Oxidative Nucleobase Modifications Leading to Strand Scission

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Contents

I. Introduction

Mustard gases were more commonly used in World War I, but it was not until after the second World War that their mutagenic activity was investigated.[1] As alkylating agents, the nitrogen- and sulfur-based mustards readily react with the electron-rich sites of nucleobases, especially guanine's N7 and adenine's N1 and N3.[2−6] Early studies went on to show that guanine N7 alkylation leads to depurination of the nucleobase and ultimately strand scission at that site.[7,8] Heat and alkaline conditions speed the depurination and cleavage reactions. This information led to the use of one of the war gases, dimethyl sulfate (DMS), as a reagent for investigating DNA−protein contacts[9−11] and quickly thereafter to the development of the Maxam−Gilbert sequencing reactions, the G-specific reaction being based upon DMS.[12,13]

A parallel story exists in nucleobase oxidation. While radiation-induced illness was recognized in the early part of the 20th century[14,15]—indeed, Pierre and Marie Curie and Irène and Frédéric Joliot-Curie all suffered from it—only after World War II were the effects of ionizing radiation on DNA investigated.[1−3,16] Like alkylation, oxidation chemistry is often focused on the nucleobase guanine, and most oxidative damage leads to strand scission after treatment with heat and alkali.[17] In fact, the analogies between nucleobase oxidation and alkylation both leading to DNA strand scission led to the term "radiomimetic" being applied to the mustard mutagens.[18]

The electron-rich purine and pyrimidine heterocycles are prime targets for reaction with electrophiles: alkylating agents, halogens, and oxidizing agents. These reagents rarely lead to direct (or frank) strand scission, but allow DNA or RNA cleavage to occur site specifically in a second chemical step, usually involving heat, base, or enzyme treatment to effect deglycosylation and β-elimination of the 3′-phosphate (Figure 1). Such reactions are

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intrinsically more site specific than those targeting the ribosyl or deoxyribosyl units since each of the nucleobases reacts differently with electrophiles. In the case of a structure probing experiment, it can be advantageous to have the DNA or RNA polymer remain intact during the first chemical step in which nucleobases are modified, so that the gross structure of the polymer is not altered until the cleavage step. Furthermore, nucleobase chemistry is inherent in both DNA-targeted mutagens and carcinogens as well as many nucleic acid-directed chemotherapeutics. In these cases, strand scission following chemical modification is often used as a means of detecting both the sequence site of reaction and, perhaps, the type of nucleobase lesion formed.

II. Scope and Organization

Direct scission of DNA or RNA by oxidizing or hydrolytic agents is covered elsewhere in this issue.[19,20] The focus of this article is on chemical events originating on the nucleobase that render the polymer subject to cleavage in a subsequent step. Reactions with electrophiles constitute the bulk of the examples, and these are roughly divided into oxidizing agents, other electrophilic agents (halogens, etc.), and alkylating agents, although some cross-referencing is necessary. DNA alkylation is a broad field that has been extensively reviewed. Therefore, the alkylation section of this review will focus only on those events that are triggered by redox processes or involve radical species. For comparison, nucleophilic additions and nucleobase reduction are briefly included in the last section. Within each broad category of nucleobase modification, the material is usually divided into sections based on site of reaction, when known, i.e., reactions of guanine C8 vs N^2, etc. The numbering scheme of nucleobases is presented

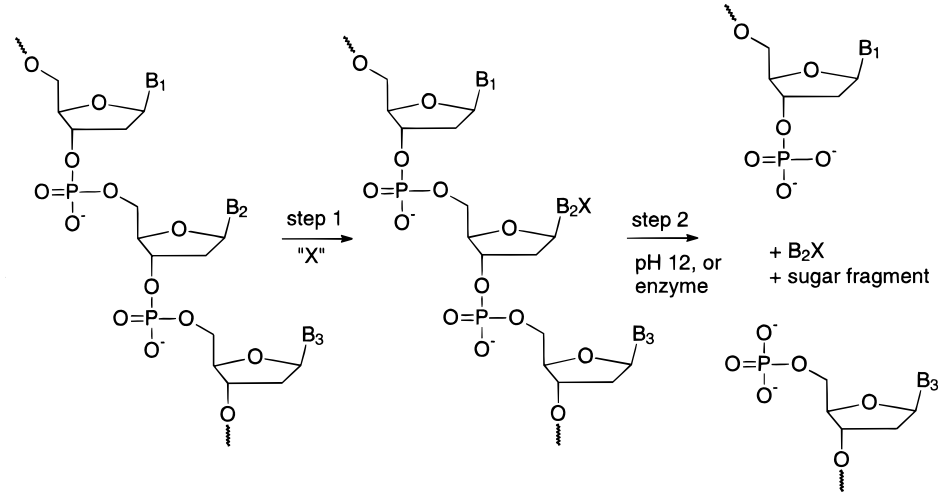

Figure 1. General scheme for nucleobase modification by reagent "X" followed by strand scission using alkaline conditions or a nuclease. The scission event usually creates two new phosphate ends on oligomers as well as a sugar fragment and a modified nucleobase.

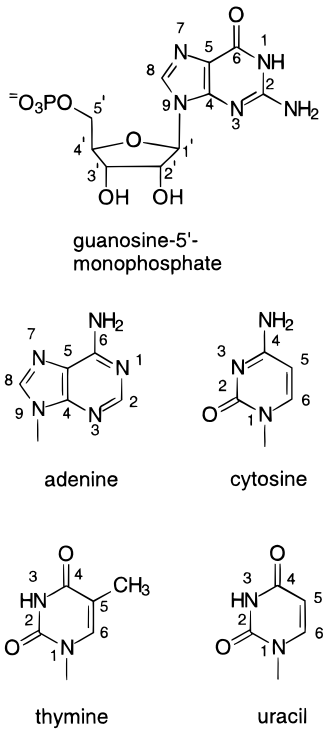

Figure 2. Numbering scheme for nucleobases and nucleosides.

in Figure 2. Note that sugar atoms bear "primes" (′) while heterocyclic atoms are unprimed. Exocyclic atoms attached to ring atoms are labeled with superscripted numbers, e.g., adenine N^6 vs N7.

III. Methods of Strand Scission

Cleavage of a DNA or RNA strand as a result of chemical reactions initially occurring on the nucleobase usually requires a second chemical step such as heat, alkali, or enzymatic treatment to effect strand scission, and we will discuss this second step first. These methods rely upon removal of the nucleobase via glycosidic bond hydrolysis or nucleophilic displacement, followed by phosphate elimination at the resulting abasic site (Figure 3). Deglycosylation can be effected by a number of enzymes,[21] by acid hydrolysis or by nucleophilic displacement. Being more basic, the purines are more sensitive to glycosidic bond hydrolysis in acidic solution than are the pyrimidines since protonation renders them better leaving groups.[22] This lability forms the basis of a sequencing reaction for A+G in which 88% formic acid is used to cleave the strand at every purine nucleotide.[23] Furthermore, there is an effect of the 2′-hydroxyl group; deoxyribonucleic acids depurinate about 500 times more rapidly than ribonucleic acids.[24] Because all the nucleobases are acid labile to some extent, acid hydrolysis is not usually used in site-specific DNA cleavage reactions. More commonly, alkaline conditions are used because nucleobases are relatively stable to these conditions unless they have been chemically modified in certain ways, as discussed in the remainder of this article. This allows unmodified nucleosides to remain intact in the polymer, and strand scission to occur only at specifically modified sites.

A. Alkali Lability

In 1953, Tamm et al. reported that abasic sites in DNA were subject to strand scission under alkaline conditions.[7] The cleavage event occurs as a phosphate elimination reaction from the deglycosylation product **3** (Figure 3). The keto form of the sugar bears acidic hydrogens at C2′, and thus high pH and heat favor the elimination to yield **4**. The other product of the reaction is a 5′-phosphorylated oligodeoxyribonucleotide representing the 3′ fragment of the original strand. The 5′ fragment **4** can undergo a second elimination because of the acidity of $H^{4'}$. The final products then include the sugar fragment as keto aldehyde **5** and additionally the 5′ fragment of the original strand that now terminates with a 3′ phosphate. Confirmation of these end groups can be obtained by high-resolution gel electrophoresis.[25]

Schiff base formation in the presence of amines greatly facilitates the elimination reaction by increas-

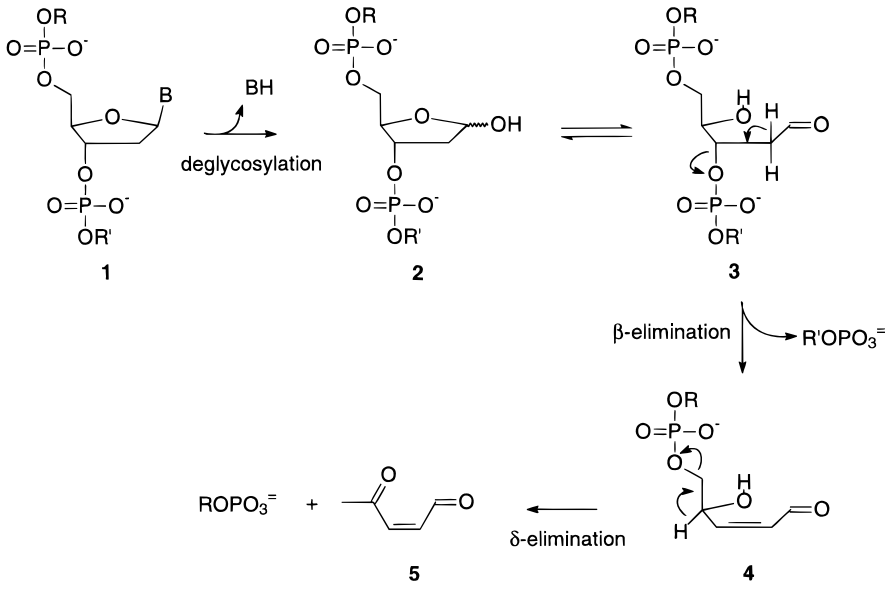

Figure 3. Mechanism of β-elimination and δ-elimination after formation of an abasic site.

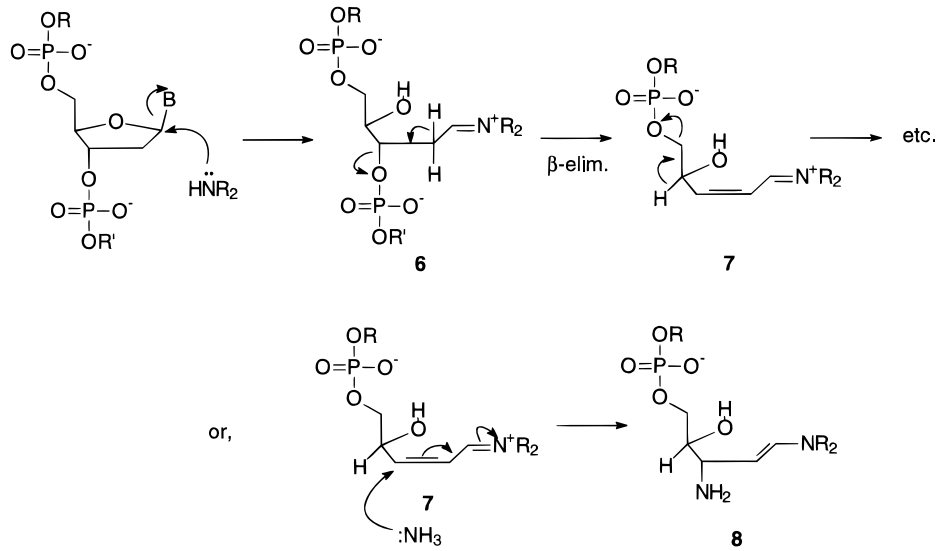

Figure 4. Participation of amines in deglycosylation and strand scission.

ing the basicity of $H^{2'}$ (see **6** in Figure 4). Furthermore, amines can accelerate deglycosylation and formation of the abasic site through nucleophilic displacement of the purine or pyrimidine. This mechanism is seen enzymatically when a lysine ϵ-amino group in an *N*-glycosylase forms an imine with the sugar aldehyde upon base release.[21,26] In principle, any primary or secondary amine will catalyze phosphate elimination. In practice, piperidine is most commonly used.[13] An advantage to piperidine is that it is not a sufficiently good nucleophile to complicate the product formation. In contrast, ammonia can participate in conjugate addition to C3′ of intermediate **7** (Figure 4) generating 3′-aminoribosyl derivative **8** at the 3′ terminus of an oligonucleotide.[27] Such a species will migrate differently in a PAGE experiment compared to a 3′-phosphate terminus.

The standard recipe for piperidine treatment to reveal abasic sites and chemically modified bases is 0.2−1 M piperidine at 90 °C for 30 min. Pyrrolidine (pK_a = 11.27 compared to 11.12 for piperidine), a nonregulated amine, may also be substituted with similar results.[28,29] The piperidine reaction was initially developed for Maxam−Gilbert sequencing,[13] and these conditions appear to be largely sufficient to reveal alkylated and oxidized bases that are piperidine labile. Rarely, however, has the piperidine-induced cleavage rate been carefully monitored for various base lesions, so it is possible that the 30-minute window represents incomplete strand scission. For example, this was recently a subject of debate for oxidized guanine lesions (see section IV.B.1.b).[30,31] For N7-alkylguanines, the strand scission event is complete within 10−15 min.[32]

In general, oxidation, alkylation, and other electrophilic reagents acting on nucleobases will remove electron density from the heterocycle, making the modified nucleobase a better leaving group in order to form an abasic site. Not all nucleobase modifications have this effect. For example, alkylation of purines at N3, N7, or C8 generally leads to alkali lability[33] although at different rates.[34,35] The N7-alkylguanine residues are not themselves alkali-labile, but rather are converted under hot alkaline conditions to imidazole ring-opened products that are.[32] Alkylation at N1 of purines might lead to alkali lability, but this lesion is less common because N1 is well-protected in duplex DNA.[6] In addition, this reaction can be confusing because alkylation of adenine that occurs initially on N1 can rearrange to N^6-alkyladenine, and this rearrangement is facilitated by basic conditions.[5] Alkylation of exocyclic groups, guanine N^2 or O^6 and adenine N^6, should not greatly influence the alkali lability of the purines since the aromaticity of the heterocycle is largely unaffected.[30,36] Oxidation of guanine in DNA at C8 to produce 7,8-dihydro-8-oxoguanine (hereafter called 8-oxoG) generates a somewhat piperidine-labile site, while the same lesion on adenine is stable.[30] For the pyrimidines, the 5,6 double bond is often the site of chemical modification. Reactions of non-carbon electrophiles (i.e., oxidations and halogenations) generating 5-substituted pyrimidines are alkali-labile.[37−39] Although less is known about 6-substituted pyrimidines, the chemical sequencing reactions employing nucleophilic attack of hydrazine at C6 do eventually lead to piperidine-labile sites.[12] In addition, conversion of the 5,6 double bond to sp^3 centers generates alkali lability. Alkylation of O^2 of all pyrimidines leads to lability of the glycosidic linkage; cleavage of this bond occurs over the pH range 4−13, but is fastest under more acidic conditions.[40] In contrast, O^4 alkylation has little effect on rates of depyrimidination. Alkylation of cytosine N3 is also a common modification, but it does not appear to generate an alkali-labile lesion, so other methods are used for its detection.[41] A general summary of alkali-labile modifications is shown by the positions of asterisks in Figure 5.

The observation of alkali-induced strand scission *may* imply that chemical modification has occurred at one of the nucleobase sites shown in Figure 5. There are, however, other interpretations. For example, all abasic sites are alkali labile, and abasic sites can also be generated from reactions occurring on the deoxyribose ring. This is one pathway available to iron bleomycin in which hydroxylation of the

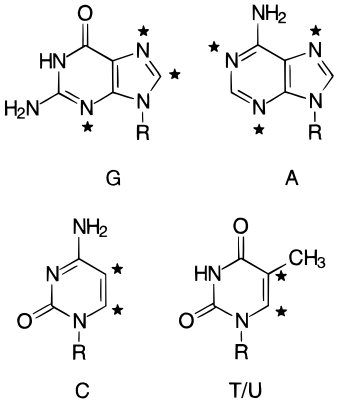

Figure 5. Sites of nucleobase modification leading to alkali lability. The degree of lability depends on the nature of the modification; see text for further details.

C4′ radical (**9**) may lead to depyrimidination (**10**) by the route shown in Figure 6.[42] The chemical structure of this lesion was elucidated with the aid of hydroxide and butylamine treatment (products **12** and **13**, Figure 6).[43] Abasic sites can apparently be distinguished from alkali-sensitive nucleobase modifications by a mild β-elimination reaction with putrescine.[44−46] Recent reaction conditions for this analysis call for the use of 100 mM putrescine, 37 °C for 1 h,[47] although this may be too short a reaction time to reveal all abasic sites since the half-life for strand cleavage can be estimated to be 1.4 h at 70 °C.[44] *N*-Methylpiperidine can also be used to detect abasic sites,[32] as well as piperazine, morpholine, ethylenediamine, and *N*,*N*-dimethylethylenediamine.[48] The latter reagents can be used at pH 7.4 and show less background cleavage at other sites. Recently, N^6-allyladenine was reported to lead to an abasic site and ultimately strand scission upon treatment first with I_2 and heating to 90 °C for 6 h, followed by piperidine treatment.[49]

Oxidation of C1′ also generates an alkali-labile lesion because of the formation of 2′-deoxyribonolactone (**16** in Figure 7). Some strand scission is also seen before alkali treatment, but hot piperidine intensifies the bands observed on a gel. The C1′ chemistry is thought to occur for a variety of radical or oxidative reagents including copper phenanthroline,[50,51] cationic metalloporphyrins,[52] and neocarzinostatin,[53] that are capable of abstracting $H^{1'}$ to form radical **14** which is in turn further reactive with O_2 to give an unstable peroxyl radical (**15**). These examples underscore the fact that observation of alkali-labile lesions or abasic sites is not necessarily proof of nucleobase modification.

All of the discussion above on alkali lability applies only to DNA. In the case of RNA, all phosphodiester linkages are subject to alkaline hydrolysis due to the participation of the 2′-hydroxyl (Figure 8), and this reaction will occur faster than creation of an abasic site and β-elimination. To effect strand scission only at the sites of appropriately modified nucleobases, one must employ mild reaction conditions along with the use of an amine catalyst, typically aniline. For example, RNA containing an N7-methylguanosine residue, **17**, is subject to depurination by either of two methods (Figure 9). In method **A**, the RNA is incubated with 0.1 M Tris buffer (pH 9.5) at 50 °C for 4.5 h followed by reaction with 0.3 M aniline, pH 3.5, 50 °C for 4 h.[54] The initial basic conditions lead to imidazole ring opening, and the subsequent treatment with aniline under acidic conditions is thought to effect β-elimination giving strand scission after loss

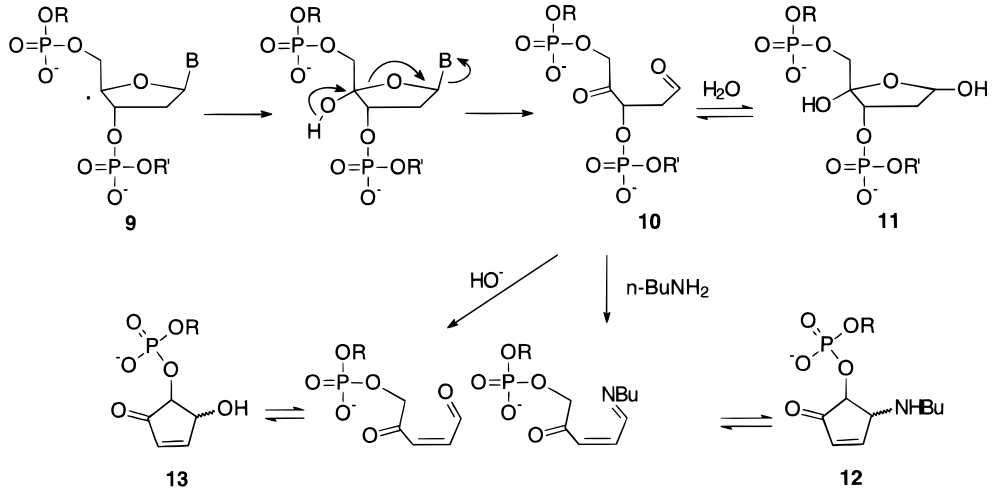

Figure 6. Products formed from H atom abstraction at C4′ followed by treatment with hydroxide or *n*-$BuNH_2$.

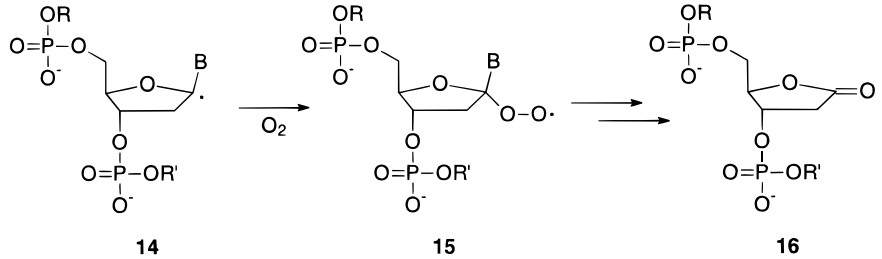

Figure 7. Formation of ribonolactone after H atom abstraction for C1′.

