1** (*77*). The damage to DNA bases by Cu^{I}(phen)$_2$/H_2O_2 is typical of those induced by HO·, suggesting that a minor reaction pathway involves free hydroxyl radicals (*78*). However, the nondiffusible active copper-centered species responsible for single-strand (ss) breaks is probably a coordinated hydroxo complex Cu^{II}–OH (or Cu^{III}–OH ?) rather than a copper–oxo species Cu^{III}=O (see Ref. *79* for a discussion by Mayer on the nonexistence of copper–oxo species, due to the filling of antibonding molecular orbitals by the too *d*-electron-rich metal–oxo complexes).

The pattern of DNA cleavage by activated Cu^{I}(phen)$_2$ is consistent with its binding in the minor groove of a right-handed double helix (*80*). Initial observation of the inhibition of cleavage by intercalators suggests that Cu^{I}(phen)$_2$ interacts with double-stranded (ds) DNA via

an intercalation mode (*73*). However, the observed cleavage specificity is related to a predominant preference for binding at 5′-TAT triplets, which is more suggestive of a minor groove binder behavior than that of an intercalator, for which GC preferences are expected (*81*). The $Cu^{I}(phen)_2$ is precluded from full intercalation by its tetrahedral geometry, but a model involving the partial intercalation of one phenanthroline ligand is also plausible (*82*). Z-DNA having a deep narrow minor groove is completely resistant to degradation by **1** (*74*). As expected for a minor-groove binder, C–H bonds at C-1′ and C-4′ of deoxyriboses, two sugar C–H bonds accessible from the minor groove, are the main targets of activated $Cu^{I}(phen)_2$ (*71*). Using 5′-^{32}P-labeled ds dodecamers, Sigman *et al.* (*70*) have shown that 3′-phosphoglycolates resulting from a C-4′ chemistry are minor DNA degradation products compared to those generated by a C-1′ chemistry, which represents 80–90% of the DNA damage (Fig. 6). After oxidation of the C–H bond at 1′, releasing the attached base, the first β-elimination liberates a 5′-phosphate end (the single-strand break event) and a damaged sugar residue still attached at the 3′ end of the DNA strand. The 5-methylene-2(5*H*)-furanone (5-MF) is released from its precursor by a thermal step (90°C for 1 min) or a piperidine treatment necessary to facilitate the second β-elimination (*83*). The released 3′-phosphomonoester termini were the potent inhibitors of *E. coli* DNA polymerase I activity as revealed by the nuclease activity of $Cu^{I}(phen)_2$, as mentioned above.

By itself, $Cu^{I}(phen)_2$ is an efficient chemical tool to probe small conformational changes of DNA. Comparison of the digestion patterns

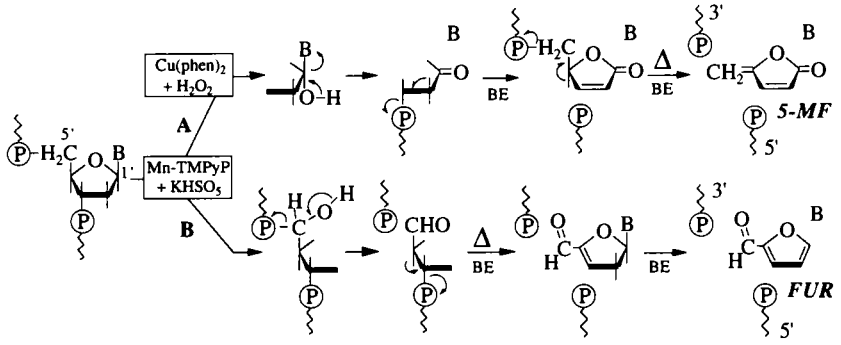


FIG. 6. Mechanism for the cleavage of DNA or DNA models after hydroxylation of C–H bonds on the 1′ and 5′ positions of deoxyribose by $Cu(phen)_2/H_2O_2$ (route A) or Mn–TMPyP/$KHSO_5$ (routes A and B) (Δ = thermal step, B = base, BE = β elimination).

generated by DNase I and $Cu^{I}(phen)_2$ of different variants of the *lac* operon control region provided information on DNA conformational variability in a region of biochemical function (*84*). Because of its size and its high reactivity, $Cu^{I}(phen)_2$ provides data complementary to that of DNase I. $Cu^{I}(phen)_2$ was also used to study the conformational changes in transcriptionally active complexes between RNA polymerase and promotor regions (*85*, *86*). The footprinting properties of $Cu^{I}(phen)_2$ were also useful to study RNA–protein complexes, in particular the interaction of TAT peptides with HIV *tar* RNA (*87*).

2. *Copper–Phenanthroline Conjugates*

This aspect has been recently reviewed by Sigman (*88*). The nuclease activity of copper–phenanthroline can be targeted by attachment of one modified 1,10-phenanthroline ligand to the 5′ end of oligodeoxyribonucleotides complementary to ss DNA (*89–91*) or RNA sequences (*92*). The phenanthroline can also be linked through N-2 of a deoxyguanosine residue (*93*). These modified phenanthroline ligands can also be tethered to oligo-α-deoxyribonucleotides resistant to nucleases (*94*) or to triple-helix-forming oligonucleotides in order to target the nuclease activity of the copper complex toward a double-stranded DNA sequence (*90*, *95*, *96*) (for a recent review on triple helices and the "antigene" strategy, see Ref. *97*). Different RNA molecules bearing uridine bases modified by a phenanthroline ligand were also used to achieve the oxidative sequence-specific cleavage of ss or ds DNA (*98*). All these conjugates contain only one phenanthroline ligand, so the coordination sphere of the chelated copper ion is probably completed by unknown extra ligands (water molecules, basic sites of DNA, or an excess of the conjugated molecule ?).

The nuclease activity of the copper–phenanthroline complex can also be directed at specific sites with DNA-binding proteins (trp, CAP, Cro, Fis, etc.) in order to study the interactions of these DNA-binding proteins with their DNA targets or to isolate long DNA fragments for sequencing, cloning, or chromosomal mapping (*99–105*). Conjugates with a minor-groove binder such as Hoechst 33258 or different peptides have also been prepared (*106*).

C. Iron Complexes

1. *Iron–EDTA*

Even without the ethylenediaminetetraacetate (EDTA) ligand, iron(II) salts are able to bind to DNA and to catalyze the formation of

hydroxyl radicals within the DNA from H_2O_2 via the well-known Fenton reaction (*107–109*), and to generate DNA damage, which can be repaired when cells are exposed to low hydrogen peroxide concentrations (*110*) (for additional information on oxidative DNA-damaging processes, see Ref. *111*).

When Fe^{II} is chelated by EDTA, its direct interaction with DNA is precluded because Fe^{II}–EDTA present as $[Fe^{II}–EDTA]^{2-}$ is a negatively charged complex, making difficult an electrostatic interaction with DNA (for the X-ray structure of the corresponding Fe^{III}–EDTA–aqua complex and a recent review on metal(III)–EDTA complexes, see Refs. *112* and *113*, respectively). This absence of direct contact of Fe–EDTA with DNA is now an advantage when using the Fe^{II}–EDTA/H_2O_2 system to generate the HO · radical in the close vicinity of DNA without or with very little sequence specificity. This hydroxyl radical footprinting method has been extensively developed by Tullius and co-workers to study DNA conformations and to map DNA–protein contacts (*114–118*). For example, a 150-base pair DNA sequence directly upstream of the thymidine kinase gene of herpes simplex virus I was found to have a helicity of 10.5 base pairs per turn, typical of a B conformation, when the DNA fragment was bound to crystalline calcium phosphate and cleaved by HO · radicals generated by Fe^{II}–EDTA/H_2O_2 (*114*). The DNase I footprinting method cannot be applied in such a case. In addition, Fe^{II}–EDTA catalyzes the scission of ss and ds DNA and RNA with the same reactivity, which simplifies the interpretation of experimental data from studies on DNA and RNA conformations (*119*).

The identity of the DNA-oxidizing species produced in the Fe^{II}–EDTA/H_2O_2 system has been an issue of debate. The presence of a weakly bound water molecule on Fe–EDTA (*112*) allows the possibility to create an oxo ligand at this position when the iron complex is oxidized by hydrogen peroxide (for the possible generation of iron–oxo species by activation of iron salts by hydrogen peroxide, see Ref. *120*). Comparison of DNA cleavage patterns generated by γ-radiolysis or by Fe^{II}–EDTA/H_2O_2 confirmed that hydroxyl radicals are responsible for DNA strand scission under the conditions used by Tullius *et al.* (*121*). The Fe^{II}–EDTA catalyzes production of HO · radicals from H_2O_2 in the presence of ascorbate, which reduces Fe^{III}–EDTA back to the Fe^{II} state according to Eqs. (1) and (2):

$$[Fe^{II}–EDTA]^{2-} + H_2O_2 \rightarrow [Fe^{III}–EDTA]^{-} + HO^{-} + HO\cdot; \quad (1)$$

$$[Fe^{III}–EDTA]^{-} + \text{ascorbate} \rightarrow [Fe^{II}–EDTA]^{2-}. \quad (2)$$

The neutral hydroxyl radicals react at a diffusion-controlled rate with bases and also with C–H bonds of deoxyriboses, leading to DNA breaks with 5′- and 3′-phosphate termini (*122*).

2. *Iron-Methidiumpropyl–EDTA*

In a pioneering work, Dervan designed a bleomycin model by attaching EDTA to an activated ester of *p*-carboxymethidium via a 1,3-diaminopropane linker (*123*). Methidiumpropyl–EDTA (MPE) is a bifunctional molecule able to interact with ds DNA by intercalation and to chelate iron(II) strongly (see Fig. 5 for the structure of Fe^{III}–MPE **2**). MPE in the presence of iron^{II}, molecular oxygen, and a reducing agent, usually dithiothreitol (DTT), cleaves a supercoiled plasmid DNA at low concentrations (10^{-8} *M*), comparable to that used with bleomycin (*123*). The postulated mechanism of DNA cleavage involves the reaction of the chelated Fe(II) with O_2 to give Fe(III) and $O_2^{\cdot-}$ [Eq. (3)]. Superoxide anions in protic media quickly disproportionate to hydrogen peroxide and dioxygen [Eq. (4)]. The H_2O_2 produced then reacts with Fe^{II}–MPE to generate HO · radicals via a Fenton reaction [Eq. (5)]:

$$Fe^{II}\text{–MPE} + O_2 \rightarrow Fe^{III}\text{–MPE} + O_2^{\cdot-}; \quad (3)$$

$$2O_2^{\cdot-} + 2H^+ \rightarrow H_2O_2 + O_2; \quad (4)$$

$$Fe^{II}\text{–MPE} + H_2O_2 \rightarrow Fe^{III}\text{–MPE} + HO^- + HO\cdot; \quad (5)$$

$$Fe^{III}\text{–MPE} + \text{reductant} \rightarrow Fe^{II}\text{–MPE}. \quad (6)$$

The presence of a reducing agent in the reaction mixture regenerates the active Fe^{II}–MPE form, making possible the catalytic production of hydroxyl radicals [Eq. (6)]. Consequently, only low concentrations of iron salts are required to efficiently cleave DNA (*123–125*). Superoxide dismutase, by depleting the medium of free superoxide anion, and catalase, by disproportionating hydrogen peroxide to water and dioxygen, are both able to inhibit DNA breaks mediated by Fe^{II}–MPE.

The DNA-binding affinities of metallo-MPE were obtained with inert metal ions. The affinity constants of Ni^{II}–MPE and Mg^{II}–MPE are 2.4×10^4 and 1.5×10^5 M^{-1}, respectively, similar to the affinity constant of ethidium bromide (*125*). As expected for an intercalator, Mg^{II}–MPE unwinds DNA (11 ± 3° per bound molecule), and the binding site size is two base pairs.

The products of DNA cleavage by **2** are the free nucleobases and

5′-phosphate termini, with equal proportions of 3′-phosphate and 3′-phosphoglycolate ends (*125*), suggesting that deoxyriboses are degraded by hydroxyl radicals via H atom abstraction at C-4′, C-1′, and C-3′. A C-4′ chemistry explains the release of nucleobases and the formation of 5′-phosphate and 3′-phosphoglycolate termini, but base propenals were not detected. The 3′-phosphate ends might be produced by a C-1′ chemistry. A C-3′ chemistry has also been proposed (*7*).

Fe^{II}–MPE cleaves ds DNA in a relatively poor sequence-specific manner, which is not unexpected because the methidium entity is an intercalator of low overall base specificity. The only specificity known for this DNA cleaver is a slight preference for GC-rich regions. Sequence specificity is lower than that obtained with DNase I, making Fe^{II}–MPE useful in footprinting experiments (for a discussion on the different merits of DNase I, Fe^{II}–EDTA, and Fe^{II}–MPE in footprinting studies, see Refs. *84* and *115*). The Dervan reagent has been used to determine the location, the size, and the relative importance of binding sites of small DNA-binding molecules on native DNA (*126*, *127*) or proteins on restriction fragments (*128*, *129*), and to study RNA (*130*, *131*), chromatin (*132–134*), and ribosome (*130*) structures.

3. *Iron–EDTA Conjugates with Oligonucleotides, Peptides, or Proteins*

The Fe–EDTA moiety has been successfully attached to various sequence-specific DNA-binding molecules such as antibiotics, polypeptides, oligonucleotides, or proteins to provide new classes of "DNA affinity cleavers." This strategy allows the design of artificial restriction enzymes with defined target sequences and binding site sizes (*135*). Accordingly, hybrid molecules have been prepared by tethering EDTA to distamycin (*136–138*), netropsin derivatives (*135*, *139*), oligopeptides (*140–142*), or oligonucleotides. Such Fe–EDTA–oligonucleotide conjugates can be targeted for cleaving (1) single-stranded DNA or RNA (so-called modified antisense oligonucleotides) (*143–147*) or (2) double-stranded DNA by triple helix formation (*148–155*). As a demonstration of this latter strategy, a large genomic DNA fragment of 340-kilobase pairs of the chromosome III of *Saccharomyces cerevisiae* was cleaved on both strands at the correct location by a 20-mer having two Fe–EDTA motifs attached at both the 5′ and 3′ ends, and was able to recognize the triple-helix site inserted within this large genomic DNA (*153*).

More recently, $tRNA^{Phe}$ has been modified with an Fe–EDTA motif attached at U47 and analysis of the autocleavage products of this

modified RNA provided a visualization of the RNA tertiary structure (*156*). By attaching EDTA to a cysteine residue of a protein, introduced by site-directed mutagenesis at different positions, mapping of its different conformations has been possible. This has been done with staphylococcal nuclease (*157*). The global structure of the protein was not modified by the presence of the Fe–EDTA entity. The Fe–EDTA was linked at the Cys-31 of ribosomal protein S4 and used to explore the 16S rRNA structure by hydroxyl radical probing (*158*). It can also be tethered to a macrocycle such as sapphyrin (*159*). 2,6-Dicarboxypyridine and *N,N*-bis(2-picolyl)amine have been used as iron chelators and tethered to oligonucleotides to cleave DNA in a sequence-specific manner (*160*).

D. Chromium, Manganese, Cobalt, Nickel, Ruthenium, Rhodium, Platinum, and Uranium Complexes

Besides copper and iron complexes, many other metal complexes are able to cleave DNA. The corresponding results will be presented in this Section, except when the ligand is a porphyrin (see Section II,E for DNA cleavage by metalloporphyrins). Complexes are classified by increasing atomic number of the metal center [for a previous review article, more focused on DNA probing by metal complexes rather than on DNA cleavage, see Pyle and Barton (*161*)].

1. Chromium Complexes

Little is known about DNA damage by Cr(III) complexes, even though these compounds are considered to be responsible for chromium mutagenicity. The $[Cr^{III}(phen)_2Cl_2]^+$ and $[Cr^{III}(bpy)_2Cl_2]^+$ complexes are both mutagenic and able to cleave a supercoiled plasmid DNA via ss breaks, whereas $[Cr^{III}(CN)_6]^{3-}$ is not mutagenic and is inefficient as a DNA cleaver (*162*).

2. Manganese Complexes

Outside of the category of manganese porphyrins, one report indicates nuclease activity of a Schiff base manganese complex $[Mn^{III}(salen)]^+$ when activated by magnesium monoperphthalate. Single-strand breaks are observed at micromolar concentrations of the complex (*163*).

3. Cobalt Complexes

Λ-Tris(4,7-diphenyl-1,10-phenanthroline)cobalt(III), a photoactivatable molecular probe for left-handed ds DNA (Z-DNA), cleaves the

genome of simian virus 40 at specific sites, in the enhancer and promoter blocks and in the region downstream of 3′ termini, suggesting local Z conformations in SV40 DNA (*164*). Binding constants of $[Co^{III}(phen)_3]^{3+}$ to calf thymus DNA were found to be $9.4 \times 10^3\ M^{-1}$ by voltammetric studies (*165*).

4. Nickel Complexes

Highly sequence-specific oxidative cleavage of ds DNA has been achieved with a synthetic 55-residue protein, containing the DNA binding domain of Hin recombinase and the tripeptide Gly-Gly-His at the terminal amine (*166*). Nickel(II) is strongly chelated by these three amino acid residues and, after activation by magnesium monoperphthalate, DNA is cleaved at a single deoxyribose position on one strand of each binding site.

Simple square planar Ni(II) complexes of tetraazamacrocycles such as Schiff bases or cyclams are active as DNA cleavers in the presence of peroxides (potassium monopersulfate or magnesium monoperphthalate) (*167*, *168*). G bases are oxidized by the active form of these nickel complexes because of their preferential binding onto N-7 of guanines. One particular complex (**3**, Fig. 5), because of its steric hindrance, is able to promote the oxidation only of highly accessible guanine residues: terminal G, G mismatch, G bulge, or G in hairpin loops (*168*, *169*). The same complex has been used to probe accessible guanine N-7 positions in folded RNA structure (*170*).

5. Ruthenium Complexes

Barton *et al.* have developed the use of photoactivatable chiral ruthenium–phenanthroline complexes as probes to study DNA structures (*161*, *171*). The $[Ru^{II}(bpy)_3]^{2+}$ complexes are known to cleave DNA by photoactivation without (*172*) or in the presence of potassium peroxodisulfate (*173*, *174*). The Λ-tris(3,4,7,8-tetramethyl-1,10-phenanthroline)ruthenium(II) complex cleaves A forms of DNA helices under visible light irradiation (*171*).

The mode of interaction of such $[Ru^{II}(phen)_3]^{2+}$ derivatives with DNA has been a matter of some debate. Both intercalation and surface binding in the major groove were initially proposed for each of the enantiomers Δ and Λ, with intercalation as the predominant mode of interaction (*175*) (see also Ref. *176* for an interesting presentation of molecular recognition and chemical reactions in restricted spaces such as micelles, dendrimers, and DNA). However, viscosity data are against a classical intercalation interpretation (*177*). Additional results obtained by absorbance, fluorescence, and circular dichroism

have indicated that both stereoisomers of $[Ru^{II}(phen)_3]^{2+}$ have little selectivity for right-handed B-DNA and left-handed Z-DNA (*178*). Both enantiomers have the same affinity constant of $10^4\ M^{-1}$ for calf thymus DNA. Both NMR and circular dichroism (CD) studies of the binding of the same two enantiomers with the self-complementary oligonucleotide $d(CGCGATCGCG)_2$ suggest that the isomers bind to DNA in the minor groove at the central AT site, the observed AT specificity being more pronounced with the Δ than with the Λ enantiomer (*179*). The chiral discrimination was found to be higher when one phen ligand was replaced by a dipyrido[3,2-*a*:2′,3′-*c*] phenazine (dppz) ligand (*180a*). This latter ligand intercalates within DNA base pairs. Photocleavage of DNA by the excited state of $[Ru^{II}(bpy)_2(dppz)]^{2+}$ is not mediated by singlet oxygen, but by an electron transfer (*180b*).

Strekas *et al.* have reported the enantiospecific cleavage of DNA by Λ-$[Ru^{II}(bpy)_2(ppz)]^{2+}$, and not by the Δ enantiomer, in the presence of Cu(II), 3-mercaptopropionic acid, and hydrogen peroxide (ppz = 4′,7′-phenanthroline-5′,6′:5,6-pyrazine) (*181*). Copper binds to the second available phenanthroline site of the ppz ligand.

Thorp *et al.* have extensively studied the nuclease activity of ruthenium bipyridine-type complexes by generating Ru^{IV}–oxo entities by electrochemical methods (*182, 183*). In the presence of DNA the reactive form $[Ru^{IV}{=}O(dppz)(tpy)]^{2+}$, prepared by electrochemical $2e^-$ oxidation of $[Ru^{II}(H_2O)(dppz)(tpy)]^{2+}$ (**4**, Fig. 5; see Ref. *184* for the X-ray structure), has been shown to decay within few seconds with concomitant DNA cleavage (*184–186*). Studies of DNA degradation products generated by $[Ru^{IV}{=}O(tpy)(bpy)_2]^{2+}$ indicate that this Ru^{IV}–oxo complex cleaves DNA by attacking H-1′ on deoxyribose units, as previously observed for the $Cu(phen)_2/H_2O_2$ (see Section II,B) and Mn-TMPyP/$KHSO_5$ systems (see Section II,E). The sugar degradation product 5-methylene-2(5*H*)-furanone (5-MF, Fig. 6) was identified (*187a*). The Ru^{IV}–oxo complex is also able to oxidize guanine residues to produce piperidine-labile cleavages. It should be noted that the corresponding ruthenium(III)–hydroxo complex $[Ru^{III}{-}OH(tpy)(bpy)_2]^{2+}$ is only able to oxidize G bases, and is unable to attack deoxyribose units. Covalent adducts are formed between CT DNA and ruthenium complexes, when the excited states of these complexes are able to oxidize guanine residues (*187b*).

6. *Rhodium Complexes*

The complexes $[Rh^{III}(phen)_2(phi)]^{3+}$ (**5**, Fig. 5) and $[Rh^{III}(phen)(phi)_2]^{3+}$ (phi = phenanthrenequinone diimine) cleave supercoiled

DNA on photoactivation in the region 310–360 nm, which corresponds to the ligand-to-metal charge transfer band (*188*). The complex **5** cleaves ds DNA at 5′-pyrimidine–purine steps such as 5′-CCAG-3′. The single-site cleavage patterns suggest the absence of diffusible species mediating the strand scission, and the asymmetry in cleavage pattern oriented to the 5′ side on each strand indicates that **5** attacks B-DNA from the major groove. NMR studies support the specific intercalation of the phi ligand of the Δ enantiomer of **5** (*189*). Intercalation of **5** through the major groove is also consistent with data on DNA cleavage products. This is also true when the phen ligand is replaced by a polyamine such as ethylenediamine: the corresponding $[Rh^{III}(polyamine)_2(phi)]^{3+}$ complex intercalates within a 5′-GC base pair in the major groove of B-DNA for the Δ enantiomer and within a 5′-AT base pair for the Λ isomer (*190*).

The initial DNA cleavage step involves H-3′ abstraction by the excited state of the phi ligand of **5**. Free nucleobases are released and 3′- and 5′-phosphate ends are detected, as well as base propenoic acids and 3′-phosphoglycaldehyde residues. These latter products are consistent with the reaction of dioxygen with the 3′-centered radical (*191a*). The formation of the 3′-phosphoglycaldehyde fragment is proportionally higher with two ethylenediamine ligands and one phi ligand (*191b*).

The three-dimensional folding of $tDNA^{Phe}$ was compared to native $tRNA^{Phe}$ by using **5** as a chemical probe (*192*). Both nucleic acids are cleaved at the same sites, suggesting that the global tertiary structure of $tDNA^{Phe}$ resembles that of $tRNA^{Phe}$ (for recent studies on DNA propeller twisting, sequence-specificity recognition of DNA, and recognition based on sequence-dependent twistability with enantiomers of **5**, see Refs. *193–195*, respectively).

7. *Platinum Complexes*

A platinum dimer $[Pt_2(P_2O_5H_2)_4]^{4+}$, containing diphosphonates as assembling ligands, cleaves supercoiled DNA when photoactivated (*196, 197*). The long-lived triplet state (9.5 μsec) of this platinum complex is probably responsible for its oxidative nuclease activity.

8. *Uranium Complexes*

Uranyl(III) salts such as $UO_2(OAc)_2$ or $UO_2(NO_3)_2$ have been used as photochemical agents for DNA cleavage and for probing of protein–DNA contacts (*198–199*). Single-strand DNA breaks are induced by uranyl acetate or uranyl nitrate under irradiation in the 420-nm region. The binding constant of the UO_2 dication is estimated to be of

the order of 10^{10} M^{-1} at pH 4 and preliminary data on DNA cleavage products suggest that the UO_2^{2+} chromophore binds in the minor groove by bridging phosphate groups of opposite strands. Photocleavage by UO_2^{2+} is not oxygen dependent and piperidine treatment does not significantly increase the number of DNA breaks, and free nucleobases are generated. All these data are in favor of H-1′ abstraction by the excited state of the uranyl salt, which is able to oxidize alcohols and olefins in aqueous medium (*199*).

E. Metalloporphyrin Complexes

The following discussion focuses on (1) the different ways of interaction of metalloporphyrins with DNA, (2) the two major modes of activation, by light or by oxidants, and the corresponding reactive species that are generated, and (3) the mechanism of DNA cleavage, which will be mainly detailed for the manganese derivative of the most extensively studied DNA-binding porphyrin, namely *meso*-tetrakis(4-*N*-methylpyridiniumyl)porphyrin (H_2TMPyP).