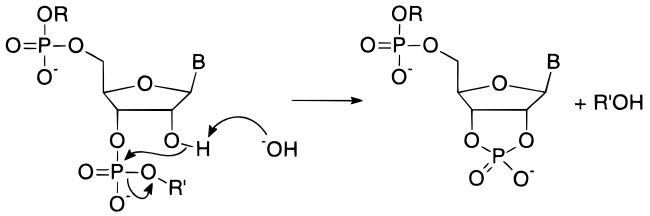

Figure 8. RNA is readily hydrolyzed under alkaline conditions due to participation of the 2′-OH.

of the modified base **19**. In method **B**, N7-methylguanosine is first reduced to aminal **20** with 25 mM $NaBH_4$ in 0.01 M KOH at 0 °C for 30 min. A second treatment with 0.3 M aniline·HCl, pH 4.5, 25 °C for 3 h then leads to strand scission.[55] Depending upon the base modification, the reduction step may not be necessary. For example, nickel-catalyzed oxidation of guanines in $tRNA^{Phe}$ can be observed by strand scission after reaction with 1 M aniline·HOAc, pH 4.5, 60 °C for 20 min.[56] A variety of related protocols exist.[57−59]

In summary, if a chemical reagent demonstrates no direct DNA strand scission but does show base-specific, piperidine-labile (or aniline-labile for RNA) scission, it is a good indication that nucleobase modification is the primary event. On the other hand, if piperidine (or aniline) only serve to enhance the strand scission already observed in its absence, the chemical modification is most likely centered on the sugar moiety. These general rules will apply to reactions of oligonucleotides or restriction fragments studied by PAGE. In contrast, little (if any) mechanistic information can be gleaned from plasmid nicking studies since even well characterized nucleobase reagents will lead to some plasmid cleavage (form I to form II conversion) that might be interpreted as direct strand scission.[60,61]

B. Enzymatic Cleavage Following Nucleobase Modification

The enzymatic analogue to piperidine treatment is the use of DNA glycosylases having endonuclease activity. DNA nucleobases are constantly subjected to chemical modification by environmental toxins; fortunately, an elaborate system of DNA repair enzymes recognize and excise the damaged bases.[21,62−64] When this occurs by the base excision repair (BER) mechanism,[65] there is not necessarily a strand scission event accompanying the repair. On the other hand, nearly all of the enzymes that follow the nucleotide excision repair (NER) pathway cleave the damaged DNA strand. The scission event typically occurs by hydrolysis of the glycosidic bond to the damaged base to create an abasic site followed by β-elimination. The overall repair process is quite complex, but the general events leading to strand scission parallel those of a chemical modification plus alkaline treatment. Thus, one can imagine the design of a site-specific DNA cleavage agent to be based on a first step of site-specific damage to a nucleobase, followed by treatment with an appropriate glycosylase with β-lyase activity. In fact, the sites of platination in DNA by (*cis*-dichlorodiammine)-platinum(II) were first detected by use of Exonuclease III, an AP endonuclease.[66,67]

A wide variety of DNA repair enzymes are currently under study. Certain enzymes seek out oxidative base damage[68] while others are specific for alkylated, or dimerized bases. Many common nucleobase lesions are shown in Figure 10 and include oxidized, alkylated and dimerized bases. Table 1 lists repair enzymes with known β-lyase or endonuclease activity along with their source and substrates.[63] A recent example of the use of a DNA repair enzyme to detect chemically generated 8-oxoG lesions comes from the work of Spassky and Angelov who used formamidopyrimidine DNA glycosylase (Fpg) to cleave

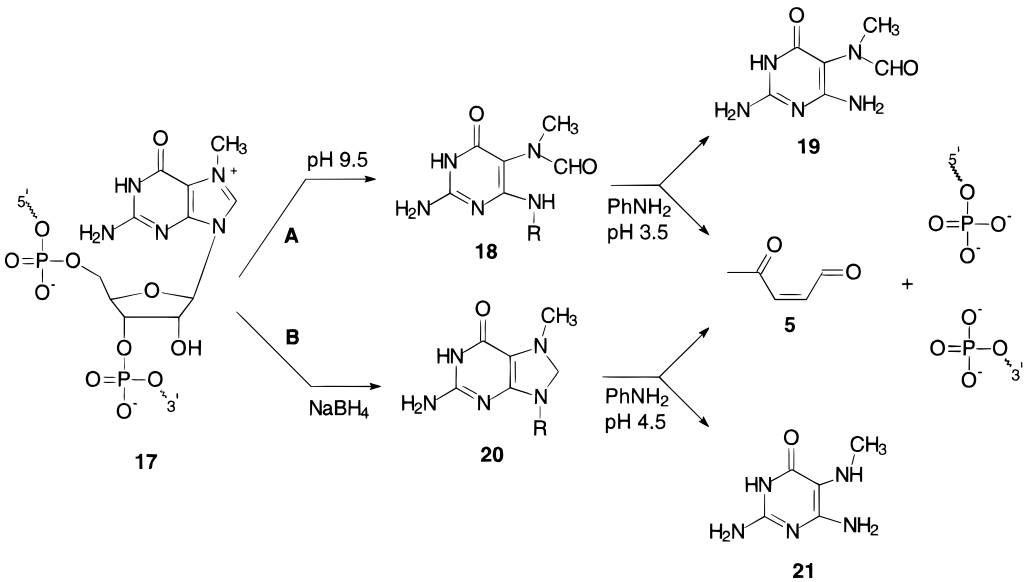

Figure 9. G−N7 methylated oligoribonucleotides undergo strand scission under two different reaction conditions. Method **A** is based on base-mediated ring opening followed by deglycosylation and β-elimination. Method **B** involves reduction to generate a labile modified base.

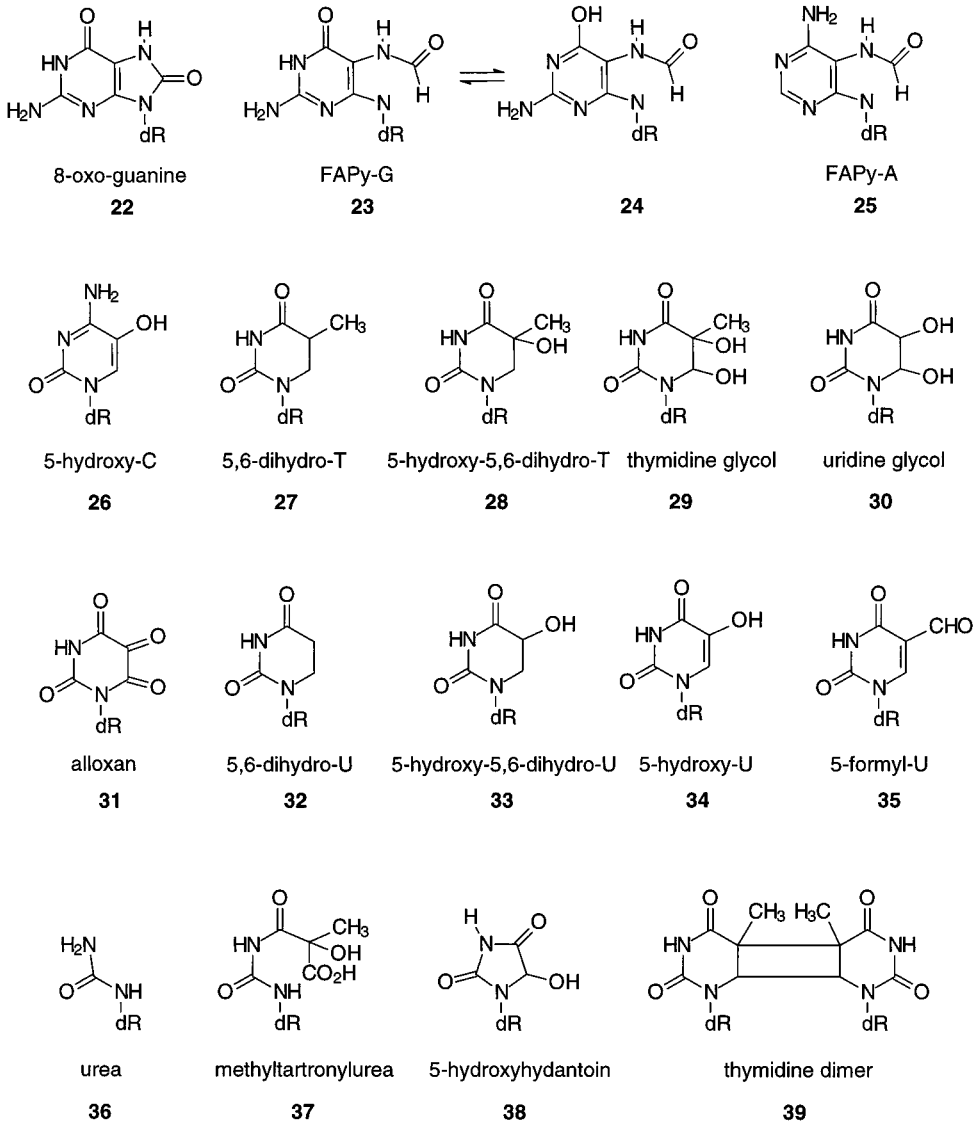

Figure 10. Modified nucleobases that are substrates for DNA repair enzymes with β-lyase activity. See also Table 1. (Adapted from ref 63).

Table 1. DNA Repair Enzymes with Lyase Activity[a]

enzyme	source	substrates
Endo III	*E. coli*	**26, 34, 36−38**
NTG1	*S. cerevisiae*	**23−25, 29**
nth	*S. pombe*	**29, 36**
Endo VIII	*E. coli*	**27, 29**
Endo IX	*E. coli*	**36**
Fpg	*E. coli*	**22−25**
OGG1	*S. cerevisiae*	**22** (opposite C or T)
OGG2	*S. cerevisiae*	**22** (opposite G or A)
T4	*E. coli*	**24, 39**
pdg	*M. luteus*	**39**

[a] Adapted from ref 63.

oligodeoxynucleotides at photooxidized guanines.[69] These lesions were then compared to those obtained by hot piperidine treatment in order to assess the relative amounts of 8-oxoG vs an imidazolone product. In both cases, the observation of strand fragmentation on gel electrophoresis was indicative of chemically modified bases. Although enzymes are an efficient cleavage method, some caution must be exercised in interpreting products based on enzymatic cleavage. Fpg, like many glycosylases, will operate on many more substrates than just 8-oxoG including several imidazole ring-opened purines, oxidized pyrimidines, and abasic sites.[26] The mechanism of action of Fpg is illustrative and appears to involve formation of a Schiff base by an amino group at or near the N-terminus of the protein with the aldehyde of the abasic site, in a fashion analogous to piperidine-induced DNA cleavage (Figure 4).[26] Fpg has both β and δ-lyase activity; that is, a second elimination occurs 5′ to the initial one leaving behind 3′- and 5′-phosphorylated fragments as in piperidine treatment.[70]

In addition to knowing the substrate specificity, one must understand how the cleavage site relates to the site of nucleobase damage. For example, T4endoV and UV endonuclease recognize thymine dimers, and an enzyme nucleophile attacks at C1′ of the 5′ T.[63,71] On the other hand, some enzymes cut out a short oligonucleotide patch surrounding the site of damage, thus the length of the remaining oligonucleotide analyzed on a gel does not allow easy prediction of the nucleotide that was initially modified. In addition, these excision repair systems

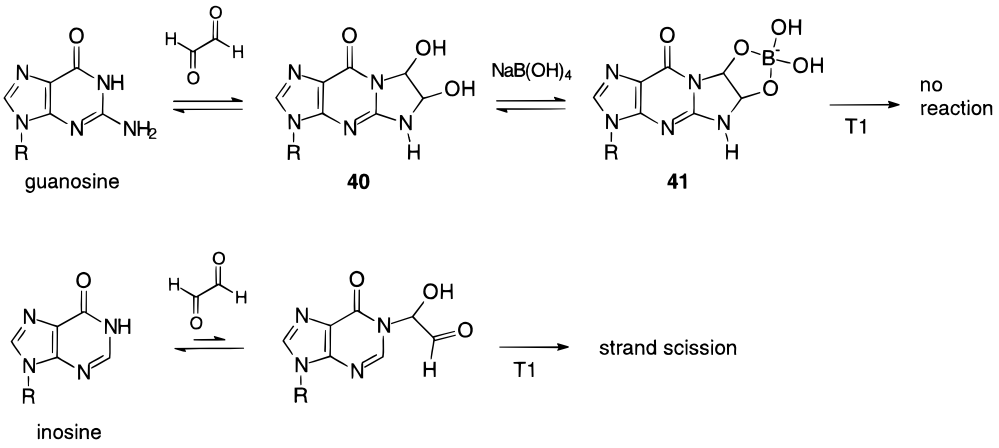

Figure 11. A chemical/enzymatic method for detection of inosine in RNA.

involve a multiprotein complex that would be cumbersome to employ as a cleavage agent.[72]

Certain classes of DNA repair enzymes are not applicable as cleavage agents because they operate on the nucleobase itself without excision. For example, photolyase reverses the thymine dimer cyclobutane formation without cleaving DNA strands.[73] Similarly, the O^6-methylguanine DNA methyltransferases (MGMT) dealkylate guanine without excision.[74]

The enzymatic methods described above are applicable to DNA modification followed by strand scission with a DNA repair-type enzyme. Because there is little biological need for RNA repair, a complementary set of RNases for nucleotide repair does not exist. Base-specific RNases do exist, however, and an example is ribonuclease T1 which hydrolyzes 3′ phosphodiester bonds following both guanine and inosine residues. An application of this enzyme is found in the recent work of Morse and Bass who combined RNA base modification with enzymatic cleavage.[75] In this case, all inosines in an RNA target could be identified by converting all guanines to glyoxal−borate adducts (**41**, Figure 11), which were then no longer recognized by RNase T1. Subsequent cleavage of RNA occurred specifically at inosine residues.

C. Direct Strand Scission Following Nucleobase Modification

The majority of nucleobase lesions do not lead to direct (or frank) strand scission. Nevertheless, a few examples have been reported in which a reactive moiety of a nucleobase leads to deoxyribosyl hydrogen atom abstraction and, in turn, strand scission, without a subsequent alkaline or enzymatic treatment. Most of these examples involve the study of suitably modified pyrimidines to generate radical centers at predictable sites such as C5 or C6. Such radicals may be relevant to DNA damage that occurs as a result of ionizing radiation, since both H• and HO• are thought to add to thymine's 5,6 double bond to generate either 5-yl or 6-yl species.[17]

5-Bromodeoxyuridine (5-BrdU) mimics thymidine in size and hydrogen-bonding ability, but it is photoreactive in a different manner than is thymine.[76] Irradiation of a 5′-dA-(5-BrdU)-3′ sequence leads to cleavage of the C5−Br bond, forming the C5 radical (**42** in Figure 12) and an adjacent adenine radical cation.[77] (5′−G-(5-BrdU)-3′ sequences show 8-fold less reactivity.) In duplex DNA, the C2′ hydrogen atom of the adjacent nucleotide is near the C5 radical, and isotope studies have shown that $H^{2'}$ is the major site of reaction.[78] The C2′ radical formed (**43**) could then be oxidized by the adenine radical cation to produce **44**. A 1,2 hydride shift from either C1′ or C3′ (illustrated in Figure 12) leads to an easily hydrolyzed species. Overall, a photochemical event initiated on a uracil base generates a sugar radical, leading to direct strand scission. Alkali-labile products are also observed. The same general mechanism is proposed to result from oxidative damage to uracil by sulfate radical ($SO_4^{\bullet -}$) in which radical damage is transferred from the base to the 2′ position in the adjacent ribosyl moiety.[79] This apparently does not occur for the equivalent deoxyribosyl analogue.

A C5 thymidyl radical can be produced at a saturated site (**45** in Figure 13) by Norrish type I photocleavage of an acylated precursor.[80] Dioxygen-dependent direct and alkali-induced strand breaks are observed at the nucleotide located 5′ to the thymidyl radical. A mechanism is proposed, again based on the observation of significant primary kinetic isotope effects, in which the peroxyl radical (**46**) derived from O_2 attack at **45** abstracts the C1′ hydrogen of the 5′ adjacent nucleotide.[80] Such a lesion will lead to both direct strand breaks and enhanced scission upon alkaline workup.

Curiously, direct strand scission is observed in RNA motifs containing U residues located 3′ to a GU wobble base pair under oxidative conditions.[81] Whether or not this reaction is following the same base-to-sugar radical transfer mechanism remains to be determined.

Although outside the context of this review, there are a few examples of C5′ sugar radical centers being transferred to reactive sites on nucleobases, effectively the reverse of the examples above. Adducts of the C5′ radical with the C8 position of purines (e.g., **47**, Figure 14)[82] and with the C6 position of cytosine (**48**) or thymine[83] have been reported. Note that for the cytosine adduct, the observed product is actually a uracil derivative. This is a common result of reactions leading to sp^3 hybridization of the C5

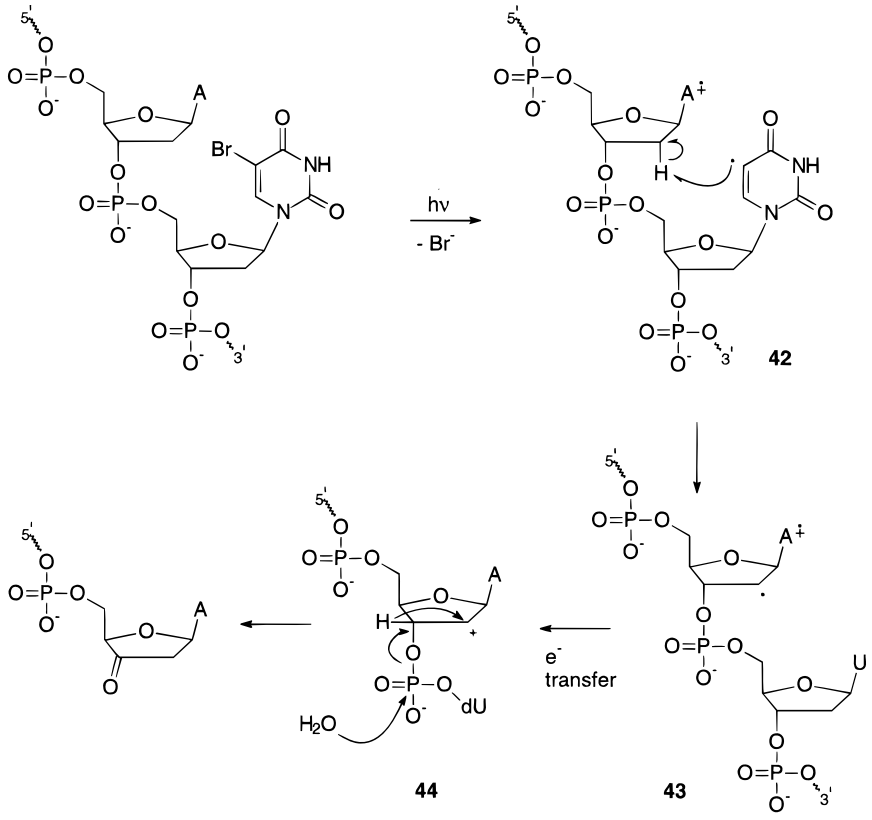

Figure 12. Direct strand scission initiated by a nucleobase radical.

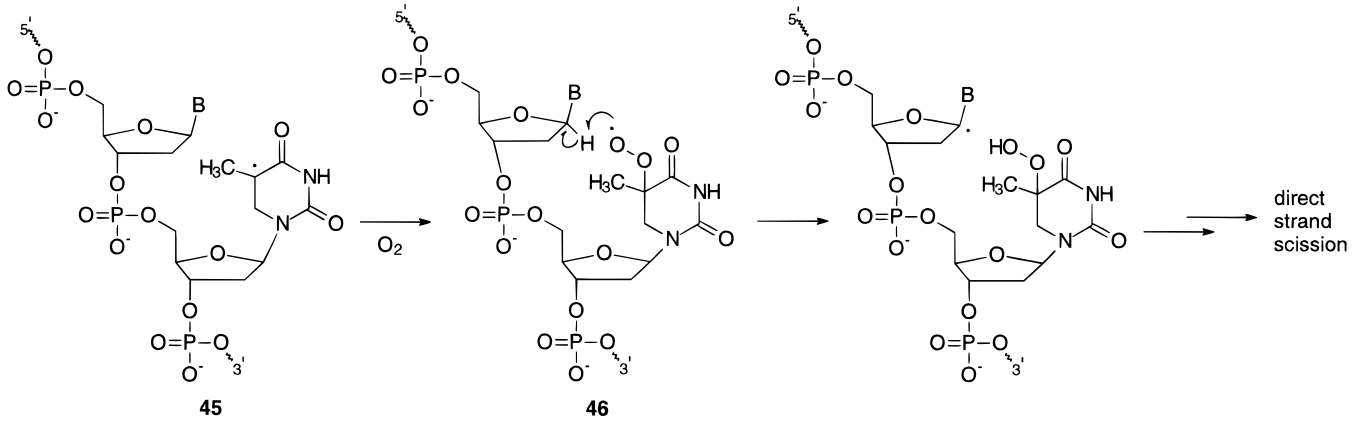

Figure 13. Direct strand scission initiated by a nucleobase radical.

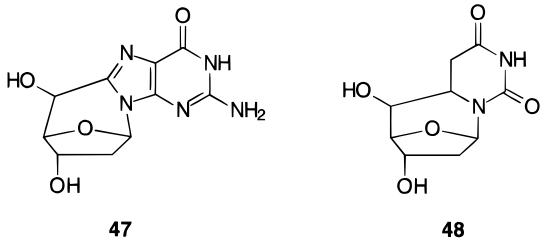

Figure 14. Nucleobase adducts formed from ribosyl radicals.

position of cytosine, in which the subsequent facile hydrolysis of the N^4 amino group provides a carbonyl group in this position.[84] The alkali lability of **48** has not been determined, but most guanine C8 adducts such as **47** are piperidine labile. Some of these products have been observed from radiation damage to DNA.[85]

Overall, direct strand scission is a minor pathway following nucleobase modification. Usually oxidation or adduct formation with the bases remains localized on the base, and alkaline or enzymatic conditions are required to effect strand scission. A number of nucleobase lesions are resistant to strand scission by these methods, and the fact that a reaction has occurred must be ascertained by other methods, as described in the next section.

D. Other Methods for Detecting Nucleobase Modification

DNA or RNA cleavage, as determined by observation of nucleic acid fragments by gel electrophoresis, does not necessarily reveal all of the chemical modification events that result from a particular reagent. A number of nucleobase adducts, notably those at exocyclic atoms of the purines and pyrimidines, are

stable to alkaline and enzymatic cleavage. Additional experiments must then be done to first detect that such adducts exist and subsequently to determine their identity. This can be investigated by (i) examining model reactions of mononucleotides, (ii) HPLC isolation of modified nucleotides from a digest, (iii) studies of short oligonucleotides by gel electrophoresis, electrospray mass spectrometry, and NMR, and (iv) primer extension analysis.