1. Interaction of Metalloporphyrins with DNA and Cleavage Activity

Since publication of the early works on the DNA intercalation and cleavage properties of some cationic porphyrin derivatives (*200–203*), a large variety of porphyrins and metalloporphyrins have retained the attention of researchers and generated a great number of works in the field of chemical and photochemical cleavage of DNA. The most frequently studied compounds in that family are natural products such as hemin [ferric protoporphyrin IX (*204–206*)], deuterohemin (*206*), coproporphyrin (*207*), or methylpyrroporphyrin (*208*, *209*), and also synthetic derivatives based on the porphyrin core bearing a large variety of hydrophobic, anionic, or cationic functional groups in the meso positions (*202*, *210–213*) (Fig. 7). All these ligands can take up various metals, such as Zn, Fe, Co, Mn, and Ni, which modulate the interaction and the reactivity with DNA. At least (see below), covalent attachment of the porphyrin ligand to a variety of suitable vectors opens the possibility to target their cleavage properties on selected sequences of DNA.

Binding mechanisms depend on the nature of the metal, its size, and its charge, and on the location of different substituents at the porphyrin periphery. In the absence of any X-ray crystallographic data on a porphyrin–DNA complex, our understanding of the nature of the binding process is based on the results of a variety of physicochemical studies: extensive NMR, equilibrium dialysis, flow dichro-

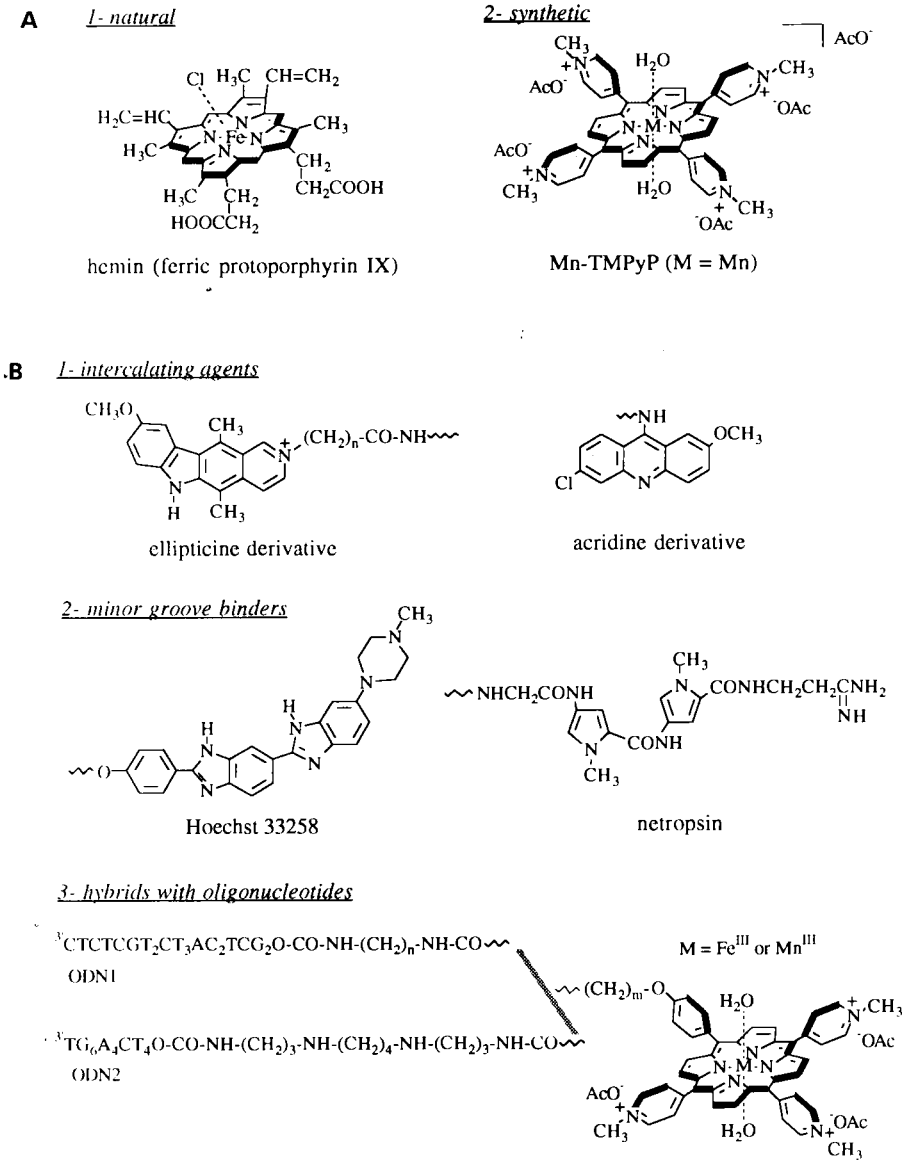


FIG. 7. (A) Examples of metalloporphyrins used in DNA cleavage. (B) Examples of vectors and hybrid molecules.

ism, and viscosimetry measurements (*214*, *215*). Some general features concerning cationic porphyrins can be pointed out, mainly deduced from the present knowledge on H_2TMPyP and its metallated derivatives:

1. The free ligand H_2TMPyP and its square planar complexes with Ni^{II} and Cu^{II} interact with poly(dG–dC)$_2$ and behave as intercalating molecules (*202*, *214*, *216*). H_2TMPyP is aligned perpendicular to the DNA helix axis (*217*, *218*). With poly(dA–dT)$_2$, these same nonaxially liganded molecules do not intercalate but bind externally, probably in the minor groove because of its higher electrostatic potential (*202*, *210*, *214*, *216*, *217*, *219–222*). The affinity constant of H_2TMPyP for calf thymus DNA is $7.7 \times 10^5\ M^{-1}$ (*210*, *223*).

2. Cationic complexes possessing (i) bulky substituents on the porphyrin framework or (ii) at least one axially bound ligand are too thick for intercalation into poly(dG–dC)$_2$, poly(dA–dT)$_2$, or DNA. As an example of the first category, due to a strong hindrance to the rotation of the ring substituents, porphyrins substituted with *ortho*-methylpyridiniumyl groups do not intercalate. In fact, only half of the porphyrin ring is necessary for DNA intercalation, and most of the porphyrins are capable of intercalative binding provided at least two *meso*-pyridiniumyl substituents in cis position can freely rotate and come in-plane with the porphyrin macrocycle (*210*). As an example of the second category, Zn-TMPyP, which is five-coordinated, does not intercalate, but lies at an angle of 62–67° relative to the helix axis and binds outside to AT sites (*202*, *224*, *225*). For the diaxially liganded complexes with Mn^{III}, Fe^{III}, or Co^{III}, intercalation is also precluded, consequently they bind in an outside fashion to DNA. The Co–TMPyP complex displays an orientation angle of roughly 45° relative to the helix axis and appears to bind to a partially melted region of DNA (*217*). These outside-binding porphyrins are believed to interact in the minor groove of DNA. A detailed study of the chemical reactivity of Mn–TMPyP (*226*, *227*) showed effectively that this metalloporphyrin cleaves DNA from the minor groove. The interaction is largely electrostatic in nature, because, as expected, it is sensitive to ionic strength. The exact nature of the ligand(s) occupying the axial site(s) of Mn–TMPyP under the conditions of most DNA binding studies is unknown. On the basis of recent X-ray studies analyses of Mn–TMPyP (*228*), axial ligand candidates are two water molecules that may serve as DNA donor/acceptor hydrogen bond sites with DNA.

Analyses of Mn–TMPyP–mediated strand scission on a restriction fragment (*224*), and on a series of short ds ODNs containing a tri-

nucleotide sequence consisting only of adenine and thymine bases (i.e., three contiguous AT base pairs, called the AT triplet) (*227*), have revealed that this AT triplet has to be considered as the minimal size of the metalloporphyrin-preferred binding site. Such cleavage selectivity is due to a tight interaction between this manganese porphyrin and AT-rich regions of DNA and is consistent with the relative affinity of Mn–TMPyP for poly[d(A–T)·d(A–T)] and poly[d(G–C)·d(G–C)] (12×10^4 and 0.2×10^4 M^{-1}, respectively) (*229*). It can be explained by electrostatic interactions of the cationic porphyrin, with the more negative potential in the minor groove of AT-rich polymers compared to GC polymers (*227*). When the AT sequence is longer than a triplet, cleavage not only affects adjacent GC or CG base pairs, but also base pairs inside the AT-rich sequence. A duplex that contains, for example, four overlapping triplets gives four consecutive cleavage sites (*230*).

Hydrophobic or anionic porphyrin derivatives do not exhibit any intrinsic affinity for DNA and so the only ways to induce DNA oxidative damage are to force them to stay close to DNA, either by linking to DNA vectors, by using dicationic metal salts in order to favor ternary associations of porphyrin/metallic cation/DNA, or by working at high concentrations with derivatives able to generate diffusing active species.

As pioneered by Dervan *et al.* (*123*, *231*), there is the possibility of targeting the activity of a DNA cleaver by attaching it to different vectors (see also Sections II,B,2, II,C,2, and II,C,3). We describe now work done in our laboratory on the different ways to recognize the cut specific sequences of DNA by linking tailored cationic manganese porphyrins to intercalators, minor-groove binders, or oligonucleotides as a rather well-documented example (see Fig. 7 for structures and Fig. 8 for DNA interaction modes).

The covalent attachment of a chelated redox-active transition metal to an intercalating agent is a way to mimic bleomycin (see Section II,2,A), as shown by Lown and Joshua, by attaching an iron(III) deuteroporphyrin IX to 9-chloroacridine via a polyamine linker (*232*). Shudo *et al.* have used a potent mutagen (resulting from glutamic acid pyrolysis) as an intercalator to be linked either to a derivative of Fe^{III}-[tris-*o*-tolyl(*p*-aminophenyl)]porphyrin, to a hydrophobic macrocycle complex, or to Fe^{III}–protoporphyrin IX (*233*). These molecules are able to cleave ds DNA in the presence of molecular oxygen and a reducing agent, but only using high amounts of probes relative to DNA. Recently, hemin was covalently connected to two 9-aminoacridines, giving a bisdentate compound

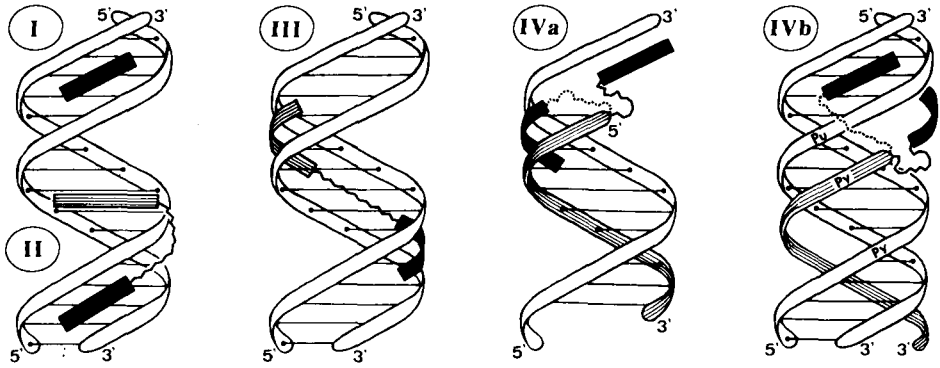


FIG. 8. Different ways to address the nuclease activity of cationic manganese porphyrins on selected sequences of DNA: free Mn–TMPyP (**I**) (*226, 227*); the vector strategy can be an intercalator (**II**) (*237, 238*), a minor-groove binder (**III**) (*240*), an ODN in antisense (**IVa**) (*246, 250*), or an antigene (**IVB**) (*247, 248, 289*). The solid black areas represent cationic manganese porphyrin; the striped areas are vector. Two possible ways of interaction of the manganese porphyrin moiety with DNA are represented by solid and dotted wavy lines.

with higher DNA binding affinity than that of the hemin (*234*). The first synthesized "metalloporphyrin–ellipticine" hybrid molecule was based on a tris(*p*-tolyl)porphyrin moiety (Fig. 7) (*235*). Because one key point in this strategy is to choose porphyrins having an intrinsic affinity for DNA, it was decided to use only cationic porphyrin precursors (*236–238*). The affinity of such cationic metalloporphyrin–ellipticin conjugates for a DNA duplex depends on the nature and the length of the linker and ranges from 2.9×10^8 to $8.2 \times 10^9\ M^{-1}$ for poly[d(A–T)] and from 1.0 to $7.0 \times 10^8\ M^{-1}$ for poly[d(G–C)] (*229, 238*). These hybrid molecules (iron or manganese complexes) are cytotoxic against L1210 cells in culture with ID_{50} values ranging from 0.7 to 1.6 μM, whereas the corresponding zinc or nickel hybrids are not cytotoxic. On the same cell line, the ID_{50} value of BLM is only 1.7 μM (*238*).