1. Mononucleotide Model Studies

Mononucleotides should, in theory, demonstrate the same chemical reactions as nucleic acid polymers. However, the reactivity of sites in folded nucleic acid structures can be dramatically different from their monomer counterparts. This is due to differences in electrostatic effects of polyanions vs nucleotide monophosphates, changes in solvation for monomers vs polymers, steric hindrance due to base pairing and helix formation, and electronic effects of base stacking. For example, generation of guanine radical cation can lead to at least two different pathways; the monomer is rapidly deprotonated and the neutral radical follows a cascade of events with dioxygen leading to imidazolone and oxazolone products (see section IV.B.1.b).[86] In duplex DNA, the deprotonation appears to be slower, and 8-oxoguanine is a major product.[69,87] An example in acylation chemistry involves the formation of an N^2-acylated guanine product, a non-alkali-labile site, from dibenzoyldiazomethane when a guanosine analogue was used as the substrate.[88] However, when DNA was the target, both piperidine-labile (assumed to be an N7 adduct) and non-piperidine-labile chemistry were observed. Thus, the monomer model study mimicked some, but not all, of the reactivity observed with duplex DNA. Monomer studies have the advantage that one can work with relatively large quantities and carry out standard experiments for structure elucidation—HPLC, MS, NMR, etc. Usually one works with the nucleotide or nucleoside, rather than the nucleobase alone, to achieve water solubility; this is particularly true for guanine. Reactions carried out in organic solvents utilize O-silylated ribonucleosides or deoxyribonucleosides which facilitate characterization by GC-MS.[89]

2. Total Digests

An alternative approach for identifying nucleobase products is to carry out chemical modifications on a large sample of nucleic acid such as calf thymus DNA. The modified polymer is then completely hydrolyzed or digested down to nucleotides that may be further converted to nucleosides by alkaline phosphatase before analysis. Most nucleobase adducts are stable to 60% formic acid hydrolysis but certain bases undergo further decomposition.[90] Enzyme cocktails including deoxyribonuclease I, spleen exonuclease, and snake venom exonuclease in conjunction with alkaline phosphatase lead to total digestion of large DNA polymers to nucleosides that can then be characterized by HPLC and mass spectral methods.[89] Most nucleobases are also stable to enzymatic digestion, but some deamination of adenosine (yielding inosine) may be observed.[91,92] To increase the sensitivity of detection, chemical and biochemical postlabeling methods may be used after enzymatic digestion.[93] These methods add either a fluorescent or a radiolabel and allow measurement of base damages at the level of one modification per 10^7 normal bases. For RNA, ribonucleases may be used, but relatively mild basic hydrolysis is most effective.

3. Synthetic Oligonucleotides

DNA restriction fragments, cut from plasmid DNA by restriction enzymes recognizing highly specific sequences, have long been a classical way to study the sequence specificity of DNA modification agents. Fragments of typically 100−600 base pairs present a "library" of target sequences, and so much of sequence space can be sampled in such an experiment. On the other hand, it is difficult to determine whether chemical adducts have been formed with restriction fragments because the relatively small percentage change in molecular weight and gel mobility makes it impossible to see a higher molecular weight adduct on a gel. For this reason, short, synthetic oligonucleotides (DNA or RNA) are better targets for modification.

Both DNA and RNA synthesis are straightforward using automated instruments, so specific sequences and structures can be designed into the target. For DNA modification studies, it can be helpful to compare single-stranded vs double-stranded substrates; single-stranded DNA normally shows much higher reactivity due to the accessibility of the reactive sites on nucleobases to external reagents. Formation of high molecular weight adducts that are not alkali labile can be readily detected if the oligonucleotide is relatively short, e.g., 12−20 nucleotides. Shorter oligonucleotides can more easily be studied by HPLC; longer ones are better behaved if duplex structures are examined (T_m's will be higher) and also are more amenable to purification by precipitation, if that is necessary.

An example of a high molecular weight adduct formed under oxidative conditions is shown in Figure 15.[94] The nickel(II)CR complex **49** and the nickel(II)salen derivatives **50** and **51** undergo one-electron oxidation in the presence of a strong oxidant, monoperoxysulfate (HSO_5^-). For **50**, the nickel(III) complex that results is unstable with respect to ligand oxidation,[95] and the electron-deficient site resides on the phenolate ligand making, in effect, a phenoxyl radical. Lane 2 in Figure 15 shows the formation of a high molecular weight adduct with a hairpin-forming 17-mer, as well as a band comigrating with starting material. To examine piperidine lability, the high MW band was isolated from the gel (its purity is shown in lane 3), and subjected to hot piperidine treatment (lane 4). Some, but not all, of the high molecular weight adduct was piperidine labile and shows cleavage sites at guanines, particularly those in the single-stranded loop region. The lower band of lane 2 was shown to be unreacted starting material by isolation from the gel (lane 5 shows its purity) and piperidine treatment (lane 6). The reaction of a G-oxidizing reagent (**49**) is shown in lane 7 after

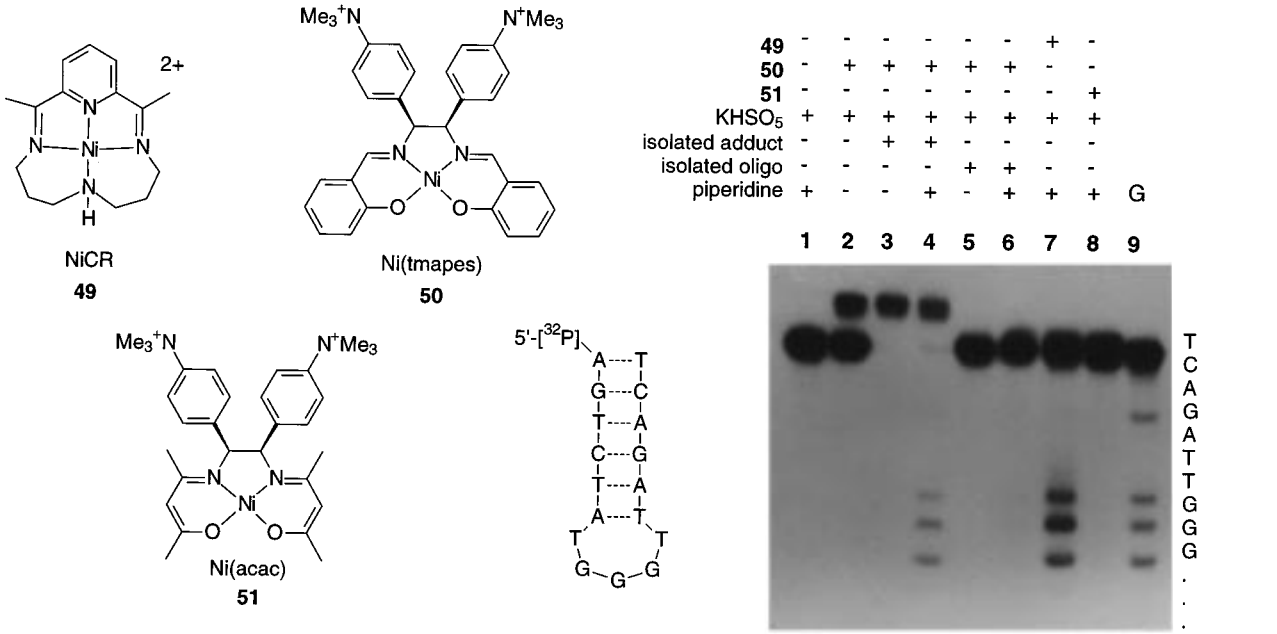

Figure 15. Nucleobase adducts can be detected as high molecular weight bands by gel electrophoresis. Reactions of complexes **49**, **50**, and **51** with a 17-base, hairpin-forming oligodeoxynucleotide in the presence of $KHSO_5$ as oxidant led to formation of an adduct only for complex **50**. Analysis of the piperidine lability of the adduct (lane 4) and of the starting material after reaction (lane 6) can be compared.

piperidine treatment for comparison. From this type of study, one can draw the following conclusions. The phenol-containing complex **50** undergoes oxidation to form a reactive species capable of coupling with guanines. Two types of adducts are formed, one piperidine-labile, one not. The piperidine-labile adduct is formed to guanines, especially those exposed in a loop region. It has been suggested in the literature that this might be an addition of the phenol radical to the C8 position of G.[96] (See also section VI.B.4.) A role for the phenol moiety is confirmed by the lack of reactivity of the corresponding acetylacetonate-type complex **51**. The site of adduct formation in the non-piperidine-labile product cannot be deduced from this experiment. Additional work must be done to characterize this adduct, but the site of reaction on DNA or RNA can be determined from a primer extension experiment, as described in the following section.

A further advantage of working with short, synthetic oligonucleotides is their ease of analysis by electrospray ionization mass spectrometry (ESI-MS).[97,98] The technique provides molecular ions without fragmentation, although care must be taken to remove hard cations (Na^+ and K^+) since they will considerably complicate the spectrum.[97] In the example shown in Figure 15, it was assumed that the high MW band (lanes 2−4) represented a 1:1 adduct of the oligodeoxynucleotide and complex **50**. This was later confirmed by ESI-MS.[99] In principle, the sequence site of modification can also be determined by relatively new techniques involving secondary ion mass spectrometry.[100]

4. Primer Extension Analysis

An excellent method for detecting sites of chemical modification makes use of the ability of polymerase enzymes to build a complementary copy of a DNA or RNA strand given a "primer" oligonucleotide and all of the deoxynucleotide triphosphates (dNTPs) with which to build, as shown in Figure 16.[101,102] Virtually any length of DNA or RNA may be used as the subject for study as long as it substantially exceeds the length of the primer used. After reaction with a chemical modification reagent, the nucleic acid target is denatured and allowed to anneal to a short 15- to 20-nucleotide primer, present in excess, that is complementary to a segment downstream of the region to be analyzed on the template strand. The primer is typically DNA although the template strand may be either DNA or RNA. In the presence of either polymerase I (for complements to DNA) or reverse transcriptase (for DNA complements of RNA) and the dNTPs, the primer will be chain extended in the 3′ direction until a lesion is reached on the target. Most, but not all, chemical modifications to nucleobases will slow or stop the action of the polymerase leading to an accumulation of a deoxyoligonucleotide whose length represents the primer plus the distance to a modified site on the template. Under ideal single-hit conditions, each template strand is modified only once but the collection of all strands contains a population of modified sites that generates, in turn, a population of extended oligodeoxynucleotides. The extended primers are then ready to be analyzed by denaturing gel electrophoresis.

Interpretation of the bands is somewhat confusing; an example is shown in Figure 16. A control experiment with an unmodified template strand will show some unreacted primer at the bottom of the gel, a few bands just above it representing partial extension (even in the absence of a polymerase enzyme) and a dark band at the top of the gel representing full, uninterrupted extension (Figure 16, lane 1). For reference, sequencing lanes are obtained also by primer extension using the dideoxy method.[103] To do

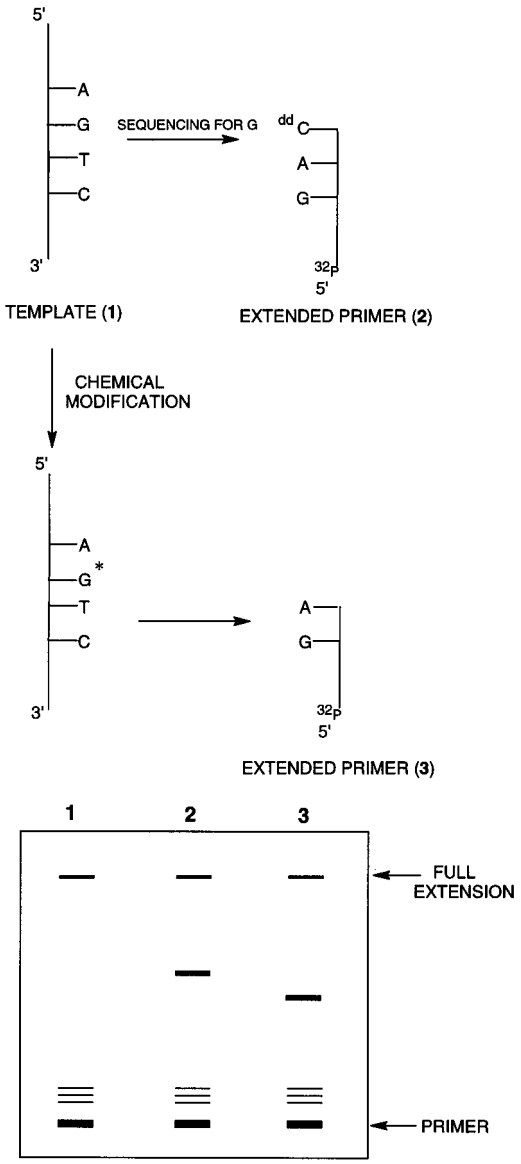

Figure 16. Schematic diagram of a primer extension experiment. For example, a G sequencing lane is obtained by extending a complementary primer in the presence of all dNTPs plus 2′,3′-dideoxy-C (lane 2). On the other hand, a modified guanosine will not incorporate C into the extended primer, giving a oligonucleotide that is one nucleotide shorter and a band that is one position lower on a gel (lane 3).

this, the primer is extended using the unmodified template strand in the presence of dNTPs that are doped with the appropriate amount of 2′,3′-dideoxynucleotide triphosphates, ddNTPs, which act as chain terminators. For example, a G lane would be obtained from the random incorporation of ddC opposite Gs on the template strand. This is shown for one G site in Figure 16, lane 2. If the template strand is subjected to a chemical reaction that occurs at that same G, the band resulting from primer extension complementary to the modified template will actually be one base shorter, since dC will not (usually) be incorporated. The extended primer thus analyzed will give a band one nucleotide lower on the gel, as in Figure 16, lane 3. It is also possible that a sterically large adduct at a particular base will prevent extension at an earlier site, and thus bands corresponding to even shorter extended primers might be observed in some cases.

The primer extension assay has some distinct advantages over other methods. The analysis can be performed on an extremely small sample size of DNA or RNA, and the extended primers may be amplified by PCR. Either strand of duplex DNA may be studied and different regions of a large biopolymer may be examined simply by choosing a different primer sequence. Furthermore, there is less need to purify the synthetic primer; incorrect sequences do not anneal as well and will not be amplified as readily. Importantly, primer extension is capable of finding most types of covalent chemical modifications, not just those leading to alkali-labile lesions, and it is particularly useful for finding the site of covalent attachment of drugs. For example, guanines were found as the platination site in DNA with (*cis*-dichlorodiammine)platinum(II) by use of a primer extension experiment.[101]

Some lesions, such as methylation of guanine with dimethyl sulfate, do not slow the action of polymerases, and so go undetected.[102] On the other hand, methylation with DMS of N1 of adenine gives "stops" in primer extension although it is not an alkali-labile site.[13] The common oxidative lesions, 8-oxoguanine and 8-oxoadenine do not give primer stops when used in conjunction with the high-temperature polymerases employed in PCR. Despite these problems, this assay is the method of choice for most analyses of long DNA or RNA oligomers. Numerous kits are commercially available for DNA analysis and dideoxy sequencing.

As discussed in the previous section, complex **50** forms both piperidine-labile and non-piperidine-labile covalent adducts with DNA. To determine which nucleobase was responsible for the non-piperidine-labile adduct, a primer extension experiment was carried out using an RNA target, the L-21 Sca fragment of the *Tetrahymena* group I intron.[104] The nucleotides of positions 57−315 were examined, and it was found that all of the modification sites corresponded to guanines, especially those in exposed loops or bulges. Thus, both the non-piperidine- and piperidine-labile sites were found to be due to guanine modification. Further discussion of the probable sites of guanine modification is found in section VI.B.4.

IV. Oxidation of Nucleobases

Oxidative damage to nucleobases is a major pathway for DNA and RNA cleavage. Much attention has focused on oxidative events in the deoxyribose moiety because this can lead to direct strand scission.[52] Indeed, many of the common DNA-targeted chemotherapeutics utilize this mechanism—bleomycin[105,106] and the enediynes[107] being the best studied. It is interesting that these natural products have evolved to bind DNA very specifically, and to position their reactive group in a precise location for abstraction of a sugar hydrogen atom.[108] In the absence of such specific-binding interactions, one would expect oxidants and free radical species to react with the electron-rich nucleobases. For example, it is esti-

mated that over half of the DNA damage inflicted by hydroxyl radical occurs on nucleobases,[109] despite its application in the direct scission mode with ribosyl hydrogens as a DNA cleaver.[19,110] This section begins with a summary of oxidizing agents and then covers the specific base lesions that are known to lead to strand scission.

A. Oxidizing Agents

1. Hydroxyl and Related Radicals

a. HO•. Hydroxyl radical damage to nucleobases has been widely reviewed,[17,109,111−113] and a summary will be presented here. Hydroxyl radical, HO•, can be produced in a variety of ways (Figure 17). It is a product of ionizing radiation (γ radiolysis) which also produces hydrogen atoms (Figure 17a). Solvated electrons are an intermediate in this process and can also effect DNA damage. Hydroxyl radical-like species are also produced from the Fenton reaction of iron(II) with hydrogen peroxide (Figure 17b).[114,115] Although there has been some debate concerning the formation of free vs metal-ligated species in the Fenton reaction, recent work by Pogozelski et al. demonstrates that the deoxyribosyl-targeted chemistry of the aqueous Fenton reaction is identical to that of HO• formed by γ radiolysis, implying that free hydroxyl radical is responsible for the sugar damage observed by Fe(II)−EDTA + H_2O_2.[25] On the other hand, reaction conditions do play a role in the oxidation DNA damage observed with HO•. In the presence of excess reductants, such as Fe(II), oxidized nucleobases (base radicals and radical cations) may be reduced back to undamaged species.[86] Thus, one will observe a higher proportion of nucleobase damage in the absence of added reductants. With reductants present, larger amounts of sugar chemistry occur. This explains why the Fenton chemistry used in DNA and RNA footprinting yields a major amount of sugar hydrogen atom abstraction and direct strand scission, while radiation chemists typically observe predominantly nucleobase chemistry.

Other transition metals may produce hydroxyl radicals by mechanisms similar to the Fenton reaction.[116] The question always arises, however, as to

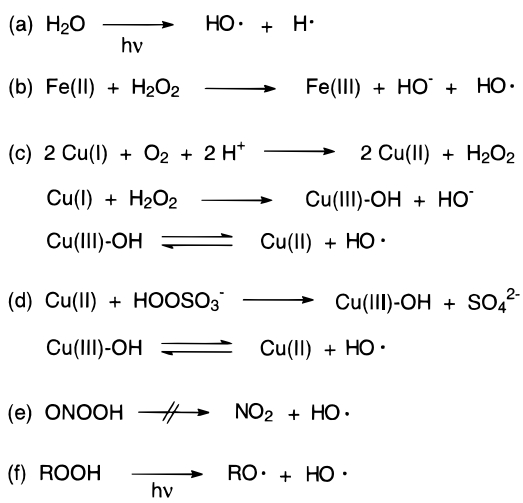

Figure 17. Reactions generating hydroxyl radical.

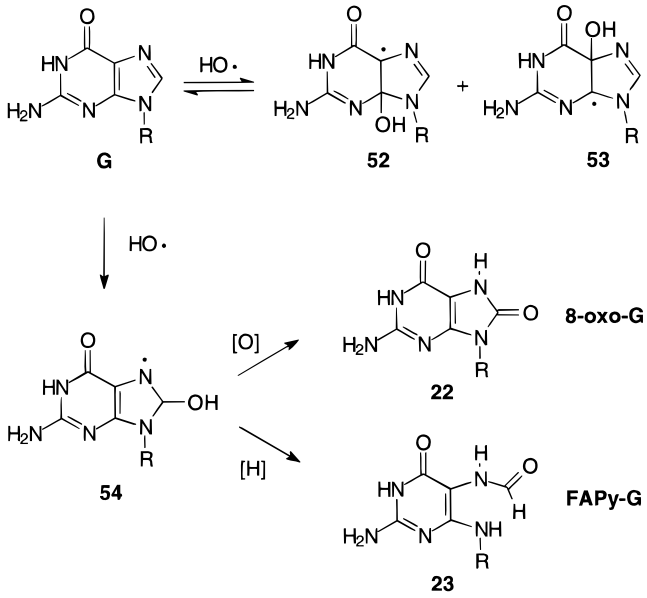

Figure 18. Addition of hydroxyl radical to guanine.

whether the active oxidant is actually free hydroxyl radical or a metal bound species, such as a high valent M^{n+1}—OH or deprotonated M^{n+2}═O (Figure 17c). This question is still unresolved for Cu(I)/H_2O_2.[117,118] The metal-catalyzed decomposition of peracids can also lead to HO•-like species (Figure 17d).[119] The decomposition of peroxynitrous acid does not require transition metal catalysis, but it is pH dependent. It has been used as a hydroxyl radical DNA footprinting agent,[120] but recent studies argue strongly that HO• is not formed in its decomposition (Figure 17e).[121] (The chemistry of peroxynitrite with nucleobases is discussed further in section IV.A.4.a.) Finally, hydroxyl radical has also been reported as a product in photolytic decomposition of alkylhydroperoxides (Figure 17f).[122−129]

There are three major intermediates that result from hydroxyl radical attack at a guanine nucleobase, the C4 adduct **52**, the C5 adduct **53**, and the C8 adduct **54** (Figure 18). The chemical fates of these radical intermediates have been investigated extensively,[113,130,131] and a thorough discussion appears by Breen and Murphy.[109] In general, adducts **52** and **53** revert back to guanine by gaining an electron from the medium (or from cellular thiols, in vivo). The C8 adduct **54** leads to two forms of DNA damage that are both heat and alkali labile, at least to some extent. The major pathway under oxidative conditions yields 7,8-dihydro-8-oxoguanine, **22** (8-oxoG), sometimes referred to by its minor tautomer, 7,8-dihydro-8-hydroxyguanine.[113,132] Under reducing conditions, gain of an electron and a proton leads to imidazole ring-opening, and the isolated product is 2,6-diamino-5-formamido-4-hydroxypyrimidine, **23** (FAPy-G). The scheme is further complicated by the observation of imidazolone and oxazolone products, **56** and **57**.[133] These are proposed to be formed from the neutral radical intermediate **55** (Figure 19) that can result from dehydration of **52**, **53**, or **54**, or from deprotonation of guanine radical cation (see section IV.B.1.b.).[86] Intermediate **55** is converted to the ring-

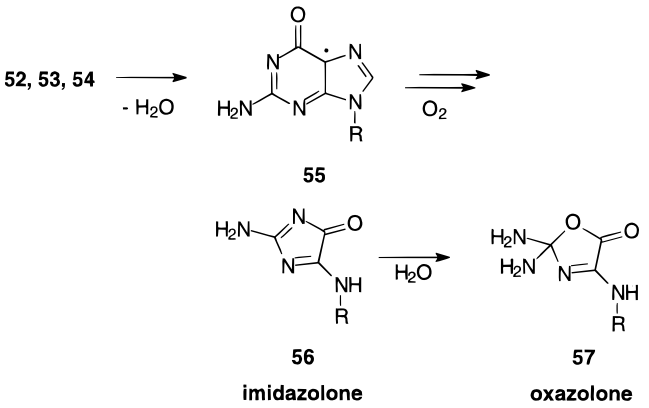

Figure 19. Formation and fate of guanine radical **55**.

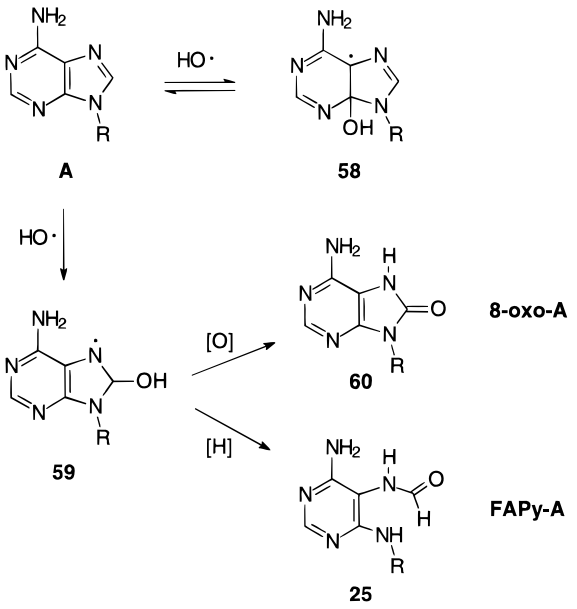

Figure 20. Addition of hydroxyl radical to adenine.

opened product **56** in the presence of dioxygen, and **56** slowly hydrolyzes to **57**.

The chemistry of adenine with HO• parallels that of guanine, although oxidative A lesions are less prevalent in DNA damage. The key addition products are the C4 adduct **58** and the C8 adduct **59** (Figure 20).[134] Radical **59** undergoes a further one-electron oxidation to yield 8-oxoA (**60**) or reductive ring opening to give the FAPy-A product **25**. 8-OxoA is not alkali-labile while FAPy-A is.

Thymine displays a rich variety of reactions with hydroxyl radical.[17,86,109,112,135] The principal adducts occur at C5 (**61**) and C6 (**62**), and hydrogen atom abstraction is observed from the exomethyl group, yielding radical **63** (Figure 21). These intermediates are formed in the ratio 56:35:9 for **61**:**62**:**63**.[136] For uracil, the ratio of C5:C6 HO• adducts is 80:20.[136] These radicals can potentially abstract hydrogen atoms from adjacent ribosyl groups leading to direct strand scission, as already described (section III.C). Subsequent competing reactions lead to oxidized thymine products **29**, **67**, and **68**. Thymidine glycol, **29**, results from either of two pathways—direct one-electron oxidation of **61** followed by hydration of the cation or intermediate formation of hydroperoxide **64**. Similarly, hydroperoxides **65** and **66** lead to rearranged hydantoin **68** and to the aldehyde, **69**.[137] The latter product is probably not an alkali-labile lesion in DNA, while products **29**, **67**, and **68** are.

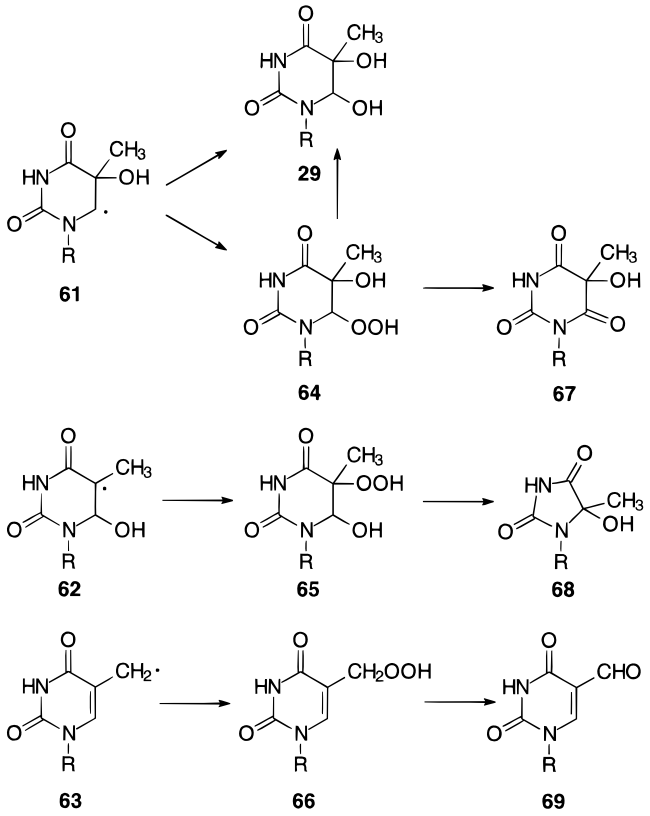

Figure 21. Reactions of thymine with hydroxyl radical.

The chemistry of cytosine with hydroxyl radical is complicated by the fact that adducts to the 5,6 double bond make the N^4 amino group more susceptible to hydrolytic deamination.[84] Thus, uracil derivatives are common products of C oxidation. The two principal HO• adducts are formed at C5 (**70a**) and C6 (**70b**) in an 87:10 ratio,[138] and they lead to a cascade of products that depend on oxidative or reductive conditions as well as the rate of deamination (Figure 22). 5,6-Dihydroxy-5,6-dihydrouracil (**71**) is one of the major products,[139] and it has been noted that addition to the 5,6 double bond labilizes the N-glycosidic bond, facilitating release of modified nucleobases and creating an abasic site.[140] The mechanistic pathways are discussed in Breen and Murphy[109] and elsewhere.[17,135,138]

b. RO•. Alkoxyl radicals, RO•, by analogy to hydroxyl radical have been studied for their ability to inflict DNA damage. The mechanistic interpretations of strand scission and nucleobase oxidation are complicated by the fact that alkoxyl radicals are usually generated from hydroperoxides, and so chemistry might also arise from other species such as ROO• or HO•. It might be expected that tBuO• would be capable of both hydrogen atom abstraction from sugars leading to direct strand scission as well as radical reactions with nucleobases.[141] RO• generated by radical ring opening of an epoxide led to plasmid DNA nicking,[142] but the direct strand scission could have initiated on the nucleobase, as previously discussed. However, studies by Hazlewood and Davies showed that nucleobase chemistry predomi-

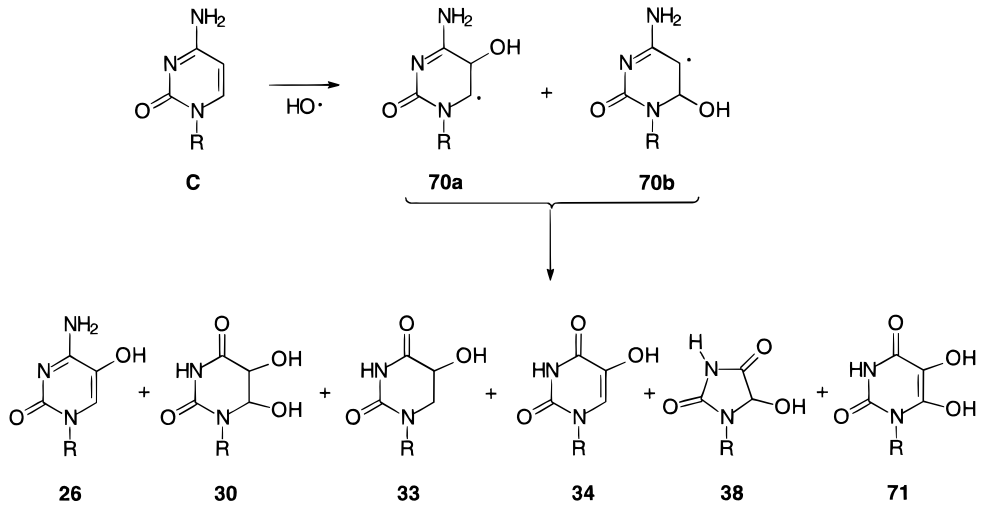

Figure 22. Reactions of cytosine with hydroxyl radical.

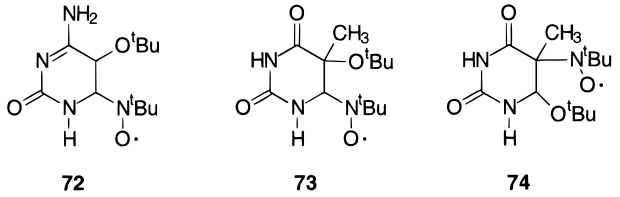

Figure 23. Spin-trapped products of tBuO• addition to pyrimidines.

nates with tBuO•, and that the regiochemistry of RO• differs somewhat from that of HO•.[143,144] For uracil or cytosine, an adduct is formed at the C5 position, generating the 6-yl radical that can be spin-trapped using 2-methyl-2-nitrosopropane (**72**, Figure 23). For thymine, adduct formation at C5 (**73**) predominates over C6 (**74**) by 13:1 at pH 7.4, and no ESR signals were detected that indicated hydrogen atom abstraction from the methyl group of T.

c. ROO•. The chemistry of alkylperoxyl radicals (ROO•) with nucleobases is particularly relevant to DNA damage since lipid hydroperoxides are a common result of cellular exposure to oxidative stress. Exposure of lipid hydroperoxides to nickel(II) and glutathione results in plasmid nicking by an unknown mechanism.[145] 8-OxoG (**22**) was detected as a product of this reaction, and it is proposed that ROO• adds to C8 of guanine. An alternative route, electron transfer from G to form guanine radical cation, is not plausible based on reduction potentials measured for the guanosine radical vs ROO•.[146] 8-OxoG formation may also account for the G→T transversions observed in M13mp19 single-stranded plasmids on exposure to 2-amidinopropylperoxyl radicals generated from AAPH.[147] The mutagenicity of the lesion was reduced by alkaline treatment, which might be consistent with 8-oxoG formation. In a related experiment, the same peroxyl radical studied with a restriction fragment showed G-specific cleavage.[148] In another study with AAPH, the thymidine nucleoside was shown to react primarily at the 5-methyl group to form 5-(hydroxymethyl)-2′-deoxyuridine (12%), 5-(hydroperoxymethyl)-2′-deoxyuridine (17%) and 5-formyl-2′-deoxyuridine (6%).[149] In contrast, thymine residues were completely unreactive with 2-hydroperoxytetrahydrofuran, an autoxidation product of THF.[150] Instead, C-specific DNA strand scission was observed using THF−OOH and 10 mM dithiothreitol (DTT) after heat and piperidine treatment. When the DDT concentration was increased, a switch to G>A scission was observed. ESR studies suggest that the major radical species in solution is a carbon radical (see section VI.B.1), and its reactivity may be quite different from the ROO• and RO• examples above.

d. $O_2^{\bullet-}$. The unsubstituted perhydroxyl radical has also been studied with DNA. HOO• has a pK_a of 4.8,[151] so the conjugate base, $O_2^{\bullet-}$, superoxide, is the major species present at physiological pH. Superoxide disproportionates rapidly to H_2O_2 and O_2, but it can be generated in situ by xanthine oxidase and acetaldehyde. The direct strand scission mediated by superoxide can be attributed to hydrogen atom abstraction from C5′ of the deoxyribose.[148] Curiously, the cleavage reaction has some sequence or site-specificity. Reactions have been reported to occur with the nucleobases, but no details are given.[148] In contrast to the site-specificity observed by Dix et al.,[148] the natural products tetrazomine and quinocarcin that apparently generate superoxide cause nonspecific direct strand scission analogous to HO•, but their nucleobase chemistry has not been investigated.[152] Hydroxyl radical, a much stronger oxidant than superoxide, is also a side product of $O_2^{\bullet-}$ chemistry. Thus, reagents that generate superoxide, especially those that are bound to DNA, should also display some of the purine, pyrimidine, and ribose chemistry of HO• (see section IV.A.1.a).

Hydrogen peroxide has been developed as a C-specific sequencing reaction for single-stranded and double-stranded DNA.[153] No details are available concerning the actual products of cytosine oxidation, but the reagent leads to piperidine-mediated strand scission after reaction with 250 mM H_2O_2 in triethylammonium acetate buffer.

2. Photochemical Oxidations

The direct irradiation of DNA with ultraviolet light leads to a variety of lesions, many of which are centered on the nucleobase and are alkali labile (Figure 24).[154] The wavelength and intensity of light

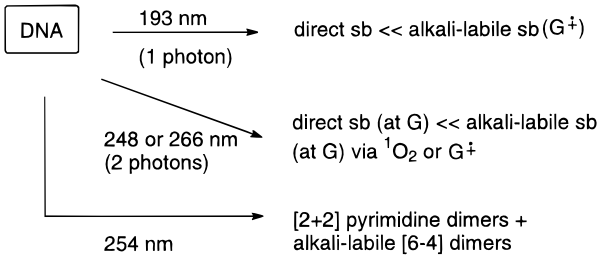

Figure 24. Chemistry resulting from direct irradiation of DNA (sb = strand breaks).

determine to some extent the type of lesion obtained. By using a 193-nm laser source, a monophotonic process is observed that gives principally alkali-labile guanine lesions through one-electron oxidation of guanine, although direct strand breaks are also observed.[155] Since guanine is the most easily oxidized base, much of the initial photoionization is thought to occur at Gs.[156] In addition, hole migration from other bases to guanine has been observed.[156−158] The guanine radical cation produced then yields alkali-labile products including 8-oxoG (**22**) and others (see section IV.B.1.b). The source of the direct strand breaks on photoionization is not well understood (see next paragraph), but may result either from C4′-hydrogen atom abstraction by nucleobase radicals or from formation of phosphate radicals. The involvement of guanine radical cation is implied from the fact that direct strand breaks occur preferentially at guanines.[159,160]

A two-photon photooxidation of DNA using either 248- or 266-nm light sources also leads to alkali-labile guanine oxidation, but this process may be due to formation of singlet oxygen. Curiously, the two-photon process, like the monophotonic, 193-nm irradation, also leads to direct strand breaks that occur on the 3′ side of a guanine. When this fragment is further treated with hot piperidine, the gel band shifts by approximately one nucleotide, suggesting that the initial lesion caused scission, but that a piperidine-labile ribose fragment was still attached.[161] Guanine radical cation was proposed as an intermediate in this process, but the lack of a 5′-GG-3′ sequence dependence would argue against this (see section IV.B.1.a).

When 254-nm light is used to mimic photolesions formed from sunlight, the principle products are pyrimidine photoadducts rather than photooxidation (Figure 25). The commonly studied [2 + 2] cyclobutane dimer products, T< >T, T< >C, and C<>C (formed in that order of preference), are not alkali-labile lesions but are repaired by various enzymes. On the other hand, the pyrimidine(6−4)pyrimidone dimer (**75**) is an alkali-labile lesion,[162] and this product is formed with about 50% of the frequency of cyclobutane dimers.[86] The mechanism appears to involve a [2 + 2] photocyclization, leading to an oxetane which later decomposes.[154] The 6−4 adduct involving a C residue further undergoes deamination to finally produce a uracil derivative. Strand scission

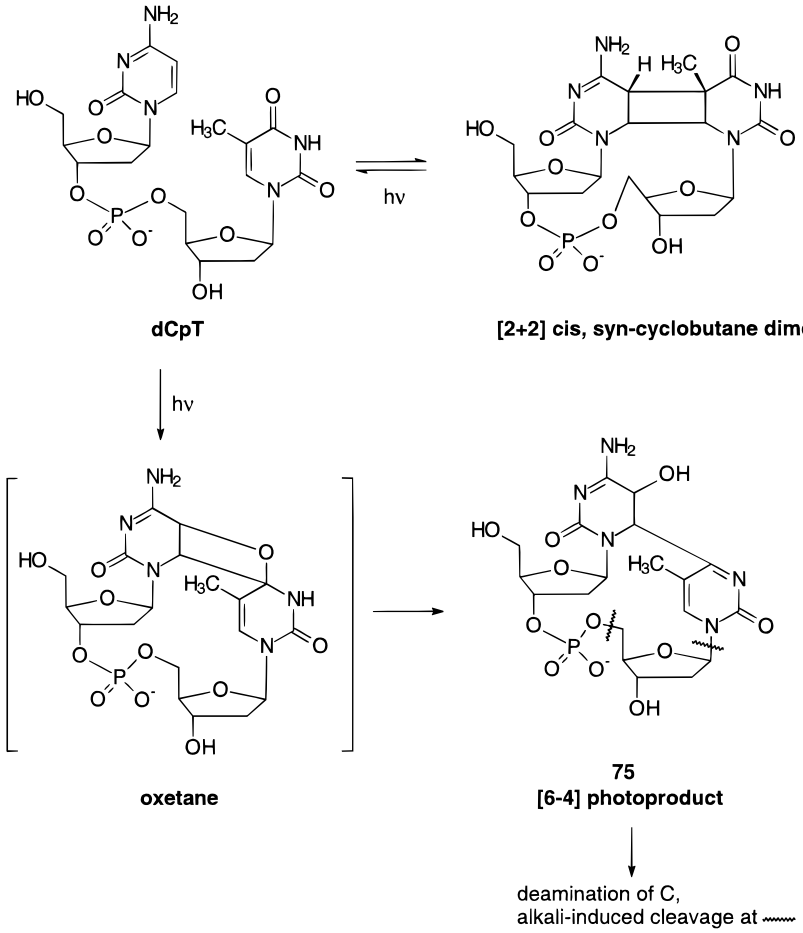

Figure 25. Photodimerization of adjacent pyrimidines creates non-alkali-labile cyclobutane dimers and alkali-labile [6−4] adducts.

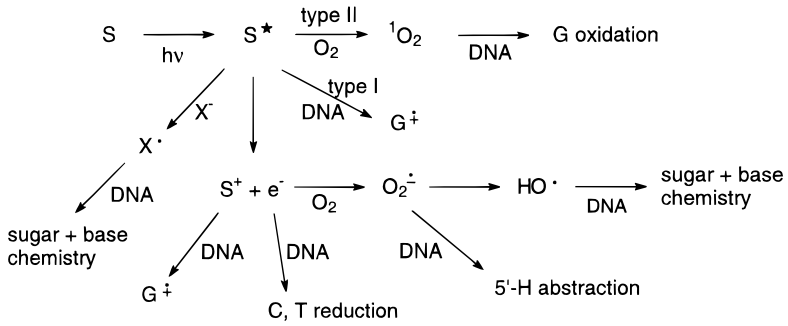

Figure 26. Chemistry resulting from photosensitized irradiation of DNA. (Adapted from ref 166.)

Table 2. Photosensitizers for Nucleobase Chemistry

structure	DNA target	mechanism[a]	ref(s)
riboflavin (**76**)	GG	e.t.	168,169
anthraquinones (**77**)	GG	e.t.	170,171
naphthalimides (**78**)	GG	e.t.	172
(**79**)	T	CH_3 oxidation	173
benzophenones (**80**)	GG	e.t.	174
benzotriazoles (**81**)	GG	e.t.	175,176
Rh complexes (**82**)	GG	e.t.	177,178
(**83**)	G	e.t.	179
Ru complexes (**84**, **85**)	GG	e.t.	180,181
(**86**)	G	e.t.	182
(**87**, **88**)	G	$^1O_2$	183,184
methylene blue (**89**)	G	$^1O_2$	185
porphyrins (**90**)	G	$^1O_2$	186
(**91**)	G	$^1O_2$	187
fullerenes (C_{60})	G	$^1O_2$	188,189
V complexes (**92**)	G?	$^1O_2$	190,191
Co complexes (**93**)	G?	$^{\bullet}CH_3$	192
isothioronium salts (**94**)	G	alkylation	193
diazomethanes (**95**)	G	alkylation	88
monothioacetals (**96**)	A	alkylation	194

[a] Abbreviations: e.t., electron transfer.

by hot piperidine was found to occur predominantly at the 3′ side of a 5′-T-C-3′ adduct.[162,163] The bands on a gel resulting from cleavage of a 5′-end-labeled oligonucleotide containing the 6−4 lesion do not quite line up with sequencing lanes, because the nucleobase dimer is attached to the 3′ end.[164] (Bond cleavage occurs at sites marked "∼" in Figure 25.) Overall, the lesson from pyrimidine photoadducts is that modification of the 5,6 double bond to form new carbon−carbon bonds (i.e. [2 + 2] thymine dimers) does not generate piperidine lability, but bond formation to the C4 position (6−4 adducts) apparently does.

Photosensitized irradiation of DNA leads to a complex array of reactions, but again much of the chemistry is centered on guanine. This subject is covered in detail by Armitage in this issue[165] and will only be briefly summarized here. An overall scheme for photosensitized DNA modification is discussed by Meunier et al.,[166] and an expanded view is shown in Figure 26. Guanine oxidation products, in addition to others, can arise from a variety of pathways including type I reactions that involve electron transfer from G to a photoexcited-state oxidant, type II reactions involving singlet oxygen production, or the generation of other oxidants including halogen free radicals ($X^{\bullet}$),[167] oxidized photosensitizer (S^+) or secondary species derived from oxyl radicals ($O_2^{\bullet-}$, $HO^{\bullet}$). A large number of photosensitizers have been studied with DNA and RNA. Representative examples that mediate nucleobase damage are shown in Figure 27, and their targets and mechanisms, when known, are listed in Table 2. The chemistry of both type I and type II nucleobase modifications will be discussed in more detail in sections IV.B.1 and IV.B.2, respectively.