Recently, potential sequence-specific DNA cleavers have been prepared by attaching a cationic manganese porphyrin motif and minor-groove binders such as the antibiotic netropsin (*239*) or Hoechst 33258, a bisbenzimidazole dye having a strong preference for AT-rich regions (see Fig. 7 for structure) (*240a*). Preliminary results obtained with a Mn–cationic porphyrin/H33258 hybrid showed that the dye was directing the selectivity of cleavage (*240b*).

Several examples of sequence-selective cleavage of DNA by various cleaving agents covalently attached to oligonucleotides have been de-

scribed in the literature (see Sections II,B,2 and II,C,3). First, hybrid molecules based on metalloporphyrin-substituted ODNs were prepared with hydrophobic derivatives of methylpyrroporphyrin XXI tethered to $(dT)_7$ ODNs (*208*). The corresponding iron(III) compound activated by H_2O_2 induced, on the ss DNA target, direct breaks and cross-linked products; these latter lesions were partly transformed to DNA breaks after piperidine treatment. Damage appears to cover 10 bases around the position of the reactive moiety on the target sequence, but only limited yields of cleavage were attained (10–20%). In a similar approach, antisense ODNs linked to proto- and methylpyrroporphyrins and their zinc derivatives were shown to create cross-links on an RNA target at G bases located close to the photosensitizer in the hybrid complex (*209*). Iron(III)–hemin or deuterohemin conjugates, on activation with H_2O_2 or reductants, gave alkali-labile lesions and alkali-labile cross-links (*206*, *241*). Also described is a palladium–coproporphyrin I conjugate (*207*) that, by photochemical activation, leads to 30% of DNA cleavage over 10 bases on the target sequence. The iron(III) *meso*-tetra(4-carboxyphenyl)porphyrin–ODN hybrid in the presence of a reductant mediates an efficient direct DNA cleavage at low concentration, with a reagent:target ratio of 1:1 (*242*).

Cationic metalloporphyrins, due to their high intrinsic affinity for nucleic acids, appear as the most reasonable candidates to be coupled to ODNs. The metalloporphyrin moiety (bottom of Fig. 7) usually carries three cationic pyridiniumyl groups (*243*) but derivatives bearing four cationic substituents, as it is the case in the parent compound Mn–TMPyP, have recently been described (*244*, *245*). Such conjugates were used to target the initiation codon region of the *tat* gene of the HIV-1 genome in an antisense strategy (*246*) or to target one possible triple helix site in the *rev* and *pol* genes of HIV-1 in an antigene strategy (*247*, *248*) (Fig. 7, ODN1 or ODN2; for a review on the triple-helix strategy, see Ref. *249*). Development in this field presumes a careful design of the tether of the conjugate, depending on the cleaving agent and on what is known about its interaction with the target as well as chemical modifications to improve their stability (*206*, *208*, *229*, *241*, *245*, *248*, *250*).

Other hybrid molecules have been prepared wherein porphyrin is attached to the intercalating agent acridione (*251*), to a nucleoside (*252*), to chlorambucil (a DNA cross-linking agent) (*253*), or to dextran (a water-soluble polymer) (*254*). The synthesis of a bis(arginyl) tricationic porphyrin designed to target the major groove of DNA has been reported (*255*). The concept of hybrid porphyrin vector molecules has

been extended to pyropheophorbide–polyamine molecules (*256*), which are able to cleave DNA by electron transfer when activated at 690 nm, a suitable wavelength in phototherapy.

2. *Modes of Activation and Reactive Species*

Depending on their structure, porphyrin derivatives can be either chemically or photochemically activated to degrade biological molecules and especially to produce DNA breaks.

a. Photoactivatable Porphyrin Derivatives: Singlet Oxygen or Electron Transfer? Two different categories of photoactivatable porphyrin derivatives are currently under study as photosensitizers in the photodynamic therapy approach: (1) derivatives of hematoporphyrin, such as HpD, which is a modified protoporphyrin IX with two α-hydroxyethyl groups instead of two vinyl groups, and (2) fully synthetic porphyrin derivatives having a noticeable affinity for DNA.

The first category corresponds to porphyrin molecules having hydrophobic substituents at the periphery of the tetrapyrrole ring or substituents bearing hydroxy or carboxylic acid functions. Some of these HpD derivatives have been proposed for phototherapy of cancers in human clinical trials, including Photofrin, which is currently used in the treatment of superficial bladder cancer (*257–260*). The toxicity of these porphyrin derivatives on tumor cells is mainly due to their affinity for membranes (*257*) and may be explained, when they are activated with red light at 630 nm, by the generation of reactive singlet oxygen in a Type II photochemical reaction. Current efforts are focused on the preparation of new macrocyclic conjugated molecules having a strong absorption between 600 and 700 nm, in the region corresponding to the maximum of light penetration through tissues. Phthalocyanine or pyropheophorbide derivatives are potential new photosensitizers (*261–263*).

The second category of porphyrins that can be photoactivated corresponds to those molecules exhibiting a reasonable affinity for DNA or RNA, and as a result are potential DNA or RNA cleavers. The paradigm molecule in this domain is the *meso*-tetrakis(4-*N*-methylpyridiniumyl)porphyrin (H_2TMPyP, Fig. 7, M = H_2), which exhibits high quantum yields for formation of singlet oxygen (*264*). The photonuclease activity of H_2TMPyP was initially reported by Fiel *et al.* in 1981 (*265*). In further studies, it was confirmed that H_2TMPyP and Zn–TMPyP are able to photolyse supercoiled plasmid DNA to open circular and linear DNA by irradiation at 436 nm (*266, 267*). Single-stranded breaks are the main damage induced on ds DNA by

photoactivated H_2TMPyP, occurring mainly at guanine bases and also on thymines. Inhibition of cleavage by nitrogen bubbling (dioxygen removal) suggests that the Type II photoprocess (mediated by singlet oxygen) occurs with significantly higher yields compared to Type I reactions (electron transfer reactions between porphyrin excited states and DNA bases) and are responsible for the formation of alkali-labile sites in aerated systems (*268*).

With photoactivatable cationic zinc porphyrin–ellipticine conjugates (*269*), both the fluorescence yield and production of singlet oxygen are lower compared to those of Zn–TMPyP, the parent porphyrin molecule, due to the quenching of the singlet state of the porphyrin moiety by the attached ellipticine. Interestingly, these photochemical properties are dramatically enhanced in the presence of DNA as a result of a conformational change: the ellipticine moiety intercalates in ds DNA and so can not quench the zinc–porphyrin singlet state. On irradiation at 436 nm, the photocleavage of supercoiled ΦX174 DNA is 50-fold greater than that of HpD and involves singlet oxygen as a probable main species responsible for DNA cleavage (*270*).

It has been shown that phosphorus(V) tetraphenylporphyrin is also capable of inducing DNA cleavage via a photoreaction involving singlet oxygen, and an electron transfer from DNA to the short-lived singlet excited state of the phosphorus (*271*). Water-soluble sulfonated porphyrins such as H_2TPPS or Zn–TPPS are good photosensitizers, but very poor DNA cleavers, probably because of their weak interactions with DNA due to their anionic groups (*265*, *267*).

b. Activation by Oxidants and Reactive Species Involved: Diffusing Oxygenated Reactive Species or Nondiffusible Metal–Oxo Entities? The oxidative cleavage of DNA with a cationic water-soluble porphyrin was first demonstrated by Fiel *et al.* with Fe-TMPyP (Fig. 7, M = Fe) activated by a reducing agent in the presence of molecular oxygen (*200*). Then Dabrowiak *et al.* (*224*, *272*) showed that not only reducing agents such as ascorbate or superoxide anion in the presence of oxygen but also oxygen atom donors such as iodosylbenzene are able to activate the complex with an efficiency depending on the metal. Several other activating agents were subsequently investigated: potassium monopersulfate ($KHSO_5$) (*272–274*), magnesium monoperphthalate (MMPP) (*275*, *276*), and, more recently, peroxynitrite ($ONOO^-$) (*277*). Hydrogen peroxide appears 10^3- to 10^4-fold less efficient as cofactor compared to $KHSO_5$ for Mn–TMPyP activation (*230*, *274*). These single-oxygen-atom donors are expected to react directly with the metalloporphyrin in a one-step process to yield a cata-

lytically active species and it is thought, by analogy with cytochrome *P*-450 modeling studies (*36*), that the reactive species involved in DNA breaks is a high-valent oxo intermediate [Eq. (7)]. Under treatment with ascorbate in the presence of molecular oxygen, reductive activation of O_2 by manganese or iron porphyrins can also yield high-valent metal–oxo species [Eq. (8)]. Alternatively, H_2O_2 and peroxy radicals are possible intermediates, as suggested by the use of inhibitors and scavengers, but diffusible hydroxyl radicals can be excluded. However, hydroxyl radicals produced in close proximity to a DNA target might be involved [Eq. (9)] (*278*):

$$\text{Por–M}^{\text{III}} \xrightarrow[\text{MMPP, or } H_2O_2]{+\ \text{PhIO, KHSO}_5,} \text{Por–M}^{\text{V}}\text{=O}; \tag{7}$$

$$\text{Por–M}^{\text{III}} \xrightarrow{+\ e^-} \text{Por–M}^{\text{II}} \xrightarrow[-\ H_2O]{+\ O_2 + e^- + 2H^+} \text{Por–M}^{\text{V}}\text{=O}; \tag{8}$$

$$O_2 \xrightarrow{+\ \text{Por–M}^{\text{II}}} O_2^- \xrightarrow{+\ H^+} O_2 + H_2O_2 \xrightarrow{\text{Por–M}^{\text{II}}} + HO^- + HO\cdot; \tag{9}$$

$$\text{Por–M}^{\text{III}} \xrightarrow{H_2O_2} \text{Por–M}^{\text{IV}}\text{–OH} + HO\cdot. \tag{10}$$

For Mn–TMPyP activated by $KHSO_5$ (*272*, *273*), the quasi-absence of additional weaker bands close to the main fragments in PAGE analysis strongly supports a nondiffusible active species, namely, a high-valent manganese–oxo porphyrin complex similar to the iron–oxo intermediate involved in cytochrome *P*-450 chemistry [Eq. (7)]. An investigation using Mn–TMPyP/$KHSO_5$ in aqueous solution to oxidize carbamazepine, an analgesic and anticonvulsant drug, shows that, through a "redox tautomerism" mechanism involving a coordinated water molecule on the metalloporphyrin catalyst, the oxidizing entity can be localized on one or the other face of the activated metalloporphyrin (*279*). Subsequently it was reported that oxidation at C-1′ of DNA deoxyribose results from such a mechanism, supporting a Mn^V–oxo as the reactive species involved in a cytochrome *P*-450-type hydroxylation reaction during DNA cleavage (*280*). With hydrogen peroxide as cofactor, breaks are as well-defined, as in the case of $KHSO_5$, and probably result from the same chemistry (*230*), suggesting that H_2O_2 is able to generate Mn^V–oxo species via a heterolytic cleavage of the peroxidic O–O bond [Eq. (7)]. However, because the reaction is much less efficient, the question of a homolytic cleavage of H_2O_2 giving hydroxyl radicals and M^{IV}–OH species with an intrinsic lower reactivity [Eq. (10)] is still open in this particular case.

In similar conditions with $KHSO_5$, Fe-TMPyP is less active compared to the manganese derivative: such a difference is not fully understood at present. Electrochemical methods were also used to activate Mn^{III} and Fe^{III} complexes of TMPyP to bring about DNA cleavage (*281*).

Anionic porphyrins are usually much less reactive, as it has been reported for Fe–TPPS activated with DTT (*200*). Cleavage experiments with anionic manganese porphyrins activated by $KHSO_5$ failed, even in the presence of polycations (*274*). In the case of hemin (ferric protoporphyrin IX chloride), for which some of its effects on gene expression might be mediated by nicking of DNA, strand scissions require the presence of O_2, a reducing agent, and transition metals salts, e.g., Co^{II}, Zn^{II}, Ni^{II}, used to favor a ternary association, porphyrin/metallic cation/DNA (*204*, *205*). In another strategy, hemin or deuterohemin linked to ODNs showed unexpected selective cleavage (30 to 50% of the target) after activation by either H_2O_2 or reducing agents and subsequent piperidine treatment (*206*, *241*). Linked to intercalating agents, deuterohemin activated by molecular oxygen and reducing agents appears to be a weak DNA-cleaving agent. Because DNA cleavage was partially inhibited by catalase and superoxide dismutase, this suggests that these BLM models produce reduced oxygen species (superoxide, hydrogen peroxide, hydroxyl radicals) rather than metal–oxo entities.

3. *Mechanism of DNA Cleavage*

The mechanism of DNA damage may involve either diffusing oxygen-reactive species, implying that the damage spans over several bases, or a nondiffusible oxidative species coordinated onto metalloporphyrin, inducing then a more localized damage. Oxidation of bases or deoxyriboses can lead to direct breaks, to alkali-labile lesions, and also to the formation of cross-linked products that can be converted (partially or totally) to DNA breaks after alkali treatment. The mechanism of DNA cleavage is now well-known for the Mn–TMPyP/$KHSO_5$ system. Additional and partial information exists for few other porphyrin derivatives.

a. Deoxyriboses as Main Targets for the Mn^{III}–TMPy / $KHSO_5$ System. When produced in the proximity of DNA, one (at least) of the reactive species evoked above attacks deoxyribose moieties and produces oxidative lesions, leading to breaks of the sugar phosphate DNA backbone, according to the mechanism detailed below. Gel electrophoresis analyses of DNA binding and cleavage specificity indi-

cated that cationic manganese or iron porphyrins bind to the minor groove and cleave DNA at all four possible nucleotide positions, strongly suggesting that deoxyriboses are the primary sites of attack (*225*). Other preliminary data were obtained by reference to the mechanism of BLM: neither Mn^{III}, Fe^{III}, or Co^{III} complexes of H_2TMPyP activated by ascorbate, superoxide ion, or iodosylbenzene (*224*) nor Mn–TMPyP in the presence of $KHSO_5$ (*276*) produce base propenals, the main characteristic products of DNA damage induced by BLM. At least, these data suggest that, if H-4′ is the target for the high-valent manganese–oxo species as observed for metallo-BLM-mediated DNA breaks, then the oxygen-dependent route is not involved (see Fig. 4). Alternative targets accessible from the minor groove are the tertiary C–H bond at C-1′ and two secondary C–H bonds at C-2′ (pro-R H-2′) and C-5′ (pro-S H-5′) (see Ref. *7* for a discussion on the accessibility of sugar C–H bonds with respect to groove binding). Further studies evidenced two main mechanisms:

1. First, a simplified model of DNA, polydeoxyadenylic acid, was shown to be readily cleaved at neutral pH by Mn–TMPyP/$KHSO_5$ with spontaneous release of free adenine and, after heating, formation of a rather unstable sugar degradation product identified as 5-methylene-2(5*H*)-furanone, supporting a C-1′ oxidation pathway. The first β-elimination created a 5′-phosphate end and the second one was accompanied by 5-MF release. The two DNA strands were terminated by phosphate groups (Fig. 6, route A) (*282*).

2. On extension to other polydeoxynucleotides, ds copolymers, and calf thymus DNA (*226*, *276*) it was shown that (i) a preliminary amount of free bases was released at ambient temperature; (ii) after heating, two other products were detected and have been identified as 5-MF and furfural (FUR) by HPLC; (iii) the appearance of FUR was correlated with an enhanced release of the corresponding bases during the thermal step and provided evidence for a C-5′ oxidative pathway (Fig. 6, route B): hydroxylation at C-5′ gives a C-5′ aldehyde at one strand terminus and a 3′-phosphate at the other at ambient temperature. On heating, two β-eliminations result in the formation of FUR and in free base release, leaving a phosphate at the 5′ terminus (*227*, *230*, *283*). The α,β-unsaturated aldehyde intermediate produced by the first β-elimination was formally identified during the course of cleavage reaction on poly(dA)·poly(dT) (*284*). Both routes A and B gave rise to the same phosphate termini, but the sugar degradation products were different and the chronology of base release and DNA break was inverted. The two monophosphate esters (at 3′ and

5′ ends) on both sides of the cleavage site were characterized by ^{31}P NMR analysis (*285*).

The FUR : 5-MF ratio is indicative of the relative reactivities of the activated Mn–TMPyP complex toward C-5′ and C-1′ for various DNA and DNA models. The C-1′ target is the main hydroxylation site for calf thymus DNA and GC polymers when selective attack at C-5′ is observed for AT polymers or AT-rich sequences in DNA, which suggests that the mechanism of DNA cleavage is highly sequence dependent. Hydroxylation at C-1′, which is located in the minor groove, also confirms that the cationic manganese porphyrin interacts with this groove. Subtle changes in the interactions of the cleaving reagent with DNA explain that reactivity changes from one target to the other: the largely opened single helix of ss polydeoxynucleotides or the widened minor groove of GC-rich ds polymers allows the more reactive tertiary C-1′ carbon to be oxidized. For AT-rich ds polymers, the narrow minor groove restricts the access to C-1′, deeply located in the groove, and then C-5′, which is located at the entrance of the groove, is preferred. Until now, oxidative chemistry at C-1′ of deoxyribose has been demonstrated only for $Cu^{I}(Phen)_2/H_2O_2$ (*7*, *83*, *286*), enediyne antibiotics such as neocarzinostatin in the presence of thiols (*7*, *287*), and more recently for ruthenium–oxo complexes (*187a*). Oxidative chemistry at C-5′, besides the case of Mn–TMPyP, represents the major mode of DNA cleavage by neocarzinostatin (*287*), but, because of the presence of reducing agents, the end-product FUR could never be observed (*7*).

b. Nucleobases as Possible Alternative Targets. In the case of hydrophobic or anionic metalloporphyrins linked to oligonucleotides, induced damage to the DNA target included chain scission, alkali-labile lesions, and cross-links that were partly transformed to DNA breaks (*206–208*, *241*). Hemin or deuterohemin conjugates show unexpected selective cleavage, at guanine positions in the close vicinity of the metalloporphyrin location on the target sequence (*206*, *241*). This guanine-selective DNA cleavage is due to complete transformation of cross-linked products on piperidine treatment, but nothing is known on the chemical nature of this base damage. In the case of cationic metalloporphyrins linked to oligonucleotides, an ODN–manganese porphyrin hybrid was shown to recognize and cut selectively the ss target sequence, even in the presence of a large excess of random DNA (*246*). The vectorized metalloporphyrin cleaved the labeled 35-mer target at 10 n*M*, a concentration two orders of magnitude order lower than the "free" metalloporphyrin Mn–TMPyP. Because the

cleavage pattern shows an intense smear near the junction of ss and ds regions of the duplex, the mechanism, in this case, must not be restricted to the specific 5′ or 1′ attack, as previously observed for Mn–TMPyP (*246*, *288*). On hot piperidine treatment, these smears are transformed into cleavage products at G residues, the intensity of the cleavage sites revealed by piperidine decreasing with the distance from the location of the metalloporphyrin moiety. The exact nature of the damage still remains to be elucidated, but it is clear that guanine damage strongly suggests that the active manganese–oxospecies is not acting only as a deoxyribose cleaver, as observed when the cationic manganese porphyrin is not tethered to an ODN (*245*, *250*, *289*).

III. Metal Ions and Nucleic Acid Hydrolysis

A. Role of Metal Ions in Enzyme-Catalyzed Nucleic Acid Hydrolysis

Nucleases are able to catalyze the hydrolysis of P–O bonds of phosphodiesters within a few seconds (*290*). This efficiency is impressive, because we know that the same noncatalyzed hydrolysis is a very slow reaction at ambient temperature (the half-life of a phosphodiester linkage of DNA being estimated to be 200 million years at pH 7), making very challenging the rational design of metallonuclease mimics.

During the past decade, many crystal structures of phosphate-hydrolyzing enzymes have been determined at high-level resolution, allowing a precise determination of metal ion binding sites. These ions, calcium, magnesium, zinc, iron, and manganese, play a key role in two catalytic events, in neutralizing the negative P–O charge by cation complexation and in assisting the nucleophilic attack of a coordinated H_2O/OH^- (or an amino acid residue) onto the tetragonal phosphorus atom of the phosphate to be cleaved.

The role of the divalent metal ions present in natural phosphodiesterases became clear in bovine pancreatic deoxyribonuclease I (DNase I), the first endonuclease structure determined by X-ray crystallography. The nucleophilic attack of a water molecule activated by a histidine residue is facilitated by the interaction of a calcium ion with the phosphate group to be cleaved (*291*). Glutamic and aspartic residues involved in magnesium binding have been identified in the crystal structure of four type II restriction enzymes: *Eco*RI (*292*), *Eco*RV (*293*), *Pvu*II (*294*), and *Bam*HI (*295*), as well as in that of the repair

enzyme *E. coli* exonuclease III (*296*). Recent studies on *Eco*RV report that the apparent affinity of the enzyme for magnesium(II) is much higher at the recognition site (GATATC) than at nonrecognition sites (*297*, *298*). Two metal ion binding sites have been identified in the structure of the exonuclease catalytic site of the Klenow fragment of DNA polymerase I of *E. coli:* one for zinc (two aspartic and one glutamic residues) and the second one for magnesium (two aspartic residues) (*299*). A similar spatial distribution of acidic amino acid residues was found in the structure of *E. coli* DNA topoisomerase I, an enzyme involved in DNA topology by breaking and religating ds DNA (*300*). Alkaline phosphatase (*301*), phospholipase C (*302*), the ribonuclease H domain of HIV-1 reverse transcriptase (*303*), and P1 nuclease (*304*) are additional examples of hydrolytic enzymes having at least two metal ions at their active site. The crystal structure of the kidney bean purple acid phosphatase has been determined (*305*). This enzyme catalyzes adenosine triphosphate hydrolysis and its active site contains a dinuclear Fe^{III}–Zn^{II} motif. In this case, a transition metal is involved in the active site of the enzyme and its role in the phosphate hydrolysis is still an open question. One possibility is that the phosphate ester coordinates at the more labile Zn^{II} and that Fe^{III}–OH is then involved in inducing hydrolysis at the phosphorous. With all the detailed crystal structures now available, it is possible to better understand the role of the metal ions acting as cofactors in DNA cleavage catalyzed by hydrolyzing enzymes.

When at least two metal centers are present, the mechanism of DNA cleavage is referred to as "the two-metal-ion assisted phosphoryl transfer." The pair of metal ions could serve as a Lewis acid, polarizing the P–O bonds of the substrate to make a better electrophile, orient the two reactants (the phosphorus center and the nucleophile), and stabilize the pentacoordinate transition state. One metal would activate an incoming nucleophile (e.g., a coordinated water molecule) by lowering its pK_a and facilitating its attack on the phosphorus atom, the other one would stabilize the leaving oxygen atom linked to the 3′-carbon of the sugar residue (Fig. 9). The phosphate ester to be cleaved is not always bridging two metals; it can bind to one metal only, as in P1 nuclease (*304*).

Because the metal center is usually chelated by several carboxylate residues that reduce the overall charge of the resulting complex, this might render deprotonation of a metal-bound water molecule less likely. Karlin has proposed that a water molecule or a hydroxo ligand bridging two metals could have a considerably lower pK_a and might be involved in the nucleophilic attack (*306*). Such a bridging hydroxo

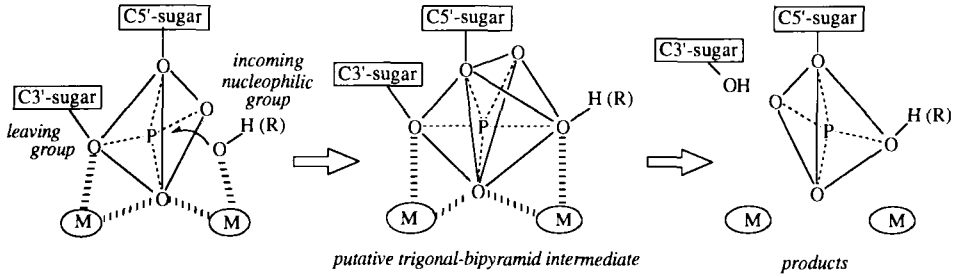


FIG. 9. Proposed DNA phosphate hydrolysis by enzymes containing dinuclear metal centers, via "two-metal-ion assisted phosphoryl transfer." Solid lines indicate the geometry around the central phosphorus atom. Charges on oxygen atoms and metals have been omitted.

ligand is present in the crystal structure of the kidney bean purple acid phosphatase (*305*), wherein the Fe^{III}–Zn^{III} distance is 3.1 Å. It is also proposed for *Penicillium citrinum* P1 nuclease, which shows two zinc ions separated by 3.2 Å but, apparently, this bridging hydroxo (in fact, the authors suggested a water molecule) is not directly involved in the described catalysis mechanism (*304*). Such nucleophilic bridging oxygen atoms are also proposed for hydrolysis of model compounds such as phosphate diesters (*307*) or monoesters (*308*).

An S_N2 phosphate nucleophilic displacement seems to prevail in biology (*309*). The concomitant approach of the nucleophile and the release of the leaving group take place at and from apical positions of a pentavalent phosphorus intermediate. The stereochemistry of the P–O bond hydrolysis is an inversion of configuration as evidenced for the first time for the 3′-exonuclease activity of DNA polymerase I (*310*). The nucleophile approaches on one face of the initial tetrahedral phosphate group and promotes the spatial rearrangement to the trigonal bipyramid transition state (pentavalent phosphorus intermediate), where the leaving group is opposite to the incoming nucleophile. The O–P–O angles must change from the initial tetrahedral value of 109° to 120° for equatorial groups and to 90° for the two apical groups with respect to the equatorial plane. A two-metal core can stabilize the pentavalent transition state or intermediate (Fig. 9).