3. Transition Metal-Catalyzed Oxidation

In addition to the transition metal complexes used under photochemical conditions, many redox-active metals mediate nucleobase damage in the presence of oxidants. Typically, an oxidizing agent is added in addition to the metal complex such as O_2, H_2O_2, or peracids, but numerous examples utilize electrochemically or chemically generated high oxidation state metals that may themselves oxidize DNA or do so in the presence of a reductant. In general, transition metal/oxidant systems that are sufficiently oxidizing to be capable of abstracting a ribosyl hydrogen will also be able to carry out one-electron oxidation of guanine, and potentially other bases.[195] However, many of the transition metal reagents designed as chemical nucleases have only been studied for plasmid nicking or in a direct strand scission mode. These are not included in this review, except for comparison in certain cases. Table 3 and Figure 28 list, by metal, the investigations of nucleobase lesions by metal complexes. Other reviews of transition-metal catalyzed DNA or RNA oxidation are also available.[195−202]

a. Cr. Much of the nucleobase modification research has been inspired by investigations of toxic metals.[256] This is particularly true for the highly carcinogenic metals, chromium and nickel, that appear to localize in chromatin and mediate oxidative DNA damage.[257−262] The interactions of Cr(VI) species with DNA were studied extensively by Wetterhahn and co-workers.[203,204,257] In the +VI oxidation state, chromium is less reactive, but upon reduction to Cr(V), DNA single-strand breaks (ssb) and formation of 8-oxoG are observed. Oxyl radicals such as $HO^{\bullet}$ may be involved,[205] and there is a report of singlet oxygen generation with Cr^{VI}.[263] In the presence of sulfite, Cr(VI) apparently oxidizes SO_3^{2-} to $SO_3^{\bullet-}$ that may be also responsible for formation of 8-oxoG.[206]

b. Mn and Re. Among group 7 metals, manganese has been the most extensively studied. Permanganate has been used as an alternative to Maxam−Gilbert sequencing for thymine; however,

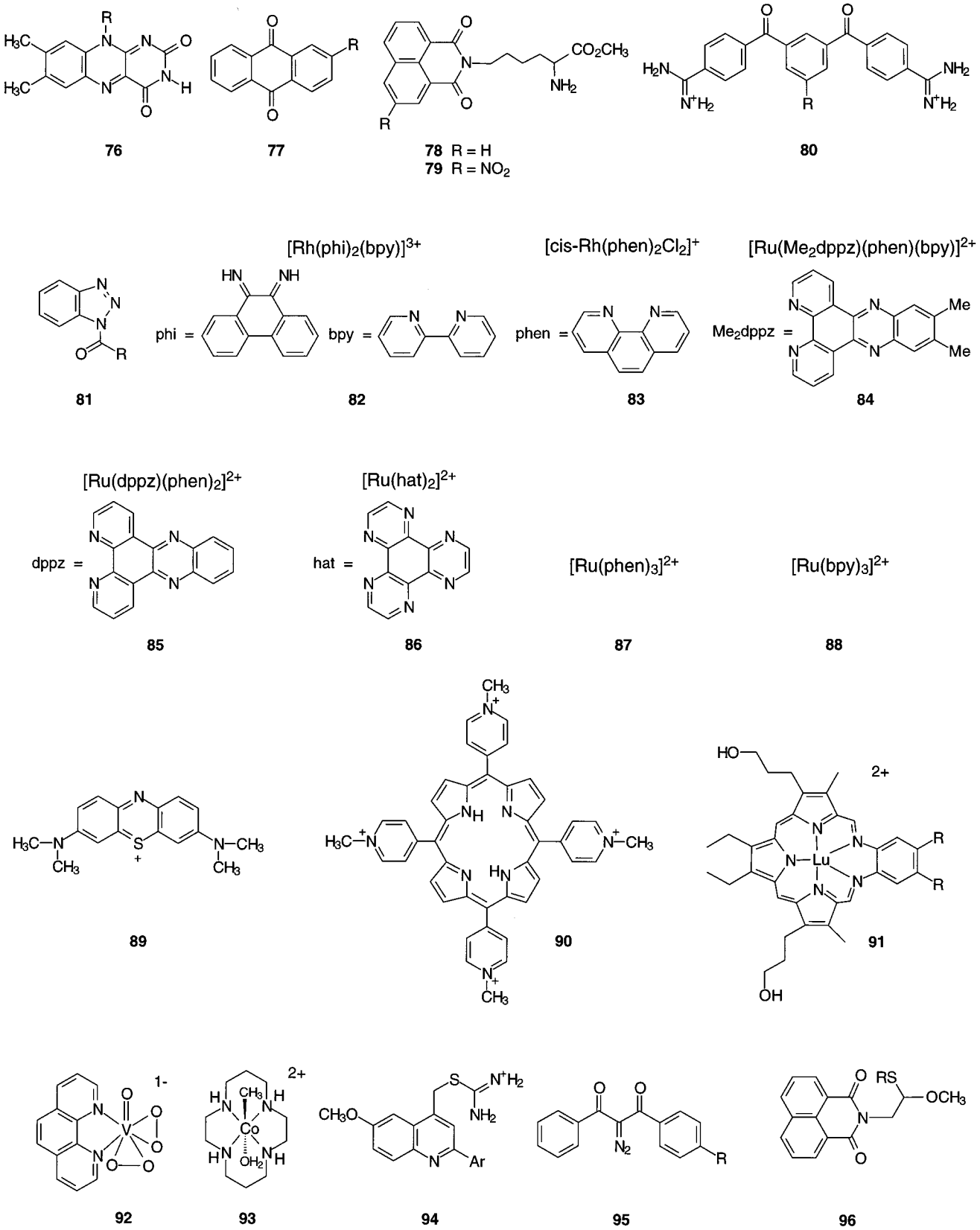

Figure 27. Photosensitizers that elicit nucleobase damage in DNA.

the reaction works best for single-stranded oligodeoxynucleotides.[38,208] Thus, the reagent is useful for detecting T residues that are part of single-stranded regions (hairpin loops, bulges, etc.; see ref 210 for examples), but is more cumbersome as a T sequencing reagent since duplex structures require heat denaturation before and/or during reaction. The reduced reactivity of duplex DNA was shown to be due to electrostatic repulsion of MnO_4^- by anionic DNA rather than conformational factors.[210] Reaction of an oligodeoxynucleotide with 50−300 μM $KMnO_4$ leads to dihydroxylation of the thymine 5,6 double bond and the products have been identified as thymine glycol (**29**) and its oxidation product **67**.[209]

Table 3. Transition Metal-Mediated Nucleobase Oxidation

metal complex	oxidant	DNA target/lesion	mechanism[a]	ref(s)
$K_2Cr_2O_7$	H_2O_2/glutathione	8-oxoG, ssb	Cr(V), oxyl radicals	203−205
$K_2Cr_2O_7$	SO_3^{2-}	8-oxoG	$SO_3^{\bullet-}$	206
$KMnO_4$	−	T	dihydroxylation	38,207−210
$MnCl_2$	O_2/RNHNH$_2$ or HAA	G, T	H_2O_2, HO$^\bullet$	116,211
$MnCl_2$	O_2/SO_3^{2-}	?	$SO_4^{\bullet-}$, $SO_3^{\bullet-}$, $O_2^{\bullet-}$	212−216
Mn^{III}−salens (**97**)	MMPP, HSO_5^-, PAA	G (+ ribose)	Mn^V=O	217,218
Mn^{III}−porphyrins (**98**)	HSO_5^-	G (+ ribose)	Mn^V=O	219
Re^VO_2(4-OMe-py)$_4^+$ (**99**)	$-e^-$	G	e.t.	220
Fe^{III}L, L = NTA, EDTA, etc.	O_2/reductant	8-oxoG + ssb	HO$^\bullet$	221−224
Fe^{3+} (asbestos)	O_2/reductant, or H_2O_2	8-oxoG	HO$^\bullet$	225,226
Fe^{III}(salicylglycine)$_2$ (**100**)	SO_2^{2-}/O_2	various	?	227
Fe^{II}(5-Cl-phen)$_3$ (**101**)	$-e^-$	G	e.t.	220
Ru^{II}(bpy)$_3$	$S_2O_8^{2-}/h\nu$	G	e.t.	228
Ru^{III}(bpy)$_3$	none	G	e.t.	229
Ru^{IV}(tpy)(bpy)O, (**102**); Ru^{III}(tpy)(bpy)OH	none	G (+ ssb)	e.t. + O atom transfer	195,230−234
OsO_4	none	G>T	?	235
OsO_4/pyr	none	T>C, G	cyclic osmylate	200,236
$CoCl_2$	H_2O_2	G	$^1O_2$	237
$CoCl_2$	SO_3^{2-}/O_2 or HSO_5^-	G	$SO_4^{\bullet-}$	214,238
Co-bithiazole (**103**)	O_2	G	Co^{III}OH ?	239
Ni peptides (**104**)	HSO_5^- or SO_3^{2-}/O_2	G (+ AT tracts without salt)	Ni^{III}−$SO_4^{\bullet}$	240,241
Ni macrocycles (**49**)	HSO_5^-, MMPP	G (>C>T)	Ni^{III}−$SO_4^{\bullet}$	60,96,218,238,240, 242−245
Ni bleomycin	HSO_5^- or Ir^{IV}	G	Ni^{III}	246
Ni salens (**50**)	HSO_5^-	G	alkylation	94
(**105**)	HSO_5^-, MMPP	G	alkylation (oxidation?)	99,247
(**106**)	HSO_5^-, MMPP	G	oxidation	248,249
Pd complex (**107**)	H_2O_2	G	N7 binding, oxidation	250
K_2PdCl_4	none	A	N7 binding, depurination	251
$CuCl_2$	H_2O_2 or O_2 + reductant	T, G	Cu^IOOH or Cu^{III}−OH	117,252,253
$CuCl_2$ + L-DOPA	H_2O_2	8-oxoG	?	254
Cu peptides	H_2O_2 or O_2	8-oxoG	?	255
Cu-famotidine (**108**)	SO_3^{2-}/O_2	T>G>C∼A	?	227

[a] Abbreviations: e.t., electron transfer.

The oxidative chemistry mediated by simple manganese salts has also been studied in conjunction with dioxygen and certain reductants—hydrazines (including isoniazid), 3-hydroxyanthranilic acid or SO_3^{2-}.[116,211−216] Alkali-dependent strand scission is observed in addition to single-strand breaks, and the damage has been variously attributed to oxyl and sulfuroxyl radicals. These studies are particularly important in light of the presence of sulfite, hydrazine, and manganese in aerosols derived from industrial and tobacco sources.[264]

Complexation of manganese(III) to organic ligands offers the ability to control the redox chemistry, and potentially the mechanism of action, as well as the site-specificity of DNA oxidation. For example, Mn−salen and Mn−porphyrin complexes lead to formation of high-valent manganese oxo species that are capable of both hydrogen atom abstraction and electron-transfer chemistry. The abundant literature of direct strand scission via H$^\bullet$ abstraction by manganese−oxo complexes from the ribose is discussed in recent papers and reviews,[52,217] and includes work with chiral Mn−salen complexes[217,218] as well as Mn−porphyrins that may be conjugated to antisense oligonucleotides.[265] Recent studies by Meunier and co-workers have also revealed the nucleobase chemistry of the latter system in which guanine sites are apparently subject to one-electron oxidation leading to piperidine-labile strand scission.[219,266] Manganese−salen complexes show a similar behavior in producing both direct strand scission and alkali-labile lesions in conjunction with the peracid oxidants, MMPP, peracetic acid, and HSO_5^-.[217,218]

There are few reports of the chemistry of rhenium complexes with DNA. Thorp and co-workers investigated the electrochemical oxidation of DNA using *trans*-dioxorhenium(V) complexes such as **99** because these oxidants do not participate in oxo-transfer chemistry and are thus purely electron-transfer agents.[220] In agreement with this, **99** demonstrated only guanine-specific, piperidine-labile lesions. No direct strand scission was observed. Rhenium complexes have also been studied with plasmid DNA.[267]

c. Fe, Ru, and Os. Among group 8 metals, iron has typically been studied for its ability to catalyze direct strand scission, while ruthenium and osmium catalysts also display a rich chemistry with nucleobases. The chemistry of Fe−EDTA has been reviewed elsewhere.[19,198,268] Under Fenton-type conditions with H_2O_2 (or less commonly, O_2 plus ascorbate or other reductant), Fe−EDTA generates hydroxyl radical.[25] As previously discussed (section IV.A.1), the hydroxyl radical so generated should be capable of both H$^\bullet$ abstraction from the sugar as well as adduct formation and electron-transfer chemistry with the nucleobases. The reaction conditions, especially the presence of reducing agents (including Fe^{II}), affect the ratio of sugar:base damage. Interestingly, the iron content of asbestos can be up to 28 wt %, and Fenton chemistry with O_2 or H_2O_2 is thought to be responsible for the formation of 8-oxoG in human lung epithelial cells treated with asbestos.[225]

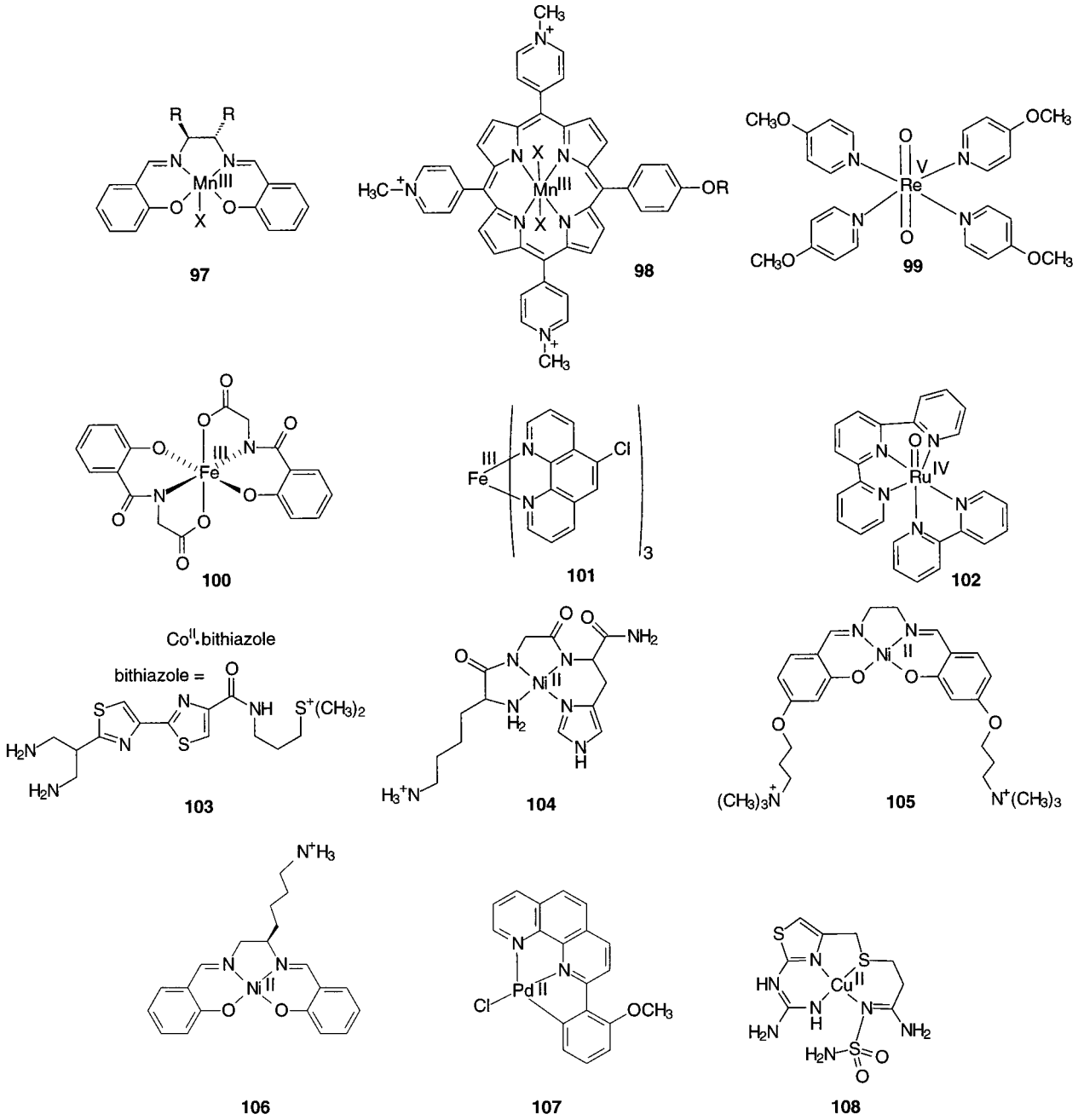

Figure 28. Transition metal complexes mediating nucleobase oxidation.

Nitric oxide (NO) might also play a role in this chemistry.[226]

For iron complexes with specific binding interactions with DNA, bleomycin for example, sugar chemistry may predominate because of precise positioning of the reactive oxidant near a ribose hydrogen. Iron(III)−porphyrin complexes have been studied as catalysts for DNA strand scission in the presence of ascorbate, superoxide or iodosobenzene,[269] but the nucleobase damage has not been assessed. Iron complexes for which alkali-labile lesions have been observed also include the iron(III) complex of salicylglycine (**100**),[227] a secondary metabolite of aspirin, and the iron(III) complex of 5-chloro-1,10-phenanthroline (**101**).[220] However, these two complexes operate very differently. Complex **101** cannot carry out inner-sphere DNA oxidation, and so functions as an electron-transfer agent, generating alkali-labile lesions at guanines.[220] On the other hand, **100** is capable of reaction with O_2 and sulfite to produce either sulfuroxy radicals or hydroxyl radical, leading to base damage in the order T > G ≈ C > A.[227] The exact mechanism has not been determined, but it is interesting that the preference for T > G modification is so different from most metal complexes whose base selectivity would normally be the reverse.

The Barton group has reported extensive investigations of ruthenium-mediated photochemical cleavage of DNA and RNA using complexes that operate either in a direct strand scission mode or weaker oxidants that lead primarily to piperidine-labile guanine oxidation.[202,270] Of the nonphotochemical cleavage methods with ruthenium complexes, most also operate by electron transfer and lead to guanine oxidation. For example, $[Ru^{III}(bpy)_3]^{3+}$ without added oxidant causes piperidine-labile guanine oxidation

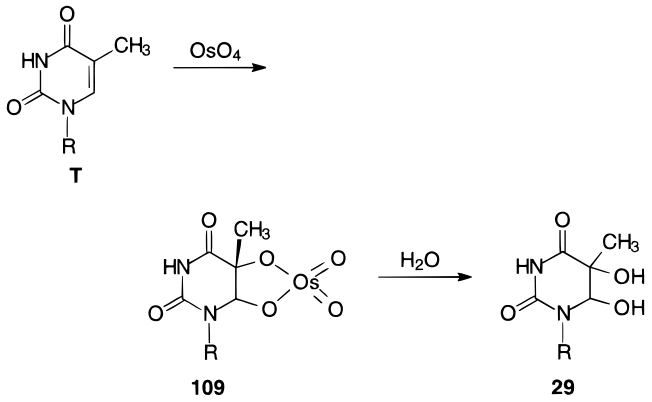

Figure 29. Thymine osmylation.

with a preference for guanines in single-stranded regions.[229] The analogous Ru^{II} complex may be used with photochemical activation in the presence of dipersulfate ($S_2O_8^{2-}$) with similar results, although some of the chemistry may be due to $SO_4^{\bullet-}$ in addition to Ru^{III}.[228]

Thorp and co-workers have investigated the effects of tuning the redox potential of a Ru complex on sugar vs base chemistry.[195,271] The $[Ru^{IV}(tpy)(bpy)O]^{2+}$ complex (**102**) is thought to oxidize guanine via oxygen atom transfer, and evidence for the formation of a Ru−O−C8(G) species has been reported.[233] This complex also mediates direct strand scission because its high potential allows sugar oxidation to compete with nucleobase oxidation, although the sugar oxidation is less favorable in RNA than in DNA.[232,234] The lower potential species $[Ru^{III}(tpy)(bpy)OH]^{2+}$ is only capable of carrying out guanine oxidation leading to piperidine-labile strand scission.[231] The effect of sterics and nucleic acid secondary structure have also been studied in this system,[230] and the Ru complexes are applicable to the study of RNA structure.[234,272]

Osmium tetroxide has been widely used as a probe for exposed thymine residues in nucleic acids,[200,236,273] and overall its chemistry is similar to that of $KMnO_4$. The site specificity of exposed thymines was shown to be dependent to a certain extent on the presence of added amines, especially pyridine derivatives (reviewed in Palecek[236]). Typically, pyridine or 2,2′-bipyridine is added in equimolar amounts to OsO_4. The reaction proceeds by formation of a cyclic osmylate, **109** (Figure 29), at the 5,6 double bond of thymine residues.[274] In some cases, the adduct is relatively stable, but it may also be hydrolyzed leading to thymine glycol, **29**, a piperidine-labile lesion. The sites of osmylation can then be identified by either alkaline treatment[37] or by enzymatic[275] or primer extension analysis.[276] Osmium tetroxide has been used as a chemical probe of DNA structure for Z-DNA tracts, B−Z junctions, and a variety of noncanonical thymine sites (reviewed in Nielsen[200]).

In DNA, T is much more reactive than C toward osmylation. Uracil is less reactive than T, and 5-bromouracil is less reactive than U, suggesting that the electron density in the C=C bond must play a role.[277] Curiously, if amines are omitted from the OsO_4 reaction with DNA, the specificity changes to G>T,C.[235] The background reactions of T and C can be completely eliminated by addition of $CaCl_2$. This finding calls into question the protocol for using OsO_4 for in vivo footprinting where it has been reported to be T specific, despite the fact that such amines are not likely to be present in sufficient concentrations inside a cell.[278]

d. Co and Rh. Oxidative nucleobase damage mediated by group 9 metals is limited to cobalt and rhodium. Rh complexes have principally been applied in a photochemical mode as noted in the previous section. Certain cobalt(III) complexes are also photochemically activated; for example, $[Co(NH_3)_6]^{3+}$ is believed to produce singlet oxygen leading to alkali-labile guanine damage.[202] Cobalt is thought to be a carcinogenic metal,[279] and simple salts of cobalt(II) have been shown to catalyze nucleobase oxidation in the presence of H_2O_2.[237,280] Some random direct strand scission has been observed, but also substantial piperidine-labile guanine oxidation that was attributed to formation of singlet oxygen or a closely related species. Co^{2+} also catalyzes autoxidation of sulfite to produce guanine-damaging species, likely $SO_4^{\bullet-}$.[214] Similarly, $CoCl_2$ catalyzes the decomposition of monoperoxysulfate (HSO_5^-) to yield $SO_4^{\bullet-}$ that primarily reacts with exposed guanines.[238] As opposed to nickel(II) complexes (discussed below), the chemistry of $SO_4^{\bullet-}$ generated from Co^{2+} catalysis leads to guanine modification that depends on the exposure of the heterocyclic face of G, and metal ion binding to N7 does not necessarily appear to play a role. Furthermore, the $CoCl_2/HSO_5^-$ system is useful under more extreme reaction conditions including high temperature,[238] allowing the study of temperature-dependent conformational changes. It is interesting that coordination of cobalt(II) to polydentate ligands or conversion to cobalt(III) complexes reduces the reactivity of the Co^{2+}/HSO_5^- system, presumably due to changes in the redox potential of the resulting complex.[238]

Cobalt(III) bleomycin with a metal-bound hydroperoxide is a good structural mimic of iron bleomycin (**103**),[108] and under photochemical conditions it carries out the same C4′ hydrogen abstraction.[281] On the other hand, a cobalt(II) complex of a bithiazole analogue of bleomycin showed guanine-specific lesions that were dependent upon dioxygen and alkali treatment but not on light.[239] Oxyl radicals and singlet oxygen were ruled out as the reactive species, and the mechanism does not appear to involve electron transfer since no effect of neighboring guanines was observed. An inner-sphere mechanism was therefore proposed.[239]

e. Ni and Pd. Nickel toxicity has been studied for more than 30 years.[282] Insoluble, particulate nickel species are thought to be more carcinogenic than soluble species because of their ability to enter cells by phagocytosis, allowing slow dissolution of the particle and nickel binding to chromatin.[257−260,283] The DNA damage mediated by nickel(II) includes DNA strand breaks, oxidative base damage, and DNA−protein cross-links.[284,285] Studies on isolated DNA with simple Ni^{2+} salts and H_2O_2 provide a similar set of data to Co^{2+}, although nickel appears much less

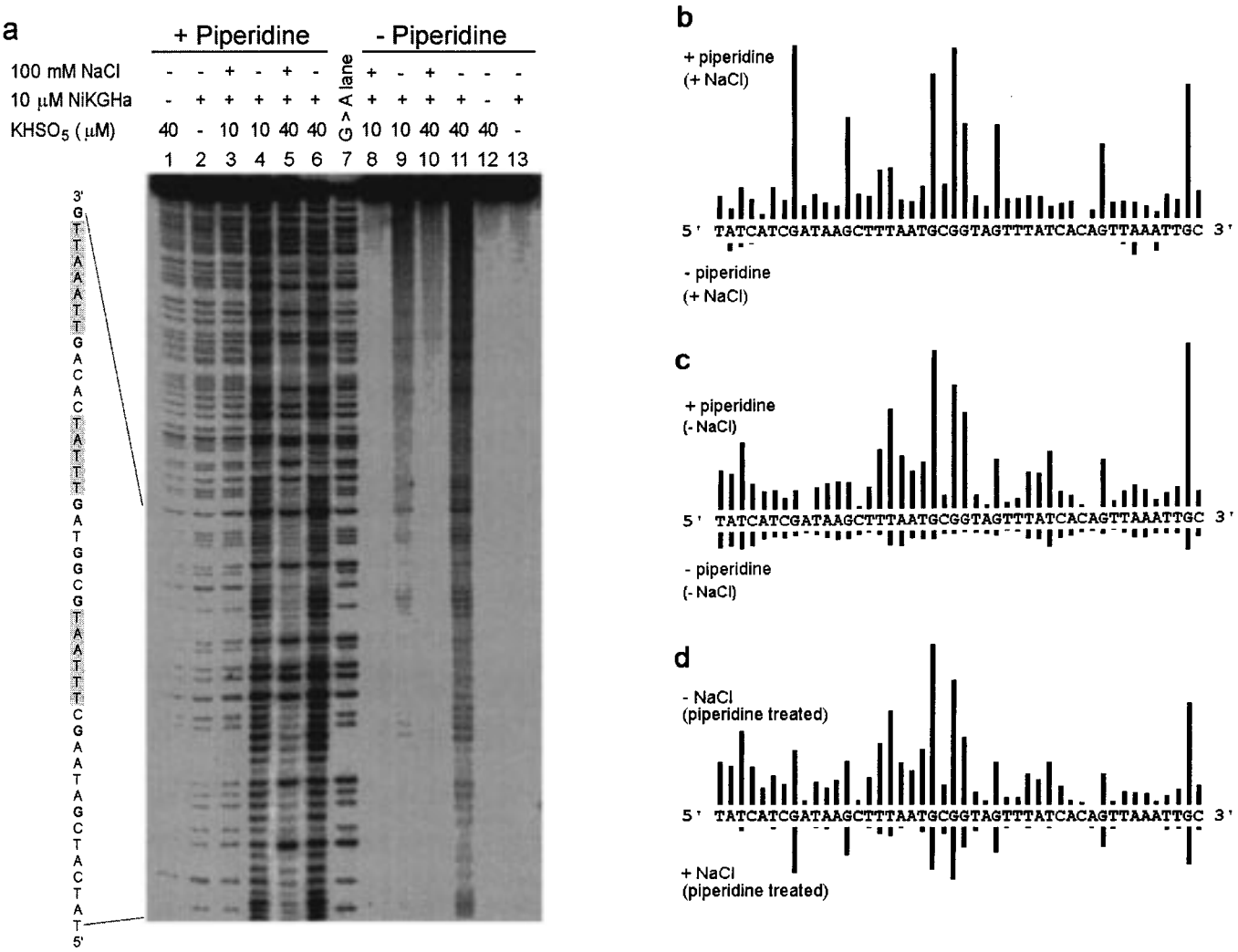

Figure 30. Studies of nickel peptide (**104**)-mediated oxidation of a restriction fragment from plasmid pBR322. (a) Phosphorimagery of gel electrophoretic experiments with 10 μM **104**, 10 or 40 μM $KHSO_5$, pH 7.1, cacodylate buffer (10 mM) with and without 100 mM NaCl and with and without piperidine treatment (0.2 M, 90 °C, 30 min). (b) Quantitative comparison of strand scission under standard salt conditions, ± piperidine treatment (lane 5 vs lane 10). (c) Quantitative comparison of strand scission under low salt conditions, ± piperidine (lane 4 vs lane 9). (d) Quantitative comparison of strand scission after piperidine treatment ± NaCl (lane 6 vs lane 5).

reactive.[280,286] 8-OxoG is a principal product of DNA reaction of Ni^{2+} and H_2O_2, although other base modifications are also observed to a lesser extent.[280,286] Coordination of nickel(II) to certain ligands, including histidine[287,288] and histidine-containing peptides, greatly enhances the metal's reactivity toward oxidation chemistry of guanine.[289−291] Indeed, nickel(II) is perhaps the metal ion most subject to control of its redox behavior by the surrounding organic ligand.

The versatility of nickel(II) as a DNA cleaving agent is aptly demonstrated in the case of square-planar tripeptide complexes of the type NH_2−XXH..., in which histidine is the third residue. The propensity of this N-terminal peptide sequence to bind nickel(II) and copper(II) has been recently reviewed.[292] This motif is found in albumins, neuromedins C and K, human sperm protamine P2a, and histatins.[292] Binding of nickel(II) or copper(II) to this type of tripeptide stabilizes the +III oxidation state,[293−295] and promotes redox chemistry leading to DNA damage. This chemistry has been exploited for site-specific DNA cleavage by synthesis of bioconjugates containing the N-terminal XXH motif appended to a DNA-binding moiety such as a protein fragment (Hin recombinase 139−190,[296] transcription factor Sp1 529−696,[297] Fos 138−211,[298] or a peptide nucleic acid fragment[299]).[300] Interestingly, all of the Ni−peptide bioconjugates led to direct strand scission under oxidative conditions using peracid oxidants such as monoperoxysulfate (HSO_5^-) or monoperoxyphthalate (MMPP), and no specific base chemistry was observed. This is in contrast to the intrinsic reactivity of the nickel peptide itself which, in the absence of a minor-groove binder or other structure-directing appendage, prefers to mediate guanine oxidation.[240]

A study of DNA oxidation with NiKGH−$CONH_2$ (**104**) and $KHSO_5$ aptly illustrates the question of direct vs alkali-labile strand scission under various reaction conditions.[301] Figure 30a shows the sites of cleavage in a 167-bp restriction fragment from pBR322. Lanes 1−6 are piperidine treated; lanes 8−13 are not. Clearly, a majority of the oxidative damage occurs at sites that are only revealed upon hot alkaline workup. Data for specific lanes are shown in the bar graphs (Figures 30b−d) and provide

further insight into the site specificity of nickel peptides. For example, lanes 5 and 10 (Figure 30b) compare the reaction of **104** with and without piperidine treatment, but under physiological pH and salt conditions. Before piperidine treatment, only a trace amount of direct strand scission is observed. After piperidine treatment, stand scission is observed primarily at guanine residues, and the higher reactivity of a 5′-GG-3′ implies an electron-transfer mechanism. If NaCl is omitted from the reaction medium, a somewhat different pattern of reactivity it observed (Figure 30c). In this case, more reactivity of the nickel peptide is seen even before piperidine treatment is observed, and this has been interpreted as enhanced binding of the peptide to AT tracts in the minor groove.[241] All of these lesions, plus a larger fraction of guanine chemistry, are enhanced upon piperidine treatment, in some cases up to 20-fold. Overall, the effect of added NaCl is to stabilize the DNA duplex and to diminish hydrophobic interactions with the nickel peptide. Thus, the oxidation chemistry mediated by **104** is highly guanine-specific in the presence of 100 μM NaCl and slight reactivity is seen at AT tracts in addition to Gs in the absence of NaCl (Figure 30d).

Nickel macrocycles such as $NiCR^{2+}$ (**49**) mediate oxidative guanine damage in the presence of peracid oxidants (MMPP or $KHSO_5$) in a fashion similar to that of nickel peptides yielding results that resemble the lower graph of Figure 30d after piperidine treatment. Unlike nickel peptides, no reactivity is seen in the minor groove under low salt conditions.[301] The majority of oxidative base damage occurs at exposed guanine residues, and this sensitivity to guanine exposure has led to the use of $NiCR^{2+}$ as a probe of DNA and RNA structure.[242] For example, guanines in bulged or hairpin loop regions of $tRNA^{Phe}$,[56] *Tetrahymena* group I intron,[56] viral pseudoknots,[302] hairpin ribozyme,[303] the *micF/ompF* antisense RNA duplex[304] and 5S rRNA (*Xenopus lavis*)[305] were correctly identified by $NiCR^{2+}$-mediated oxidation and cleavage.[96] The complex can also be used to study accessible guanine residues in DNA structures.[243,306] $NiCR^{2+}$ strongly prefers to oxidize exposed guanines, and the mechanism appears to involve binding to G−N7 since the reactivity correlates with N7 exposure in well-characterized structures such as $tRNA^{Phe}$.[56] Indeed, NMR data are consistent with the ability of Ni^{II} to bind to G−N7,[307,308] and CD studies indicate that Ni^{III} macrocyclic complexes are superior for promotion of B→Z helical changes, a process thought to involve G−N7 binding.[308] Thus, $NiCR^{2+}$ appears to deliver a reactive oxidant to exposed guanines through direct metal ion coordination to N7. When coordination to N7 is not possible, but other nucleobases are exposed in single-stranded regions, the order of reactivity is C > T > A.[309] Thus, bulged C and bulged T residues can be identified, if no exposed guanines exist in the same structure.[310] If all bases are completely paired in a duplex structure, the reactivity reverts to G, and a preference is seen for 5′-GG-3′ oxidation (Figure 31).[240] This suggests that when nickel complexes cannot reach G−N7 in the major groove, they perhaps react by an outer-sphere electron-transfer mechanism. Mechanistic studies have shown that the reaction with guanines does not involve freely diffusible radicals, and the reactive oxidant is thought to be a nickel(III) complex with a bound oxidant such as a sulfate radical (Figure 32).[238] The success of the nickel macrocycles as DNA and RNA modifying agents is highly dependent on ligand structure suggesting a key role for the ligand field and redox properties of the bound nickel ion.[244,245]

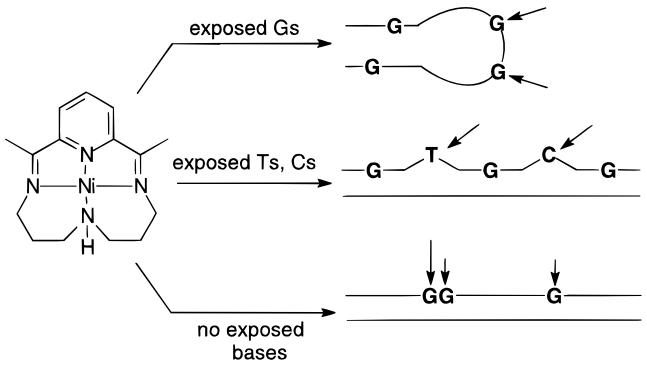

Figure 31. Base selectivity of DNA oxidation mediated by $[NiCR]^{2+}$ (**49**).

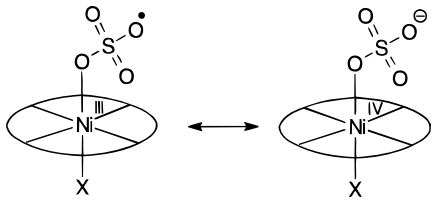

Figure 32. Proposed reactive intermediate in DNA oxidation mediated by nickel macrocycles.

Nickel(II) forms a coordination complex with bleomycin, and it has been used in a fashion similar to nickel macrocycles. In the presence of either $KHSO_5$ or Ir^{IV}, guanines in the exposed loops of a G-quartet structure are oxidized and revealed by piperidine treatment.[246]

As discussed in section III.D.3, nickel−salen-type complexes effect oxidative modification of nucleic acids, but the initial reaction is formation of a guanine adduct.[94,99] Thus, redox active ligands surrounding nickel can participate in the reaction through formation of ligand radicals that ultimately alkylate (or arylate) DNA or RNA bases. Methyl radicals are known to add to C8 of guanine leading to a labile product,[311] so this is a possible explanation for the adduct formation with nickel−salens. Salen derivatives are good candidates for this chemistry since the nickel-coordinated phenol moiety is easily oxidized.[95] The guanines so alkylated are at least somewhat piperidine labile, thus the alkylation event must be observed before piperidine treatment either by gel analysis of short oligonucleotides or by a primer extension assay. Other reports of water-soluble salen derivatives have focused on the strand cleavage events.[247−249,312,313]

There are two reports of palladium-mediated DNA strand scission. The cyclometalated complex **107** leads to both direct strand scission and piperidine-labile lesions in the presence of H_2O_2 at pH 8.[250]

Curiously, even the direct strand scission sites were concentrated on guanines, so a binding interaction with guanine preference has been suggested. Binding studies suggest both intercalative and covalent binding to DNA. The simple palladium salt K_2PdCl_4 has also been reported to effect DNA nucleobase modification at pH 2 without added oxidant.[251] Piperidine treatment is used to effect strand scission. This reaction is one of the few that is highly A specific, and therefore represents a convenient sequencing reaction for A without interference by G. A mechanism has been proposed in which binding occurs at both A−N7 and G−N7, but only the A adduct is depurinated at pH 2.

f. Cu. The chemistry of group 11 metals with nucleobases belongs to copper. The sugar-directed chemistry of copper, especially copper−phenanthroline complexes, has been thoroughly reviewed.[118,314] There do not appear to be any examples of copper complexes that solely mediate nucleobase oxidation; all appear to give direct strand scission derived from hydrogen atom abstraction from the sugar, at least to some extent. However, even $Cu(phen)_2^{2+}$, a reagent that is normally used in a direct strand scission mode, shows enhanced strand scission on piperidine treatment,[301] and 8-oxoG formation has been detected.[315] A key intermediate in nucleic acid oxidation with copper is the formation of a Cu^{III}−OH species, also termed a "crypto-HO•", although in some cases it is argued that a freely diffusible HO• is formed.

Unlike its neighbor nickel(II), copper(II) mediates a great deal of redox chemistry even in the absence of coordinated organic ligands. $CuCl_2$ with H_2O_2 leads to DNA strand breaks at T > G > C > A sites that are enhanced by piperidine.[252] Ts and Gs were also the most reactive site when O_2 plus ascorbate, reduced glutathione (or other thiols) or NADH replaced the H_2O_2 in the reaction.[117,316,317] The presence of a 3′-G led to greater reactivity. In a somewhat contrasting study it was found that preference for reaction at polyguanine sequences was seen with $CuCl_2/H_2O_2$ followed by piperidine treatment.[318] Addition of hydrazine or alkyl- or arylhydrazines to the copper-mediated oxidation led to an increased preference for T modification that is suggested to be due to hydrazyl or alkyl radicals.[253,319,320] Copper-containing metallothioneins gave a similar result.[321]

In the presence of phenolic ligands, such as L-DOPA,[254] a catechol, or alkylresorcinols,[122] greatly enhanced DNA oxidation is observed using Cu^{2+} and H_2O_2. (Note that metal complexes were not isolated and characterized.) In the case of L-DOPA, the nucleobase lesions were examined, and 8-oxoG was found to be the principal product. Amine ligands, including dien, trien, and tacn, have been studied with peracid oxidants such as HSO_5^-, and while the reactions lead to alkali-labile guanine oxidation, the overall effect of the polyamine ligand is negligible.[322] On the other hand, peptide ligands such as the N-terminal XXH motif allow Cu^{II}-mediated oxidative cleavage to be directed to specific DNA sites when incorporated into a bioconjugate.[323,324] However, these bioconjugates appear to carry out direct strand scission rather than nucleobase modification. A more recent study of the N-terminal segment of human protamine HP2 indicates that binding of Cu^{2+} leads to increased formation of 8-oxoG in the presence of either H_2O_2 or O_2.[255] Copper(II) was also studied in the presence of famotidine, an antiulcer drug, with which it forms a 1:1 complex, **108**.[325] This complex is a catalyst for sulfite autoxidation, leading to DNA damage revealed by piperidine treatment.[227] The base selectivity is low with a small preference for T and G residues over C and A. $CuCl_2$ alone is also a catalyst for sulfite autoxidation, but it is less reactive than **108**.[214]

Overall, this section on transition metal-mediated oxidation has focused on known nucleobase chemistry of the metals, although a great deal of direct strand scission, often detected as plasmid nicking, has been reported. Much more nucleobase chemistry is likely to be uncovered with transition metals as researchers investigate the complete reaction profile of a particular reagent. The general picture that emerges is this: $KMnO_4$ and OsO_4 are very T specific and carry out dihydroxylation. Nickel complexes almost always display G-specific chemistry, with little background reaction at other bases or at sugar residues; however, nickel's G chemistry is relatively diverse because of both inner-sphere and outer sphere mechanisms. Most other metals mediate a combination of H-atom abstraction from sugars, electron-transfer chemistry with guanine, and other base lesions. The extent to which the one-electron transfer pathway is followed compared to atom transfer or H-atom abstraction is a function of the metal complex whose properties can be tuned by the ligand. The oxidation potential and the presence of vacant coordination sites are particularly important. A good example of ligand control is the case of ruthenium, for which all three mechanisms (one-electron G oxidation, oxo-transfer chemistry, and H-atom abstraction) have been observed. Overall, the most common lesion observed in metal-mediated DNA oxidation is guanine oxidation since this nucleobase is both the best transition metal binding site and the most easily oxidized base.

4. Other Oxidants

a. Peroxynitrite. Peroxynitrite, $ONOO^-$, is formed by the reaction of superoxide with nitric oxide, both of which are produced in cells under conditions of oxidative stress.[326] Since the pK_a of peroxynitrous acid is near neutrality ($pK_a = 6.8$),[327] a substantial fraction of the neutral acid form is present physiologically. Model studies have shown that $HONO_2$ formed outside a vesicle can easily cross a lipid bilayer and react with metal complexes and DNA inside a vesicle,[328,329] suggesting that the same may be true for living cells. Early studies with peroxynitrite suggested that it was a hydroxyl radical generator, decomposing by the pathway shown in Figure 33a.[120] Indeed, the DNA cleavage pattern showing a non-base-selective ladder of direct strand scission was highly suggestive of HO• formation.[120] On the other hand, more recent studies show that this pathway accounts for only about 1−4% of the peroxynitrite decomposition.[330] Detailed rate studies in

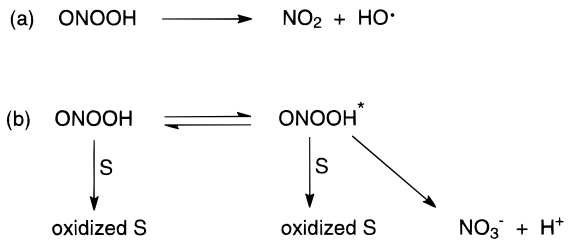

Figure 33. Possible modes of decomposition of peroxynitrous acid.