B. Hydrolysis of RNA. The Case of Ribozymes

In RNA, the 2′-OH group on the ribose unit constitutes an intramolecular nucleophile, making the attack of the vicinal 3′-phosphodiester bridge much easier than that for DNA, which is deprived of such

a possibility. A first trans-esterification step involving the 2′-OH nucleophilic attack on the 3′-phosphate leads to a five-membered ring phosphate and so to fragments of RNA, ending with a 2′,3′-cyclic phosphate on one side and a 5′-OH function on the other side. Hydrolysis of the cyclic phosphodiester occurs readily, this second step being facilitated by the fact that the phosphodiester to be attacked by water is a cyclic strained phosphodiester (*311*). Nucleophilic attack of the 2′-OH is the key step in the alkaline hydrolysis of RNA.

Nonenzymatic hydrolysis of RNA assisted by metals (*312–316*) as well as by some ribozymes (*317, 318*) presumably follows the mechanism described above. Metals may be involved in the deprotonation of the 2′-OH group (*319*). Ribozymes, now recognized as metalloenzymes (*318, 320, 321*), correspond to selected sequences of RNA able to cleave the same strand (intramolecular process) or an RNA sequence on a different strand (intermolecular process). Metal ions are necessary to ribozymal activity (*318*). They promote the folding of the secondary structure of RNA into an active tertiary structure, and it is difficult to discriminate between their structural and/or catalytic role (*322, 323*).

The so-called hammerhead ribozymes belong to the category of ribozymes that involve the 2′-OH group of adjacent ribose as a nucleophile. Recent kinetic data suggest that two magnesium ions are involved in the cleavage step (*317*). One Mg^{II} is linked to the 2′-oxygen atom of the ribose ring at the cleavage site and the second one acts as a Lewis acid by coordinating the leaving 5′-oxygen atom. Despite a large value of 4.4 found for the apparent solvent isotope effect $k_{cleav}(H_2O)/k_{cleav}(D_2O)$, a proton transfer step is not involved in the rate-determining step of the RNA cleavage. Because the concentration of Mg^{II}–OD in D_2O is severalfold lower than that of Mg^{II}–OH in H_2O, a magnesium–hydroxo species is probably the key intermediate in the ribozyme-catalyzed reaction (*317*).

The mechanism of RNA cleavage does not always make use of the advantage of the adjacent 2′-OH nucleophile, because examples exist of enzymatic hydrolysis or cleavage by trans-esterification of RNA, for which the products of the reaction are 5′-phosphate and/or 3′-OH ending fragments. This latter mechanism, involving an external nucleophile, has been observed for RNase H (*304*) or RNase P (*324*) or group I and II introns (*318, 320, 321, 325*). Whatever is the attacking nucleophile (intramolecular or external), both mechanisms involve an in-line S_N2 nucleophilic substitution at the phosphorus center (*317, 328, 325*).

An important point is the flexibility of the scissile phosphodiester. In this respect, the double-stranded structure of DNA is too rigid, as

illustrated by the failure of metal complexes to cleave an RNA substrate when hybridized onto a complementary strand of DNA (*326*). Some bent phosphates are particularly prone to hydrolysis in looped RNA molecules in the presence of metals (*312*, *314–316*). Substrate flexibility is necessary to allow the RNA molecule to adopt a conformation favoring (1) either deprotonation of the 2′-OH group by a properly oriented basic species or nucleophilic attack by an exogenous nucleophile from the RNA (3′-OH of guanosine for group I and 2′-OH of adenosine for group II introns) and (2) potential straining of the scissile phosphodiester to make it more susceptible to cleavage. The complete understanding of all parameters involved in the cleavage step is important to improve the design of highly efficient ribozymes that might have a real future as modulators of gene expression and as potential drugs in inflammatory, neoplastic, and viral diseases (*327*).

C. Hydrolytic Cleavage of RNA, DNA, and Simple Phosphodiesters by Metal Complexes

Metals can facilitate the hydrolytic cleavage of phosphodiesters by (1) neutralization of the initial phosphate charge as well as the developing charge in the pentavalent intermediate, (2) stabilization of the transition state, and (3) lowering the pK_a of a bound water (or alcohol molecule), and stabilization of the leaving group on the phosphate (acid/base catalysis). One single metal is probably not sufficient to fulfill all these requirements (see Ref. *328* for a comprehensive review on how metals can help hydrolysis), because it may only accelerate the rate of phosphate hydrolysis by affecting some of the parameters described above. A metallic cation needs to be helped by surrounding components, as in DNase I (*291*) or in the Zn/imidazole system developed by Breslow *et al.* (*329*). As previously mentioned, from X-ray data on di- or trinuclear metal clusters of protein active sites, an emerging unified concept supports the necessity of a two-metal ion catalysis for the hydrolysis of phosphomono- or diesters, because it better fulfills all the requirements of an efficient catalytic process (Fig. 9).

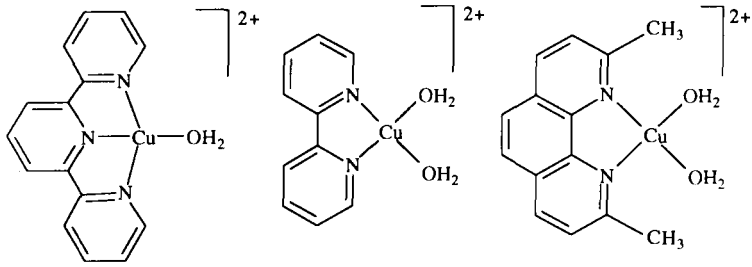
In the following discussions, recent examples of metal-promoted phosphate hydrolysis will be described, whereby one or two metals have been shown to play a key role, and metal ion participation in the hydrolysis process will be assessed. Metal-promoted hydrolysis seems to be a more suitable term than metal-catalyzed hydrolysis, because up to now one cannot find any case of an efficient metal-catalyzed nucleic acid hydrolysis in the literature. In order to hydrolyze a phosphate ester with water, with a half-life of 1 hr, the

first-order reaction rate constant must be $\sim 2 \times 10^{-4}$ sec^{-1}, whereas a half-life of 1 min requires a constant of about 10^{-2} sec^{-1}. For comparison, the half-life of DNA or RNA phosphodiesters extrapolated in physiological conditions are estimated to be 200×10^6 and 800 years, respectively. Due to this tremendous difference, results are often expressed in terms of enhancement of the rate constant of the considered phosphate hydrolysis compared to that in the absence of metal ions. The order of magnitude of the rate constant acceleration of phosphate ester hydrolysis should be in the range of 12 to 14 in order to cleave the DNA phosphodiester bonds within hours or minutes under physiological conditions (*330*). Hydrolysis assays are carried out in general in pseudo-first-order (excess of metal complex over substrate) and sometimes in second-order conditions with activated phosphate ester, RNA, or DNA models. The activity described is not necessarily relevant to RNA and even less to DNA hydrolysis. Only some examples can be found whereby a few turnovers could be evidenced for the metal species involved in the hydrolysis reaction (*331–333*). Nevertheless, with regard to the real challenge that this reaction represents to chemists, the results are significant.

The cleavage of a P–O bond of phosphomono- or diesters results in a nucleophilic substitution on the scissile phosphate. This is a true hydrolysis if a water molecule is the attacking nucleophile, or a transesterification when the nucleophile is an alcohol. These two formally distinct reactions will be discussed in this section, which is devoted to hydrolytic cleavage of nucleic acids and models, in contrast to the oxidative cleavage described in Section II.

1. Cleavage of RNA by Mononuclear Copper Complexes

Apart from the well-documented oxidative DNA or RNA cleavage (see Section II,B), some copper complexes favor RNA hydrolysis. Among them are $[Cu^{II}(typ)(H_2O)]^{2+}$ and $[Cu^{II}(bpy)(H_2O)_2]^{2+}$, two copper complexes with a bipyridine or a terpyridine ligand (**6** and **7**, Fig. 10). The monoterpyridine complex of Cu^{II} was shown to be able to promote the hydrolysis of 2′,3′-cAMP (*332*). ApA (*334*), and poly(A_{12-18}), but not the corresponding deoxypolynucleotide substrate poly(dA_{12-18}) in similar conditions (*335*). The mechanism of phosphodiester cleavage involves, as a first step, a trans-esterification due to an intramolecular attack of the 2′-OH group of the RNA riboses on the phosphate, because 2′,3′-cAMP was found as a product of poly(A_{12-18}) cleavage (*335*). Hydrolysis of 2′,3′-cyclic phosphate diester was also metal promoted and led to a mixture of 2′-AMP and 3′-AMP (*332*). This complex was able to achieve almost complete hydrolysis of



$[Cu^{II}(tpy)(H_2O)]^{2+}$ **6** $[Cu^{II}(bpy)(H_2O)_2]^{2+}$ **7** $[Cu^{II}(neocuproine)(H_2O)_2]^{2+}$ **8**

FIG. 10. Structure of terpyridyl (**6**), bipyridyl (**7**), and neocuproine (**8**) copper complexes.

poly(A_{12-18}) in 20 hr at neutral pH and 37°C [concentrations of poly (A_{12-18}) and Cu^{II} were 60 and 160 μM, respectively] (*335*). At a complex concentration of 60 μM, the pseudo-first-order rate constant was 1.6×10^{-5} sec^{-1} for the hydrolysis of poly(A_{12-18}). Cleavage of poly (A_{12-18}) by $[Cu^{II}(tpy)(H_2O)]^{2+}$ shows a bell-shaped rate constant versus pH dependence, with a maximum rate constant of cleavage (corresponding to the trans-esterification step) at pH 7.8, in agreement with a pK_a of 8 for the metal-bound water molecule. The order of the reaction with respect to the metal complex was one in the case of 2′,3′-cAMP as substrate (*332*).

The monobipyridine copper complex is less reactive than the terpyridine analogue in promoting the hydrolysis of poly(A_{12-18}) (*335*) or ApA (*334*), but this reaction involves also a transient 2′,3′-cAMP intermediate with an optimal reactivity at a pH value corresponding to the pK_a of the metal-bound water molecule (pK_a = 7.8). The yield from the cleavage is 60% for poly(A_{12-18}) at neutral pH and 37°C after 20 hr (*335*) [poly(A_{12-18}), 60 μM; complex, 160 μM], but the complex is almost inactive on ApA, which gives a half-life of 42 days in the presence of a large excess of metal complex (*334*).

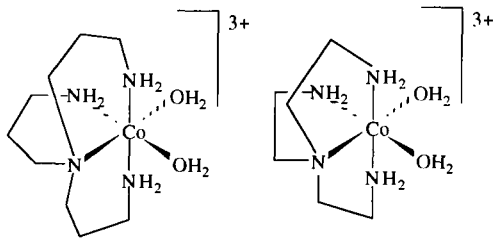
The lower efficiency of the bipyridine complex compared to its terpyridine analogue was proposed to be due to its dimerization at neutral pH. A related complex that cannot dimerize, $[Cu^{II}$(neocuproine) $(H_2O)_2]^{2+}$ (**8**, Fig. 10), was proved to be much more efficient than both bpy and tpy complexes (*334*). The half-life of ApA with respect to the trans-esterification step was 3 min (ApA, 0.5 mM; complex, 10 mM) at neutral pH and 37°C. The reaction is first order with respect to the copper complex and, as in the two previous examples, a pH dependence of rate constants is observed (optimal reactivity at pH 7). The

neocuproine complex-promoted hydrolysis is calculated to proceed 1000-fold more rapidly than in the case of the bipyridine complex.

A catalysis through a "double Lewis acid" activation of the scissile phosphate (coordination of two oxygen atoms of the phosphate onto the copper instead of one oxygen atom for a single Lewis acid assistance) was proposed by Chin (*334*) to account for the higher reactivity of **8** compared to **6**. The mechanism proposed by Bashkin *et al.* for **6** is totally different and is based on the fact that the optimal rate of phosphate trans-esterification is at a pH value close to the pK_a value of the metal-bound water molecule (*332, 335*). Because monoaqua complexes as in **6** do not form stable four-membered ring phosphate coordinates, they may just behave as acid/base coreactants like histidine residues in ribonuclease A (*336*) or like imidazole buffer (*337*).

2. *Cleavage of Phosphodiesters by Mononuclear Cobalt Complexes*

cis-Diaqua cobalt complexes can provide up to 10 orders of magnitude rate acceleration for hydrolyzing phosphate diester models with good as well as poor leaving groups (*330, 338*). Chin studied $[Co^{III}(trpn)(H_2O)_2]^{3+}$ and $[Co^{III}(tren)(H_2O)_2]^{3+}$ complexes, with pK_a values for the two water-bound molecules of 5.6 and 8 (**9** and **10**, Fig. 11) (*290*). Both complexes exhibit the highest activity at pH 7, where only one coordinated water molecule is deprotonated, giving an aqua–hydroxo–cobalt complex. The mechanism of the reaction is supposed to involve coordination of the phosphate onto the metal followed by an intramolecular attack of the hydroxo ligand on the metal-bound ester, forming a four-membered ring intermediate (Fig. 12). Despite presenting the same water-bound pK_a values and the same affinity for phosphates, **9** was 300 times more efficient than **10**. This difference might be due to the easier stabilization of the four-membered ring of the chelated phosphate with the more flexible trpn ligand com-



$[Co^{III}(trpn)(H_2O)_2]^{3+}$ **9** $[Co^{III}(tren)(H_2O)_2]^{3+}$ **10**

FIG. 11. Structures of $[Co^{III}(trpn)(H_2O)_2]^{3+}$ (**9**) and $[Co^{III}(tren)(H_2O)_2]^{3+}$ (**10**).