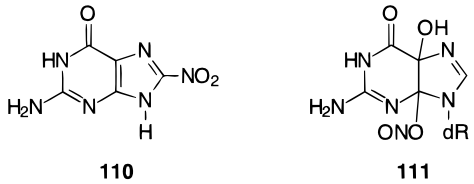

Figure 34. Guanine modifications arising from reaction with peroxynitrite.

the presence and absence of radical scavengers point to the mechanism shown in Figure 33b.[121] In this scheme, peroxynitrite can either directly oxidize a substrate, or can first isomerize to an activated species ONOOH*, which then partitions between decomposition to nitrate and reaction with substrate.[121] The identity of the activated species ONOOH* is not yet clear. It may pertain to *cis*−*trans* isomerization about the central N−O bond.[331−333] Furthermore, CO_2 appears to accelerate the peroxynitrite oxidations although it does not lead to hydroxyl radical formation.[334]

Peroxynitrite is capable of direct strand scission of nucleic acids that is likely due to hydrogen atom abstraction from sugars.[335,336] On the other hand, nucleobase lesions have been identified that lead to depurination. These include formation of 8-nitroguanine (**110**)[337,338] and 4,5-dihydro-5-hydroxy-4-(nitrosooxy)-2′-deoxyguanosine (**111**)[339] (Figure 34). The nitration product **110** is enhanced in the presence of CO_2/HCO_3^-.[340] There are conflicting reports concerning the formation of 8-oxoG in peroxynitrite oxidation of DNA. Small amounts were measured by HPLC or enzymatic analysis of calf thymus DNA[341] or plasmid DNA[46,342] treated with peroxynitrite. Subsequent studies by Pryor and co-workers[343] and by Cadet and co-workers[344] show that there is no buildup of 8-oxoG upon exposure of DNA to peroxynitrite. In a competition study, 8-oxoG was further oxidized by peroxynitrite, even in the presence of 1000-fold excess of guanine.[343] Thus, 8-oxoG is either a minor product of DNA oxidation, or it is rapidly further oxidized and so does not accumulate. It is interesting to note that another guanine oxidation product, an oxazolone (**57**) was observed in higher concentrations than 8-oxoG.[344]

Electrophilic reactions of related nitrogen oxides, NO, NO_2, and HONO, are discussed in sections V.B and C.

b. Sulfoxyl Radicals. The sulfoxyl radicals $SO_3^{\bullet-}$, $SO_4^{\bullet-}$, and $SO_5^{\bullet-}$ are linked as intermediates in an environmentally relevant process, the autoxidation of sulfite.[264,345] Sulfite is the hydration product of SO_2, an industrial pollutant, and is also found

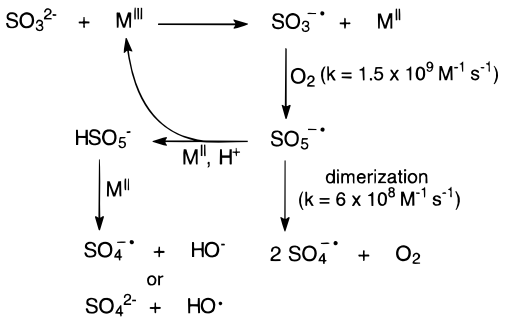

Figure 35. Mechanistic scheme proposed for metal-catalyzed sulfite autoxidation.

as a preservative in foods and beverages. The autoxidation of sulfite (SO_3^{2-}) or bisulfite (HSO_3^-, pK_a = 7.2)[345] leads to the formation of monoperoxysulfate (HSO_5^-), a species that is essentially the sulfur analogue of peroxynitrite discussed above. Transition metals catalyze the autoxidation process; the thermal and photochemical pathways being less efficient. A general scheme for metal-catalyzed sulfite autoxidation is shown in Figure 35.[264] Sulfite radical is the initial product, but in the presence of dioxygen, it reacts at nearly diffusion-controlled rates to form $SO_5^{\bullet-}$. Two pathways then lead to formation of sulfate radical ($SO_4^{\bullet-}$)—the metal-mediated, one-electron reduction to HSO_5^- (pK_a = 9.4) followed by metal-mediated decomposition, or dimerization of $SO_5^{\bullet-}$ in a Russell-type termination process to generate $2SO_4^{\bullet-} + O_2$. Thus, in considering DNA damage arising from sulfite autoxidation processes, it is not immediately evident which radical species is responsible for nucleic acid oxidation. The situation is further complicated by the fact that some metals, such as Cu^{II} [346] and perhaps some Ni^{II} complexes,[347] are thought to decompose HSO_5^- to yield hydroxyl radical plus sulfate ion.

Of the three sulfoxyl radicals, $SO_4^{\bullet-}$ is the most potent oxidant with a potential estimated between 2.5 and 3.1 V vs NHE, while $SO_5^{\bullet-}$ and $SO_3^{\bullet-}$ have potentials of 1.1 and 0.63 V, respectively.[348] On the basis of redox potentials alone, $SO_5^{\bullet-}$ and $SO_3^{\bullet-}$ should be slow or incapable of one-electron oxidation of nucleobases since guanine, the most easily oxidized base, has a potential of 1.29 V vs NHE.[146] Independent generation of $SO_4^{\bullet-}$ in metal-free solutions, either by photolysis or pulse radiolysis of $S_2O_8^{2-}$, allows the study of rate constants of sulfate radical with nucleosides and deoxyribose (see Table 4).[348] Among the nucleosides, rate constants vary over an order of magnitude with G being the most reactive. Oxidation of the deoxyribose moiety is nearly 100-fold less favorable, explaining the general observation of nucleobase damage rather than direct strand scission with sulfate radical. In comparison, hydroxyl radical reacts promiscuously with all nucleobases and with ribose and deoxyribose via one-electron abstraction, radical addition, or hydrogen abstraction pathways.[17] Sulfate radical appears to prefer one-electron abstraction as a reaction mechanism. Reaction rate constants for $SO_5^{\bullet-}$ with nucleosides are unavailable, and those for $SO_3^{\bullet-}$, which can be prepared by photolysis of $S_2O_6^{2-}$, are estimated and much slower than for $SO_4^{\bullet-}$.[348]

Table 4. Rate Constants[a] for Reactions of $SO_3^{\bullet-}$, $SO_4^{\bullet-}$, and HO• with Nucleosides[b]

	nucleoside[b]		
nucleoside	$SO_3^{\bullet-}$	$SO_4^{\bullet-}$	HO•
dA	$\leq 1 \times 10^6$	3.7×10^8	5×10^9 [c]
dC	$\leq 1 \times 10^6$	2.5×10^8	4.5×10^9 [c]
dG	$\leq 1 \times 10^6$	2.3×10^9	9×10^9 [c]
dT	$\leq 1 \times 10^6$	2.0×10^8	5×10^9 [c]
rU	$\leq 1 \times 10^6$	$\sim 1 \times 10^9$	6×10^9 [c]
deoxyribose		3.8×10^7	1.9×10^9
ribose			1.5×10^9

[a] Rate constants (in M^{-1} s^{-1}) assumed to be room temperature. [b] Data for $SO_3^{\bullet-}$ and $SO_4^{\bullet-}$ from ref 348. Data for HO• from ref 17, p 117. [c] Reported for nucleobases rather than nucleosides.

DNA damage resulting from autoxidation of sulfite in the presence of Cr^{VI},[206] Mn^{II},[214−216] Fe^{III},[214,227] Co^{II},[214,240] Ni^{II},[240] and Cu^{II}[214,227] has been reported. Of these metals, Cr, Co, and Ni lead primarily to G-specific reactions, suggesting the involvement of $SO_4^{\bullet-}$ (or its metal-bound analog), despite various literature interpretations to the contrary. Other metals in conjunction with sulfite show little nucleobase selectivity and might be due to other sulfoxyl radicals or hydroxyl radical.

The products of nucleobase oxidation with $SO_4^{\bullet-}$ have been studied. Guanine reacts by one-electron oxidation to form $G^{+\bullet}$, which goes on to form a cascade of products including 8-oxoG and other derivatives discussed in section IV.B.1.b.[86,113,349] Adenine can be similarly oxidized by one electron, but does not usually lead to an observed lesion, because of rapid hole migration to guanine.[350] Thus, only G oxidation products would be observed, despite the fact that the original base radical cations formed are $G^{+\bullet}$ and $A^{+\bullet}$ in about a 70:30 ratio. Many of the pyrimidine products of $SO_4^{\bullet-}$ oxidation are the same as those obtained from the HO• reactions, except that they presumably occur through a first step of either radical addition to the 5,6 double bond or H• abstraction by $SO_4^{\bullet-}$ at thymine's methyl group. Thus, products **29** and **35** are obtained from T oxidation with $SO_4^{\bullet-}$;[351] glycol **29** is also obtained upon direct reaction with HSO_5^-.[352] Uracil undergoes a similar reaction with $SO_4^{\bullet-}$ to produce 5-hydroxyuracil.[351] The oxidation of cytosine with $SO_4^{\bullet-}$ requires further study, but there is some evidence that deamination is the principal pathway.[351] This is curious since deamination is usually thought to result from attack by nucleophiles at C6, followed by hydrolysis of the amidinium group.[353−355] With HSO_5^-, the *N*-oxide of C is formed at N3.[356] Substituted pyrimidines have also been studied to some extent.[352,356,357]

B. Guanine Oxidation

Guanine is the most easily oxidized of the nucleobases, and it is therefore not surprising that most of the oxidizing agents listed in section IV.A attack guanine with greater frequency. One-electron reduction potentials for guanosine and adenosine neutral radicals have been recently reinvestigated, and the corresponding potentials for deoxythymidine and deoxycytidine have been reevaluated. Data also exist for common oxidized nucleobases, and these will be discussed in the sections that follow. The redox potentials are presented in Table 5 where values have been converted to potentials vs NHE for comparison. The reduction potentials are highly pH dependent (increasing at lower pH) because of the rapid deprotonation of the corresponding radical cation at neutral (or basic) pH.

Table 5. One-Electron Redox Potentials for Nucleosides

species	$E°$ (V vs NHE)	pH	ref
rG	1.29	7	146
rA	1.42	7	146
dC	1.6	7	146
dT	1.7	7	146
r(8-oxoG)	0.58	8	358
r(8-oxoA)	0.92	8	358
r(5-OH−C)	0.62	8	358
r(5-OH−U)	0.64	8	358

1. Guanine Radical Cation

a. Formation. Guanine radical cation is the initial product of DNA and RNA oxidation by a wide variety of reagents that includes pulse radiolysis to form radical species (HO• and $SO_4^{\bullet-}$), transition metal complexes (Table 3), and photooxidants (Table 2). The oxidation of the nucleoside guanosine has been studied extensively and is the subject of reviews by Steenken.[113,349] The most recent study of the nucleoside provides a $E°$ value corrected to pH 7 of 1.29 V vs NHE.[146] However, it is recognized that the oxidation potential of guanine in a DNA or RNA helical array might differ somewhat from the isolated nucleoside. Studies in various laboratories suggest a slightly lower oxidation potential for G in DNA, on the order of 1.10−1.24 V vs NHE.[220,359,360]

As early as 1985 it was observed that the reactivity of guanines toward oxidants including ionizing radiation was sensitive to sequence.[361] Specifically, Gs located 5′ to a purine, especially another G, were more reactive than those with 3′ pyrimidine neighbors.[362] This 5′-GG-3′ effect has been observed in radical, metal-mediated,[214,233,239,240] photochemical,[169−175,177,178,180,181,185,363−366] and ionizing radiation reactions,[361,362,367] but only holds true for duplex DNA as the substrate. The phenomenon has been explained by Saito et al. as being due to a π-stacking effect of purines in which the HOMO resides predominantly on the 5′-G in a purine stack,[363] thus the electron should be kinetically removed from the 5′-G of a purine sequence. The ionization potentials calculated for stacked base pairs are listed in Table 6, and for comparison, we have converted them into oxidation potentials. While the kinetic product is expected to be formation of a 5′-guanine radical cation, the most thermodynamically stable $G^{\bullet+}$ is one sandwiched by purines, especially guanines, so that a central G in G tracts of 3 or more will actually lead to more oxidized product.[174] Thus, the observed order of reactivity of G sequences is GGGG > GGG > GG > GA > GT ≈ GC, where the underlined bases are the principal sites of cleavage. This prediction has been borne out in various experiments showing quantitative results for guanine

Table 6. Calculated Ionization and Oxidation Potentials for Nucleobases

sequence[a]	I. P. (eV)[b]	$E°$ (V vs NHE)[c]	I. P. (eV)[d]	$E°$ (V vs NHE)[c]
GGG	7.07	0.64		
$\overline{G}$G	7.28	0.82	6.64	0.29
$\overline{G}$A	7.51	1.00		
$\overline{G}$C	7.68	1.15		
$\overline{G}$T	7.69	1.16		
$\overline{G}$	7.75	1.20	7.31	0.85
8-oxoG			6.93	0.53
(8-oxoG)G			6.38	0.08
G(8-oxoG)			6.51	0.18

[a] Sequences are 5′ to 3′ in stacked B-helix geometry. Underlined base is the major site of oxidation. [b] From ref 172. [c] Calculated from data in previous column using $E° = 0.827 \times$ I. P. $- 5.20$ (adjusted for V vs NHE).[369] [d] From ref 370.

reactivity.[69,174,240,362,368] In the cases studied, guanine radical cation is the implied intermediate that is initially formed upon oxidation. It is not clear if the same trend will be observed with oxidants that operate by other than one-electron transfer, e.g., oxo transfer.

Guanine radical cation can be formed by direct loss of an electron due to ionizing radiation or chemical oxidation. Alternatively, radical cations formed initially on other nucleobases may undergo hole migration leading to the more stable $G^{\bullet+}$. For example, photoionization of DNA with 193-nm light leads to 50−75% electron abstraction from Gs initially, but after electron transfer within DNA, $G^{\bullet+}$ accounts for 75−86% of the oxidized bases.[367] Hole migration is thought to be much more efficient through duplex DNA, especially B and perhaps A helices, but to be inefficient with single-stranded DNA, although hole migration to adjacent bases has been observed.[156] In GA dinucleotides, electron transfer converts $A^{\bullet+}$ to $G^{\bullet+}$.[350] Long-range electron transfer has been observed by covalently tethering an oxidant to one end of a oligodeoxynucleotide duplex and observing guanine oxidation at GGG and GG sites that are up to 11 nucleotides distant.[177] Interruption of the DNA duplex by base bulges reduces the extent of electron transfer through the helix,[178] and base mismatches may attenuate electron transfer depending on the identity of the mismatch.[180] Recent data suggest the B helix is somewhat more efficient at such long-range transfer than a protein matrix,[371] but a contrasting study indicates an insulating effect of the helix.[372] This rapidly evolving picture requires additional research before drawing a final conclusion.

b. Fate. While guanine itself is a weak acid ($pK_a = 9.4$), its corresponding radical cation is a much stronger one with a $pK_a = 3.9$.[373] Thus, isolated guanine, deoxyguanosine, or GMP undergoes rapid deprotonation after one electron oxidation to generate the neutral radical. ESR studies have shown that the N1 proton is the one lost in this process.[374] When $G^{\bullet+}$ is formed in duplex DNA, the effect of base pairing is to transfer the proton to N3 of cytosine in the base pair, as shown in Figure 36.[349] Since the pK_a of N3-protonated C is only slightly higher ($pK_a = 4.3$), the equilibrium constant for proton transfer is small, $K_{eq} = 2.5$. Thus, $G^{\bullet+}$ formed in a DNA duplex should retain more cationic character than

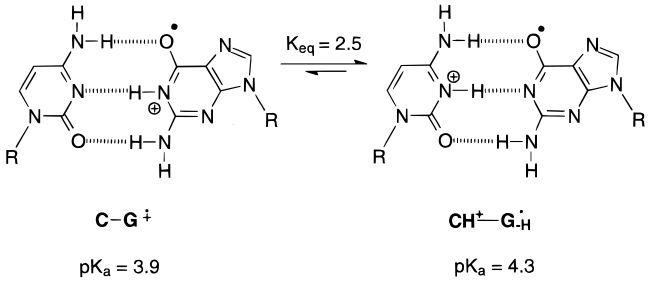

Figure 36. Proton transfer in the cytosine−guanine radical cation base pair.

would an isolated G nucleoside or nucleotide. This is a significant issue, because it is not clear that model studies performed on monomers correctly mimic the reaction pathway of guanine in duplex DNA.[87]

Guanine radical cation can lead to formation of 8-oxoG (**22**) by hydration followed by one-electron oxidation (Figure 37).[86,169] On the other hand, the deprotonated guanine radical $(G-H)^{\bullet}$ does not hydrate, but instead reacts with dioxygen to initiate a cascade of reactions leading to imidazolone and oxazolone products (Figure 37).[133,375] A mechanism has been proposed beginning with O_2 addition to a resonance form of $(G-H)^{\bullet}$ with unpaired electron density at C5, forming **112**.[133] In subsequent fast steps, CO_2 and formamide are lost, leading to the observed products with proposed structures **56**, an imidazolone, and its hydrolysis product **57**, an oxazolone. Compound **56** hydrolyzes with a half-life of 147 min at 37 °C, pH 7. Importantly, both **56** and **57** are alkali-labile products with half-lives of 11 and 21.9 min, respectively at 65 °C, pH 10. Under standard piperidine conditions (pH 13, 90 °C, 30 min), one would expect that all imidazolone and oxazolone lesions in DNA have been completely converted to strand breaks.

Product analyses of guanine oxidation in nucleoside or nucleotide monomers is expected to favor the imidazolone/oxazolone pathway because of rapid deprotonation of $G^{\bullet+}$. In contrast, significant amounts of 8-oxoG are observed in oxidation studies of duplex DNA. For example, Spassky and Angelov show in ionizing radiation studies that 8-oxoG is formed in excess of **56** and **57** in duplex oligodeoxynucleotides, but it is the minor product with the same single-stranded oligodeoxynucleotides.[69,87] Guanines present as bulges or mismatches shift the ratio toward greater formation of **56** and **57**, presumably because the stabilizing influence of a paired C on $G^{+\bullet}$ is absent.

2. 8-Oxoguanine

For the reasons outlined in the previous sections, 8-oxoG is the most common oxidative lesion observed in duplex DNA, and a family of DNA repair enzymes has evolved to deal with this lesion and its mismatches. Studies suggest that about 1 in 40 000 guanines in the genome is present as 8-oxoG under normal conditions.[376] This means that >30 000 8-oxoGs may exist at any given time in the genome of a human cell. Thus, the subsequent chemistry of 8-oxoG and its repair are of particular interest.

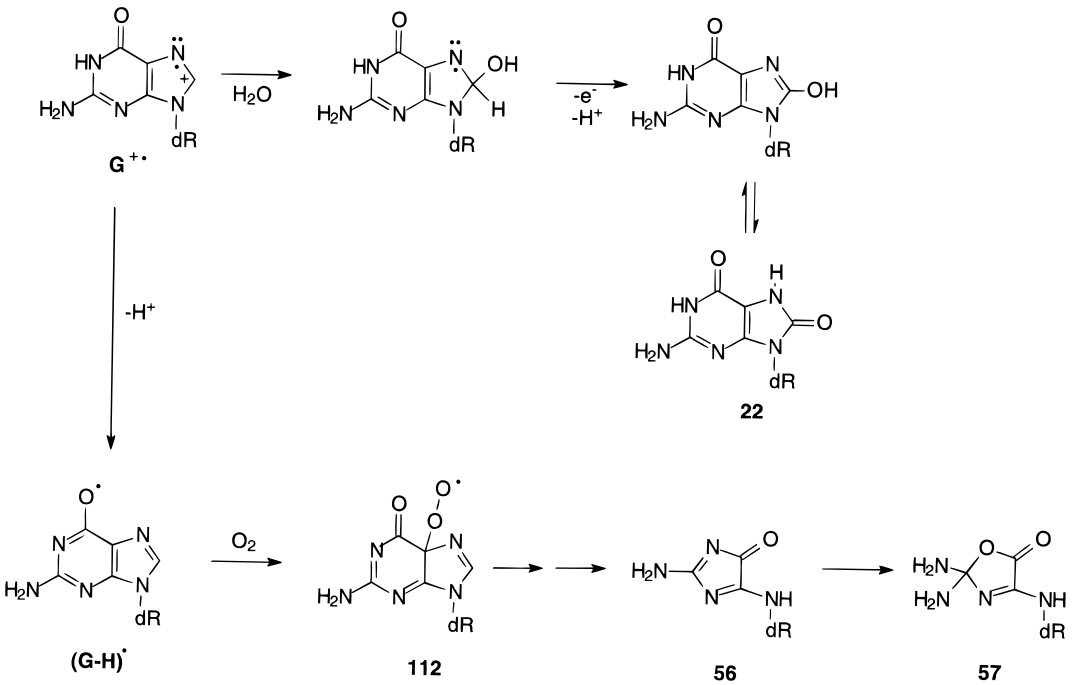

Figure 37. Fate of deoxyguanosine radical cation. Addition of H_2O is preferred in duplex DNA, but deprotonation and reaction with O_2 are observed in nucleosides.

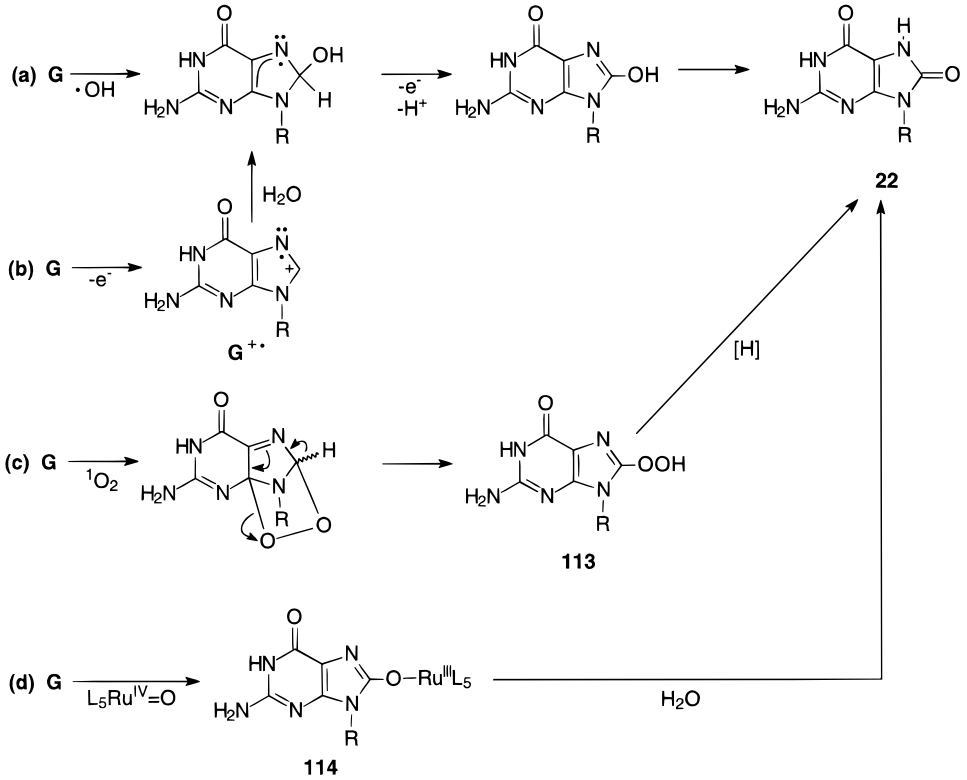

Figure 38. Guanine reactions leading to 8-oxoG.