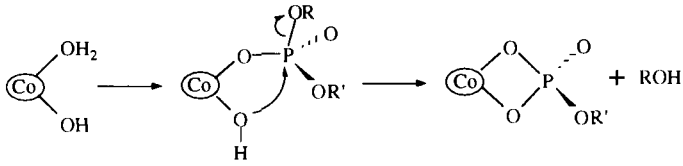


FIG. 12. Intramolecular metal-bound hydroxide nucleophilic attack.

pared to tren. These cobalt complexes are also efficient in the hydrolysis of 3′,5′-cAMP (*290*).

3. *Cleavage of DNA by Cerium Salts*

Cerium(III) salts under air or cerium(IV) salts in the absence of oxygen are able to mediate hydrolysis of DNA phosphodiester linkages. Single- and double-stranded oligodeoxyribonucleotides (*339*) as well as dideoxyribonucleotides (*340, 341*) are cleaved by $Ce^{III}Cl_3$. Under air Ce^{III} is oxidized to Ce^{IV} *in situ*, and this latter cerium salt is responsible for the cleaving activity. The pseudo-first-order rate constant was 5×10^{-5} sec^{-1} (half-life 3.6 hr) for the hydrolysis of TpT (100 μM) by $Ce(NH_4)_2(NO_3)_6$ (10 mM) at 50°C and neutral pH (*342*). Komiyama *et al.* proposed that a Ce^{IV}–hydroxo form intramolecularly attacks the phosphate of DNA, which is bound to the same cerium ion to promote hydrolysis (*342*). The reaction would also involve acid assistance by a metal-bound water molecule, also bound to the same cerium ion, and electrostatic interaction between the phosphate charge and the cerium(IV) ion.

Up to now, these mononuclear complexes do not fulfill the requirements for an efficient *in vitro* or *in vivo* DNA or RNA hydrolysis, especially because the rate constant acceleration is too slow to allow the cleavage of phosphodiester bonds of DNA within hours or minutes under physiological conditions. Efforts were made to enhance their cleavage efficiency by tethering them to antisense oligonucleotides (see Section III,C,6).

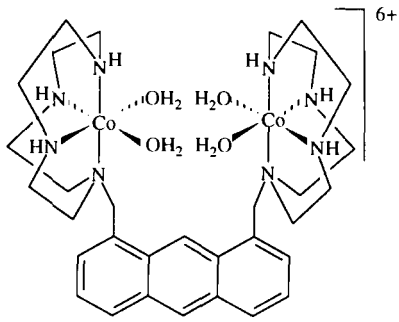
4. *Cleavage of Phosphodiesters by Homodinuclear Complexes*

A cooperative effect of two mononuclear complexes has been observed in the hydrolysis of phosphate mono- or diesters, the reaction order being then two with respect to the metal (*343–348*). Hydrolysis of methyl *para*-nitrophenylphosphate diester doubly coordinated to a dinuclear cobalt(III) complex was reported, and a crystalline compound of dimethylphosphate coordinated to the same cobalt complex was characterized $[Co_2^{III}(1,4,7\text{-triazacyclononane})_2\ (OH)_2\{O_2P(OCH_3)_2\}]^{3+}$

(*307*). Synergistic effects between two different metals could also promote the phosphate cleavage (*349–351*). All these data suggested that it was possible to prepare and to use dinuclear complexes to promote the hydrolysis of phosphodiester linkages (*308, 352–355*).

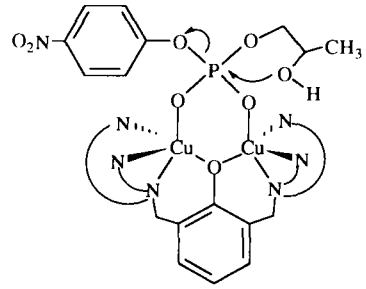
The effectiveness of the binuclear complex **11** (Fig. 13), with two mononuclear cyclen–cobalt(III) units linked together by an anthracenyl spacer (cyclen = 1,4,7,10-tetraazacyclododecane), was compared with the monomer in the hydrolysis of phosphate monoesters (*354*). The reaction assisted by this rigid binuclear complex, having a "phosphate-sized pocket," was 10 times faster than that promoted in the presence of two equivalents of the single cyclen–Co^{III} complex. In these experiments the substrate concentration was 25 μM and the total cobalt concentration was 2 mM at 25°C and neutral pH (*354*). No such cooperativity could be noted using a diester substrate because the pseudo-first-order rate constants were similar for both **11** and the mononuclear complex. With **11** as catalyst, an overall rate enhancement of 10^6 was achieved over the uncatalyzed hydrolysis of paranitrophenyl phosphate monoester as substrate.

Two different binuclear copper(II) complexes have been prepared recently, one with a bridging phenoxy ligand having two bis-benzimidazole arms (**12**, Fig. 14), and the second having a bis-cyclen–naphthalene ligand (**13**, Fig. 15) (*352, 353*). Both of them show bimetallic cooperativity for the hydrolysis of phosphate diesters, contrary to studies with the dinuclear cobalt complex (*354*). The pseudo-first-order rate constants for hydrolysis of the *para*-nitrophenylphosphate ester of propylene glycol by bis-benzimidazole-based copper complexes



dinuclear Co^{III} complex **11**

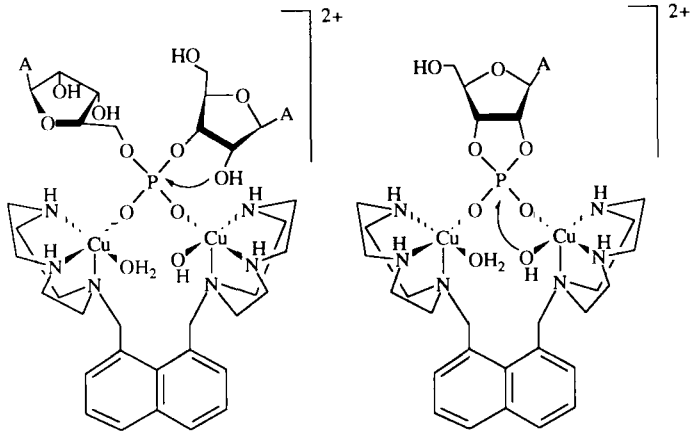
FIG. 13. Structure of the covalent binuclear Co^{III} complex with a 1,4,7,10-tetraazacyclododecane ligand (**11**).



dinuclear Cu^{II} complex **12**

FIG. 14. Double-Lewis acid activation of phosphate diester proposed for the bis-benzimidazole binuclear Cu^{II} complex **12**. The Cu–Cu distance is 3.7 Å. Charges on oxygen atoms and metals have been omitted.

at 25°C and pH 7 are 4×10^{-5} and 2.1×10^{-3} sec^{-1} (half-lives of 5 hr and 5 min, respectively) for the mononuclear (containing only one bis-benzimidazole arm) and the dinuclear (**12**) compounds (*352*). The binuclear complex **12** is therefore 50 times more active than its mononuclear analogue. This factor constitutes a significant enhancement compared to the case of the corresponding binuclear versus mononuclear cobalt(III) complexes (only a 10-fold acceleration) (*354*). The



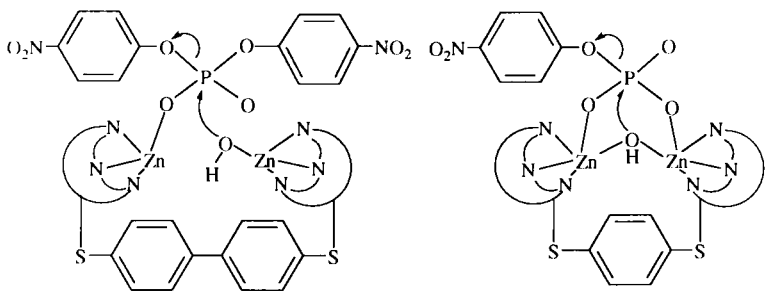
dinuclear Cu^{II} complex **13**

FIG. 15. Cooperativity of two Cu^{II} ions in the covalent binuclear complex **13** for cleaving RNA models.

double Lewis acid activation by the two metal centers was proposed to explain this higher reactivity (Fig. 14). The crystal structure of **12** with an inert dibenzylphosphate revealed a bridging phenolate (*352*). The 3.67-Å Cu–Cu distance is comparable to the distance between the two Zn centers in 3′-exonuclease (3.9 Å) (*299*). HIV RNase H (4 Å) (*303*), and alkaline phosphatase (3.94 Å) (*301*).

While complex **12** was inactive toward RNA cleavage (*352*), the complex **13** at 2 m*M* concentration cleaved the RNA models ApA and 2′,3′-cAMP (0.05 m*M*) with pseudo-first-order rate constants of 2.2×10^{-4} and 2.5×10^{-3} sec^{-1}, respectively (*353*). It therefore provides about 10^5 rate acceleration for cleavage of ApA, and 10^8 for hydrolyzing 2′,3′-cAMP over the background hydrolysis at pH 6. Bell-shaped pH–rate constant profiles, with a maximum at the pK_a value of the metal-bound water molecule, have been reported for these dinuclear copper complexes, suggesting that a metal–hydroxo species is involved in the reaction. The large difference in the rate acceleration for cleavage of ApA and 2′,3′-cAMP (10^5 and 10^8) was interpreted in terms of a better efficiency of the metal–hydroxo species when acting as nucleophile (as in the case of 2′,3′-cAMP) rather than as a base (as in the case of ApA) (Fig. 15).

Binuclear Zn^{II} complexes compared to mononuclear species were also shown to be more efficient by a factor of about 3 for hydrolyzing phosphate mono- and diesters (*308*). These complexes are based on dimers derived from 1,4,7-triazacyclododecane and 1,5,9-triazacyclotetradecane ligands (**14** and **15**, Fig. 16). A 10^3 rate enhancement over the noncatalyzed reaction was obtained. The distance between the metal binding sites influenced the reagent reactivity. Short spacers



dinuclear Zn^{II} complex **14** dinuclear Zn^{II} complex **15**

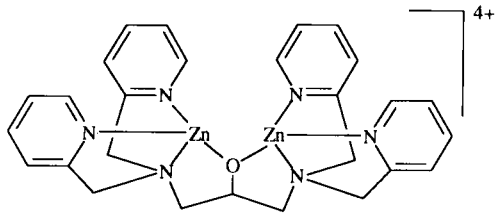
FIG. 16. Mechanism of phosphomono- or diester hydrolysis promoted by the binuclear Zn^{II} complexes **14** and **15**. Charges on oxygen atoms and metals have been omitted.

(as in **15**) were better for hydrolyzing monophosphates, whereas longer spacers (as in **14**) favor hydrolysis of diesters. The tether between the two metal ligands must also be rigid. A single Lewis acid activation associated with a metal-bound hydroxide attack was proposed for the hydrolysis of phosphodiesters. For the hydrolysis of phosphomonoesters the mechanism was rather ascribed to double Lewis acid activation by coordination of phosphate on both metals and the nucleophile was proposed to be a bridging hydroxo between the two metals (Fig. 16).

Another dinuclear Zn^{II} complex with *N,N,N′,N′*-tetrakis[(2-pyridyl)methyl]-2-hydroxy-1,3-diaminopropane was shown to be able to achieve phosphodiester cleavage of diribonucleotide ApA at 50°C and neutral pH (*355*) (**16**, Fig. 17). The hydrolytic cleavage of ApA was significantly accelerated by the cooperation of two metals in the binuclear complex because the mononuclear complex was inactive in comparable experimental conditions. The pseudo-first-order rate constant is 1.9×10^{-5} sec^{-1} (half-life 10 hr) (ApA, 100 μM; $ZnCl_2$, 5 m*M*; ligand, 2.5 m*M*). Deprotonation of 2′-OH by a metal hydroxo would be involved in the mechanism of reaction because the products of cleavage were adenosine, adenosine 2′- or 3′-phosphate, and 2′,3′-cAMP.

5. *Cleavage of Phosphodiesters through Cooperation of Two Different Metal Ions*

Some homogeneous solutions of mixed hydroxo clusters of appropriate combinations of two metal ions have been prepared (by avoiding the precipitation of polymeric aggregates of metal–hydroxo species) (*349–351*). The two combined metals, in a 1:1 mixed cluster, were located close to each other and were shown to participate in a cooperative bimetallic mechanism of phosphate hydrolysis. The metal



dinuclear Zn^{II} complex **16**

FIG. 17. Structure of the dinuclear Zn^{II} complex with *N,N,N′,N′*-tetrakis[(2-pyridyl)-methyl]-2-hydroxy-1,3-diaminopropane ligands (**16**).

pair could be a lanthanide ion with a nonlanthanide ion (La^{III}/Fe^{III}), or two nonlanthanide ions (Zn^{II}/Sn^{IV}) (**17** and **18**, Fig. 18). The La^{III}/Fe^{III} (**17**) or La^{III}/Sn^{IV} associations were shown to be efficient in RNA and, to a lesser extent, in DNA models of hydrolysis (*349, 351*), whereas Zn^{II}/Sn^{IV} (**18**) promoted hydrolysis only of RNA models (*350*). One of the metal ions of the association must have a metal-bound water molecule with a relatively high pK_a value (La^{III} or Zn^{II}) and the other metal center should be a strong Lewis acid (its metal-bound water has a low pK_a value) (Fe^{III} or Sn^{IV}) (Table I). The association of two strong acidic metals was inefficient (Fe^{III}/Sn^{IV} or Lu^{III}/Fe^{III}) (*351*). Associations between a lanthanide and a nonlanthanide ion (La^{III}/Fe^{III} or La^{III}/Sn^{IV}) were the most active on RNA and DNA simplified substrates (*349, 351*). The ions La^{III}, Eu^{III}, Ce^{IV}, and Lu^{III} were tested in association with Fe^{III} (*351*). Among them, only La^{III}, and to a lesser extent Eu^{III}, showed cooperativity with Fe^{III}.

The overall efficiency of these "nonmolecular" bimetallic sysems to promote phosphate ester hydrolysis was illustrated with ApA as RNA model (0.1 m*M*); hydrolysis occurred with a mixture of $LaCl_3$ and $FeCl_3$ (10 m*M* each) to more than 70% at 50°C and neutral pH in 5 min (*351*). The products of the reaction included adenosine and 2′- and 3′-monophosphate adenonsine, indicating that the mechanism involved a first trans-esterification step by the vicinal 2′-OH of ribose. With the same mixture of metal ions, the DNA model TpT (0.1 m*M*) (Fig. 18), left, B = thymine) was converted to thymidine and 3′- and 5′-monophosphate thymidine with 36% of conversion in 24 hr at 70°C at neutral pH. The important point is the notion of cooperativity between the two metals.

In the second example of cooperativity of two metal ions described by Komiyama *et al.*, the two nonlanthanide metals, the Zn^{II}/Sn^{IV} sys-

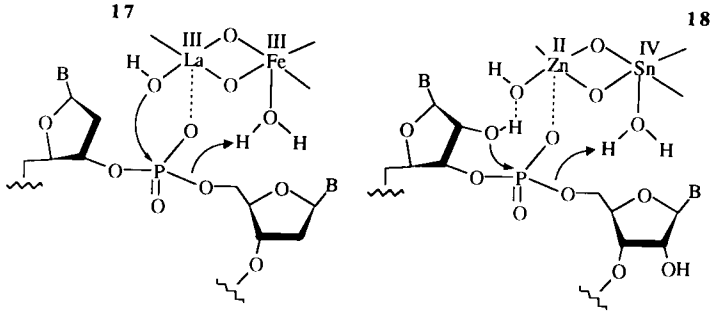


FIG. 18. Proposed mechanism of DNA or RNA hydrolysis mediated by La^{III}/Fe^{III} or Zn^{II}/Sn^{IV} systems (**17** and **18**).

TABLE I

VALUES OF pK_a FOR METAL-BOUND WATER MOLECULES

Molecule	pK_a
$Sn^{IV}(H_2O)$	-0.6^a
$Ce^{IV}(H_2O)$	0^b
$In^{III}(H_2O)$	$2.1–4.4^a$
$Fe^{III}(H_2O)$	$2.5–3.1^a$
$Al^{III}(H_2O)$	$4.3–5^a$
$La^{III}(H_2O)$	7.4^c
$Zn^{II}(H_2O)$	8^a

[a] From Ref. *350*.
[b] From Ref. *342*.
[c] From Ref. *349*.

tem (10 m*M* of each metal ion), the half-life of the RNA model ApA (50 m*M*) is 3 hr at 50°C and neutral pH (*350*). The products of the reaction are adenosine, 2′- and 3′-phosphate adenosine, and some 2′,3′-cyclic phosphate adenosine (Fig. 18, right, B = adenine). The authors showed that the best association with Zn^{II} is observed when the bound water molecule on the metal is most acidic. The order of decreasing cooperativity is $Sn^{IV} > In^{III} > Fe^{III} > Al^{III}$ (see Table I for respective pK_a values).

The mechanism of the phosphate hydrolysis promoted by these metal associations was proposed to involve the La^{III}– or Zn^{II}–hydroxo ligand as nucleophile attacking the scissile phosphate (for the TpT substrate), or as a Brønsted base able to deprotonate the 2′-OH of the ribose, respectively. The water molecules bound to Fe^{III} or Sn^{IV} are believed to act as a Brønsted acid (proton source) with respect to the 5′-oxygen atom of the leaving alcohol (Fig. 18).

6. *Cleavage of RNA by Oligonucleotides Modified with Metal Complexes*

Linking artificial RNases to oligodeoxynucleotides by a covalent tether has emerged as an important topic due to the lack of sequence-specific natural RNases and to the potential therapeutic issue of reactive antisense oligonucleotides (*327*). The promotion of RNA cleavage by such hydrolyzing reagents is attractive due to the easier hydrolysis of the RNA target compared to that of oligodeoxynucleotide carrier. This represents a considerable advantage for the strategy compared

to the "oligonucleotide–oxidative cleaver" approach, because the latter cleavers do not discriminate between the oxidaton of the nucleic acid target and the oligonucleotide carrier. However, this strategy is dependent on robust complexes that are inert to metal release and would be optimized if they could undergo several reaction turnovers. The first promising metal complex in this respect consisted of a hexadentate Schiff base ligand of lanthanide ions (*331*). Provided that special attention is given to mediate hydrolytic cleavage of RNA in the hybridized zone of the duplex ODN vector/RNA target, the conjugated ODN could behave as a catalyst. After the cleavage of a first RNA target, the melting of the nicked duplex would release two RNA fragments and a free conjugate, which would be available again to bind and cleave another RNA substrate. This would considerably lower the concentration of antisense conjugated ODN necessary for biological effects.

Covalent attachment of RNA-hydrolizing metal complexes to ODNs involved, up to now, essentially Eu^{III}, Lu^{III}, and Cu^{II} complexes. The *in vitro* sequence-specific hydrolytic cleavage of RNA by metal complexes conjugated to ODNs, at physiological pH, was reported (1) with a terpyridine–Cu^{II} entity (*356*) mediating 18–25% of target cleavage at 45°C within 72 hrs (2) with an iminodiacetate–Lu^{III} or –Eu^{III} entity (*357*) mediating 17% of target cleavage in 8 hr at 37°C, and (3), more interestingly, with a stable texaphyrin–Eu^{III} (*358*) or robust terpyridine-derived hexadentate–Eu^{III} entity (*359*). The latter achieves near quantitative cleavage of target RNA (88%) in 16 hr at 37°C, where the complex moiety is remarkably resistant toward decomposition. In general, cleavage of RNA occurs in the single-stranded zone of the RNA target in the vicinity of the metal complex.

Attempts to position the metal complex in the middle of the oligonucleotide vector have been reported (*360*). A mono-bpy–Cu^{II} complex was covalently bound to the uracil C-5 position of 2′-deoxyuridine nucleoside in order to incorporate this synthon into an ODN sequence. One of the various nucleoside–bipyridine synthesized conjugates was able to mediate 65% of cleavage of poly(A_{12-18}) in 24 hr, whereas the free mono-bpy-Cu^{II} complex degrades 78% of the same polymer in 48 hr [poly(A_{12-18}), 60 μM; complex, 160 μM; 37°C, pH 7.1]. In contrast to the Eu^{III}–ODN conjugates described previously, the Cu^{II} conjugates were inactivated by 0.5 mM EDTA and consequently are not relevant for an *in vitro* utilization.

Beside RNA targeting, a monoiminodiacetate–Ce^{IV} complex conjugated to an ODN has been reported to cleave sequence selectively a ss DNA target (60% yield after 12 hr) (*361*), but its reactivity toward RNA or ds DNA has not been described.

Although these ODNs conjugated to artificial hydrolyzing reagents do not show a reactivity comparable to that of their analogues coupled with oxidative reagents, improvement of their reactivity may be possible. They constitute an interesting alternative in nucleic acid cleavage that is worth pursuing. In order to be relevant as reactive antisense oligonucleotides in a cellular context, they should be able to promote the cleavage of RNA molecules more rapidly than the general turnover of mRNA molecules inside cells (20 min to 24 hr) (*362*).

IV. Conclusion

DNA and RNA cleavage, a very active field of research, has been developed in two main and complementary directions within the past decade: oxidative cleavage and hydrolysis. In some cases, these two different strategies are linked. This was the case of the pseudohydrolysis of DNA involving a selective oxidative step followed by a reduction (*363*). In general, the two different approaches are separated, but the same goals are involved. There is first the preparation of new chemical tools to study genomic DNA, because recognition sites of most of the restriction enzymes are too often limited to palindromic sequences, and it is useful to have artificial nucleases able to cleave DNA at any desired sequence. Second, the synthesis of compounds that cleave nucleic acids should help in the design of potential therapeutic agents for the treatment of cancer and viral diseases. The challenging development of new, efficient DNA and RNA cleavage agents will require a strong cooperation between chemists, biochemists, and molecular biologists.

V. Addendum

References listed below appeared in the literature after the submission of this manuscript. Devoted to the general topic of this review, three books must be mentioned (*364–366*). For work related to specific sections, see the following addenda:

Section II,A: From recent two-dimensional NMR studies on bleomycin analogs, a revisited structural model for specificity, binding, and double-strand cleavage was proposed (*367*). An investigation of the reaction of Fe^{III}–BLM with iodosylbenzene by ES–MS showed that neither hypervalent iron nor activated oxygen was involved but that hypervalent iodide I(III) was the oxidant (*368*).

Section II,B: The ss DNA region of the *lac*UV-5 promoter of *e. coli* RNA polymerase could be targeted by tetrahedral cuprous chelates of 1,10-phenanthroline, resulting in inhibition of transcription (*369*).

Section II,C: Iron–EDTA was covalently attached to pyrrole–imidazole polyamides for sequence-specific recognition and cleavage of ds DNA from within the minor groove (*370*).

Section III,A: The crystal structure of the NH_2-terminal fragment of T4 DNA polymerase was published and its complexes with metal ions and short deoxyoligonucleotides were compared to that of the 3′–5′ exonuclease domain of the Klenow fragment (*371*).

Section III,B: The molecular mechanism of the action of ribozymes was the subject of numerous papers (for some of them see Refs. *372–376*).

Section III,C: Selective recognition of phosphomonoesters and their P–O ester bond cleavage by new dinuclear Zn^{II}–cryptate was described (*377*). Cleavage of RNA by lanthanide complexes attached to oligonucleotides showed that it was possible to target the duplex region of the hybrid by creating a bulge site on the RNA target (*378*).

VI. List of Abbreviations and Definitions

A	Adenine
2′-AMP, 3′-AMP, 5′-AMP	2′-, 3′-, or 5′-Adenosine monophosphate
2′,3′-cAMP	2′,3′-cyclic adenosine monophosphate
ApA	Adenylyl-3′,5′-adenosine
BLM	Bleomycin
bpy	2,2′-Bipyridine
C	Cytosine
CD	Circular dichroism
Conjugate	A conjugate is a hybrid molecule made of two partners tethered by a covalent link, the biochemical or biological properties of one moiety being targeted toward biological macromolecules or cells, and with the second entity acting as vector
Cyclen	1,4,7,10-Tetraazacyclododecane
dG	Deoxyguanosine
dppz	Dipyrido[3,2-*a*:2′,3′-c]phenazine
ds	Double strand(ed)
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetate
G	Guanine
HIV	Human immunodeficiency virus
HpD	Hematoporphyrin
5-MF	5-Methylene-2(5*H*)-furanone
MMPP	Magnesium monoperphthalate

PAGE	Polyacrylamide gel electrophoresis
ODN	Oligodeoxyribonucleotide (oligonucleotide for short)
phen	1,10-Phenanthroline (oP has also been used)
ss	Single strand(ed)
T	Thymine
TMPyP	Dianion of *meso*-tetrakis(4-*N*-methylpyridiniumyl) porphyrin
TPPS	Dianion of *meso*-tetrakis(4-sulfonatophenyl)porphyrin
TpT	Thymidylyl-3′,5′-thymidine
tpy	2,2′:6′,2″-Terpyridine
tren	Tris(2-aminoethyl)amine
trpn	Tris(3-aminopropyl)amine

Acknowledgments

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