8-Oxoguanine, or its minor tautomer 7,8-dihydro-8-hydroxyguanine, can be formed by at least 4 different pathways (Figure 38). C8 appears to be especially susceptible to radical addition, and this is a major route proposed in HO• oxidation (path a).[109] The majority of oxidants react via the one-electron abstraction pathway (b) to provide $G^{\bullet+}$, which is then hydrated and further oxidized to form **22** (see Tables 2 and 3). Singlet oxygen might react by a one-electron mechanism, but an alternative pathway (c) has also been proposed.[377] In this mechanism, the imidazole ring of guanine undergoes a [2 + 4] cycloaddition to produce an endoperoxide that can ring open to 8-hydroperoxyguanine, **113**, which should undergo facile reduction to **22**. Finally, two-electron oxidation of guanine should be possible for metal−oxo species capable of oxygen atom transfer reactions (d). Evidence for intermediate **114** generated from oxo transfer from a ruthenium(IV) complex has been reported.[233]

Since 8-oxoG is such a key lesion in toxicology studies, it is imperative to have reliable methods for its detection and quantitation. One option is complete digestion of DNA (or RNA) to nucleotides with nuclease P1, dephosphorylation to nucleosides with alkaline phosphatase, and analysis by HPLC.[376] 8-OxoG does not markedly differ from guanine in its UV−vis absorbance, but it is easily detected with an electrochemical detector (see section b below). Enzyme assays can also be used to detect 8-oxoG, but they are complicated by the fact that more than one guanine oxidation product may be a substrate for the enzyme (see section III.B). This is the case for Fpg, which effects strand scission at formamidopyrimidines (**23**−**25**) in addition to 8-oxoG.[26] Alternatively, 8-oxoG can be analyzed by polyacrylamide gel electrophoresis, but its alkaline lability was recently shown to be much lower than previously believed, as discussed below.[31]

a. Alkaline Lability. Chung and co-workers reported in 1992 that 8-oxoG in a synthetic oligodeoxynucleotide is a piperidine-labile lesion using the standard protocol of 1 M piperidine, 90 °C for 30 min.[30] In retrospect, it is significant that incomplete cleavage was observed for the strand that was presumably 100% modified as 8-oxoG. Subsequent studies have confirmed that 8-oxoG is somewhat, but not completely, piperidine-labile. Further insight was gained when Cullis et al. reported a careful study of the rate of strand scission of synthetic oligodeoxynucleotides containing 8-oxoG residues.[31] The piperidine lability appears to depend on the presence or inhibition of oxidants that further oxidize 8-oxoG to species that are more piperidine labile. The half-life for depurination of 8-oxo-2′-deoxyguanosine alone was determined to be 150 min at 90 °C in 1 M piperidine. In an oligonucleotide, about 6% cleavage is reported after 4 h under these conditions. If β-mercaptoethanol is present to inhibit further oxidation of 8-oxoG, only about 2% strand scission occurred in 4 h. A possible explanation is that the usual piperidine workup facilitates further oxidation, possibly by participation of dissolved dioxygen, that is inhibited when β-mercaptoethanol is present. Then, if the oxidation products of 8-oxoG and dioxygen are more piperidine labile (such as imidazolone **56**, oxazolone **57**, and possibly guanidinohydantoin **116**), cleavage will be observed in the form of an intense band on a gel.[133] In the end, 8-oxoG may or may not be an alkali-labile site. This will depend on the presence of remaining oxidants that may not be quenched before piperidine treatment, the radioactivity of the sample (wherein radiodecay may lead to hydroxyl radical formation), the concentration of dioxygen compared to DNA or RNA, or the presence of reductants to inhibit over-oxidation.

b. Further Oxidation. It has long been recognized that 8-oxoG is readily subject to further oxidation, since electrochemical detection is used for its analysis by HPLC.[376] A redox potential of 0.58 V vs NHE has been reported at pH 8 for 8-oxo-2′-deoxyguanosine,[358] substantially lower than that of the parent nucleoside, 2′-deoxyguanosine (1.29 V vs NHE).[146] Calculations suggest that the redox potential may be influenced by neighboring bases in a duplex helix in the same fashion as guanine itself.[370] Ionization potentials obtained from ab initio calculations of 5′-(8-oxoG)G-3′ vs 5′-G(8-oxoG)-3′ are shown in Table 6, and suggest that a 3′-guanosine neighbor will facilitate further oxidation of an already oxidized site. This remains to be confirmed by experiment for duplex DNA, although some data exist for single-stranded oligodeoxynucleotides where the effects of π-stacking are less certain.[378−380]

The products of oxidation of 8-oxoguanosine have been analyzed under various reaction conditions. Electrochemical oxidation of 8-oxoguanosine at pH 7 followed by silylation with BSTFA led to formation of a mass = 480 product that was assigned to be the silylated guanidinohydantoin **116**, presumably formed via the 5-hydroxy species **115** (Figure 39a).[381] Similarly, photooxidation studies of 8-oxo-2′-deoxyguanosine with singlet oxygen generation suggested initial formation of a dioxetane **117**, followed by a cascade of pathways.[382−384] One route leads to the 5-hydroperoxy species **118**, that was proposed to go on to intermediate **119**, a two-electron oxidized analogue of **120** (Figure 39b). Significantly, this work found that the final products of oxidation were imidazolone **56** and oxazolone **57**,[382,384] two of the same products that are observed in the initial oxidation of guanine.[133] This interesting result further complicates the overall picture of guanine oxidation, since products **56** and **57** may arise either directly from guanine oxidation, or via the intermediacy of 8-oxoG. The major pathway from dioxetane **117** is explained by ring opening to macrocycle **120**, followed by ring closure with extrusion of CO_2 yielding **121** that slowly hydrolyzes to urea plus cyanuric acid (**122**). In a report from Sheu and Foote of an organic-soluble, silylated 8-oxoguanosine under conditions of singlet oxygen generation, the two diastereomers of the 4-hydroxy product **124** were found to be major products (Figure 39c),[385] although they were the minor products reported in the Raoul and Cadet report.[382] In the former case, **124** was observed only at low temperature; upon warming it was converted to a seven-membered ring product **125** (presumably through intermediate formation of the nine-membered ring **120**) and the parabanic acid derivative **126**.[385] Peroxynitrite also oxidizes 8-oxoG, but nothing is known about the products.[343]

In summary, oxidation of guanine leads to a cascade of events. The major initial product is formation of 8-oxoG, but this species may or may not be detected because of its ready conversion to further oxidized products. Indeed, the conventional tests for 8-oxoG rely on its oxidation, either by electrochemical means, or by aerobic oxidation during 90 °C piperidine treatment. This latter process has not been carefully studied, but it is reasonable to assume that 8-oxoG should be somewhat reactive with dioxygen, since its close analogue, urate (**127**), is known to autoxidize to 5-hydroxyisourate (**128**), ultimately forming allantoin, **129** (Figure 40).[386,387] Furthermore, the identification of 8-oxoG by gel electrophoretic methods would be made easier if complete

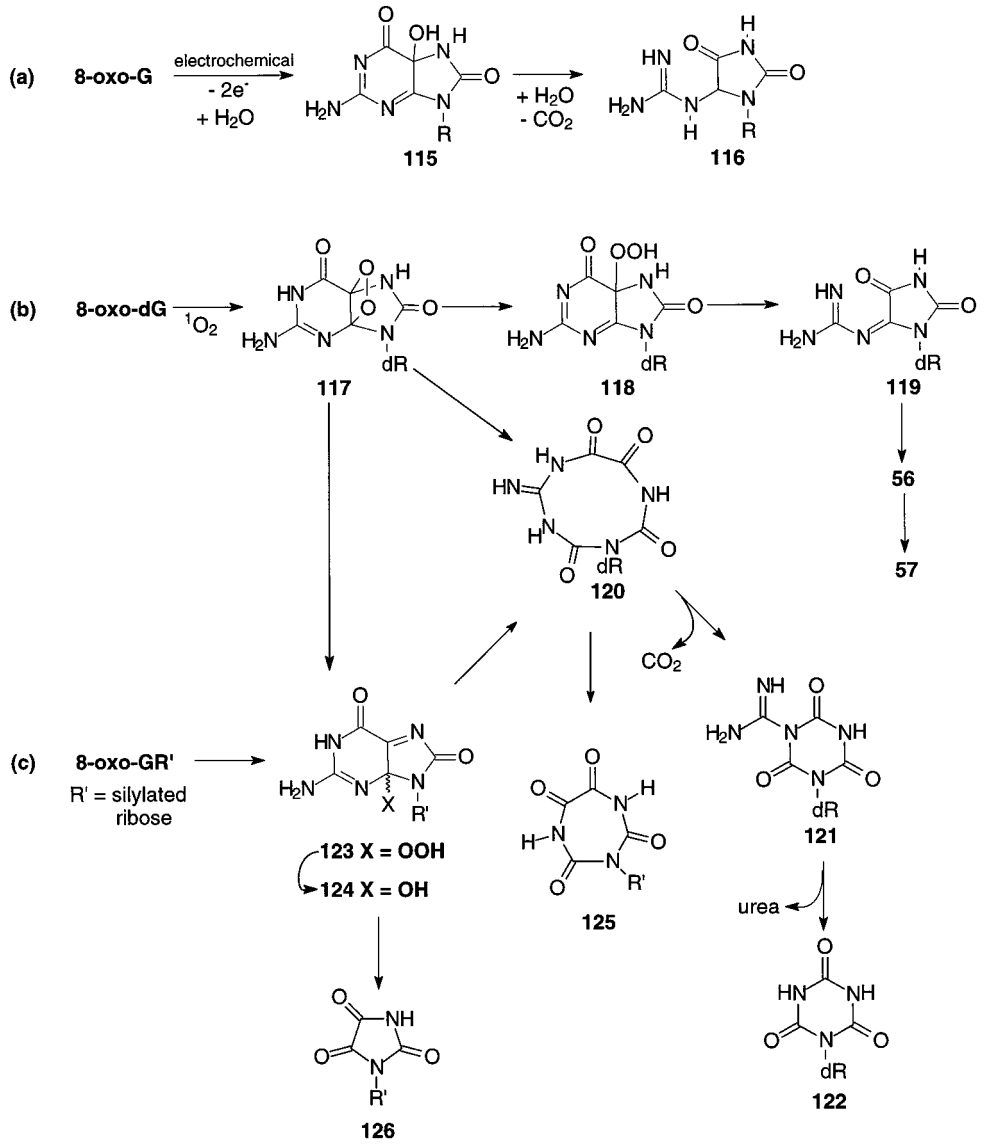

Figure 39. Oxidation pathways for 8-oxoG.

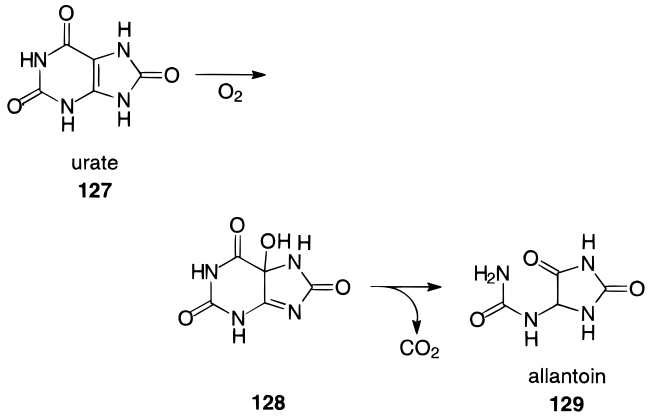

Figure 40. Autoxidation of urate, an 8-oxoG analogue.

oxidation to readily alkali-labile products (**56** and **57**) could take place. Appropriate one-electron oxidants should facilitate the over-oxidation of 8-oxoG and enhance its piperidine lability.[388] At present, $KMnO_4$ has been used in this capacity, but its utility is somewhat compromised by reaction at thymines in addition to 8-oxoG.[378,379,388]

3. FAPy-G

2,6-Diamino-5-formamidino-4-hydroxypyrimidine (FAPy-G, **23**) was isolated in 1960 as a guanine oxidation product in irradiated DNA.[389] Although it is now thought to be the minor substrate compared to 8-oxoG, certain DNA repair enzymes including Fpg and Ogg1 recognize this lesion and catalyze cleavage of DNA at that site.[63] The FAPy-G residues undergo deglycosylation upon heating to 80 °C for 1 h,[109] and thus represent an alkali-labile site under the usual conditions of piperidine or other base treatment. The FAPy-G group is also electrochemically active, although the products of oxidation have not been determined.[390]

C. Adenine Oxidation

The one-electron redox potential of adenine is somewhat higher than guanine (1.42 and 1.29 V vs NHE, respectively),[146] and thus significantly fewer oxidative lesions are observed at A. Furthermore, A does not undergo hydroxylation as readily as the 5,6 double bond of T, C, or U. The major oxidative products derived from hydroxyl radical attack at

adenine involve addition to C4 or C8.[134] Although the C4 adduct (**58**) is formed in greater abundance, it readily loses water and is reduced back to adenine. One mechanism for this in DNA is abstraction of an electron from an adjacent, or even more distant, guanosine residue.[350] It is the less abundant 8-hydroxyadduct (**59**) that leads to new product, 8-oxoadenine (**60**), a further oxidation product, and FAPy-A (**25**), a reduction product of **59**. Both are the adenine analogues of guanine oxidation. 8-OxoA is formed in about $^1/_3$ the amount of 8-oxoG during aerobic radiation (20−200 Gy) of both naked DNA and chromatin.[391,392] 8-OxoA would be the expected product under aerobic conditions compared to FAPy-A, but it is only detectable by enzymatic digestion and HPLC analysis using electrochemical detection.[393] 8-OxoA is not as easily oxidized as 8-oxoG (0.92 and 0.58 V vs NHE, respectively),[358] and might not be subject to aerobic oxidation under conditions of hot piperidine treatment. In any case, 8-oxoA is reported to be stable to hot piperidine, and is therefore not an alkali-labile site.[30] Thus, currently there is not a good gel method for detecting 8-oxoA, since strand scission does not occur with the standard reagents.[388] It is possible that a certain amount of adenine oxidation goes undetected in DNA or RNA oxidation experiments because it cannot be readily detected by gel electrophoresis.

2-Hydroxyadenine has also been observed as an oxidation product of the reaction of isolated DNA with Fe-EDTA/H_2O_2 or of human cells with H_2O_2 alone.[394,395] This lesion is formed in 1/5th to 1/40th the amount of 8-oxoG, but it is as mutagenic as 8-oxoG in *E. coli* and mammalian cells. Nothing has been reported concerning the potential alkali lability of this adenine oxidation product.

Adenine N1-oxide is formed from peracid oxidation of adenine by an oxygen atom transfer process as opposed to one-electron oxidation or HO$^\bullet$ attack. Monoperoxysulfate (HSO_5^-) is the most convenient means of preparing it in high yield from adenosine or adenosine-containing oligomers.[396] Monoperoxyphthalate has also been used with oligomers and nucleic acids. In both DNA and RNA, adenosine is the preferred site of reaction presumably via the formation of the N1-oxide, and greater reactivity is seen with single-stranded or exposed adenine residues.[397] To our knowledge, the alkali lability of this nucleobase lesion has not been reported.

D. Pyrimidine Oxidation

Formation of oxidized pyrimidine products has been discussed in previous sections, including the chemistry of hydroxyl radical (section IV.A.1.a), dihydroxylation using permanganate or osmium tetroxide (sections IV.A.3.b and c) and reactions of sulfoxyl radicals (section IV.A.4.b). Many of the products have not specifically been studied for their alkali lability. Figure 41 shows pyrimidine oxidation products that are known to deglycosylate under conditions of hot piperidine treatment, as well as products that are likely candidates to be alkali labile. In the latter

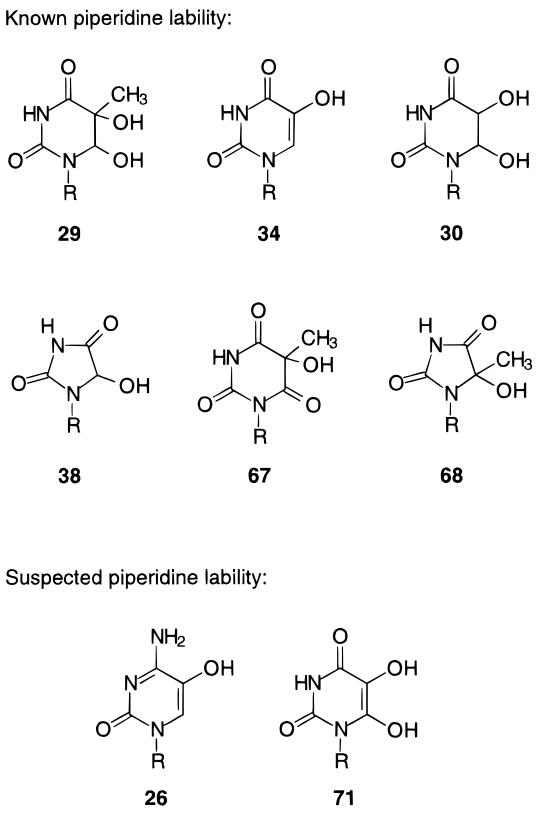

Figure 41. Summary of piperidine lability of oxidized pyrimidines.

category, analogy is made to 5-bromocytidine and 5-hydroxyuridine which are susceptible to strand scission upon piperidine treatment.[39,398] Thus, 5-hydroxycytidine and 5,6-dihydroxyuridine are suspected to be alkali labile. Furthermore, 5-hydroxyC and 5-hydroxyU are nearly as redox active as 8-oxoG (Table 5),[358,399] and their overoxidation products may likely be subject to deglycosylation leading to strand scission.

V. Electrophilic Addition Reactions

Oxidation reactions using one-electron oxidants, peroxyl radicals, or peracids, with or without photochemical or transition metal assistance, are covered in the previous section. This section describes the non-oxygen and non-carbon electrophiles that add to electron-rich nucleobases to generate species that may alter the lability of the DNA or RNA strand.

A. Electrophilic Reagents

Principally two classes of electrophiles will be considered here: halogens and nitrous acid. Little work has been done on chlorination of nucleobases compared to bromination and iodination.[400] Reagents that are effective for bromination of oligomers in water include aqueous bromine, *N*-bromosuccinimide (NBS), and NaBr/$KHSO_5$.[39] The latter pair of reagents is thought to generate Br_2 in situ, and prefers to brominate C with a nucleotide specificity of C ≫ G > T = U at pH 6.9.[39,401] Cytosines in unpaired regions of DNA, such as base bulges, are 10-fold more reactive, thus the reagent is a convenient one for detection of cytosine exposure in noncanonical struc-

tures.[39] Bromination of uracil is facilitated by somewhat acidic conditions. For NBS brominations, C is most reactive at pH 7 while G is more reactive at pH 9.[402]

Iodination can be effected by use of *N*-iodosuccinimide,[403] KI/$TlCl_3$,[404] or I_2.[405−407] Cytosine is the principal site of reaction when these reagents are used with DNA or RNA in aqueous solutions, although ICl in DMF will more readily iodinate uracil.[408] Very little, if any, iodination of guanine has been observed.[404] As with bromination, the C residues that are iodinated in folded RNA structures are those that are in single-stranded, exposed regions.[409] Similarly, denatured calf thymus DNA is more than 20-fold more reactive than the native structure.[404]

B. Guanine Reactions

Electrophiles find several possible reaction sites in guanine, depending upon the nature of the electrophile. Carbon electrophiles generally choose N7, the N^2-amino group, N3 or O^6, in roughly that order of reactivity. On the other hand, the known chemistry of halogens is restricted entirely to C8 attack, a position that also appears in oxidation chemistry and reactions with various radical species. In contrast, nitrogen electrophiles such as HONO react at the exocyclic amino group, N^2.

1. Additions to C8

8-Bromoguanosine and its 2′-deoxy analogue have been studied as synthetic nucleosides because of their biomedical properties as well as their ability to promote the formation of Z-DNA or RNA.[410] However, bromination of G in DNA or RNA constitutes a minor pathway compared to cytosine chemistry. Thus, little is known about its toxicity, alkali lability, or ability to be recognized by repair enzymes. 8-Bromoguanosine is likely to be an alkali-labile site since electron-withdrawing groups facilitate depurination, but this is yet to be tested rigorously. Some evidence for lability comes from a bromination study of single-stranded oligodeoxynucleotides with aqueous Br_2 in which some Gs were found to be cleavage sites after hot piperidine treatment.[39] Furthermore, 8-nitroguanosine (**110**), a close relative to 8-bromoguanosine formed in peroxynitrite oxidation of G, is readily depurinated with a half-life of 4 h at 37 °C at pH 7.4.[338]

2. Reactions of N^2

The exocyclic primary amino group of both guanine and adenine is subject to attack by nitrogen electrophiles such as NO^+ generated from HONO, $2NO_2 \rightleftharpoons N_2O_4$, and potentially NO alone. Nitrosation of amines leads to formation of diazonium ions and conversion to carbonyl groups after loss of N_2. For example, adenosine is converted to inosine by this mechanism. More attention has been given to guanine chemistry with nitrous acid because of the variety of toxic lesions formed which include both deamination pathways (Figure 42),[411] as well as G−G and G−A cross-linking reactions (Figure 43). Thus, diazonium ion **130** can undergo ring opening to **131**, a species thought to be more stable than **130** according to ab initio calculations.[412] Intermediate **131** accounts for many of the products formed, including xanthine (**132**), the major product, and oxanosine (**134**), a significant product.[413] Either intermediate **131** or **135** could account for other minor pathways which include trapping by NO_2^- to generate 2-nitroinosine **136**[413] (Figure 43) or nucleophilic attack by an adjacent guanine N^2 on the opposite strand of duplex DNA.[414,415] This latter reaction produces a covalent interstrand cross-link (**137**); an analogous reaction can occur with the N^6 amino group of an adjacent adenine residue.

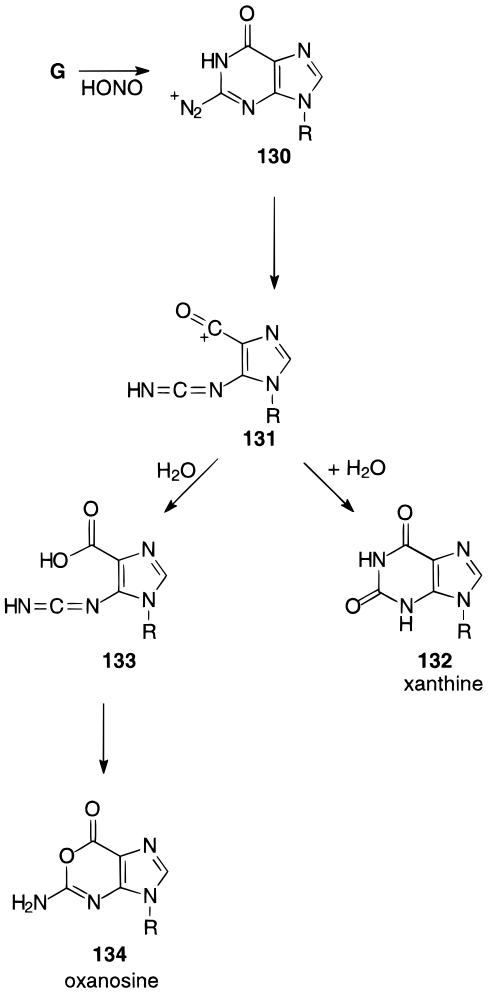

Figure 42. Pathways to xanthine and oxanosine from reaction of guanine with nitrous acid.

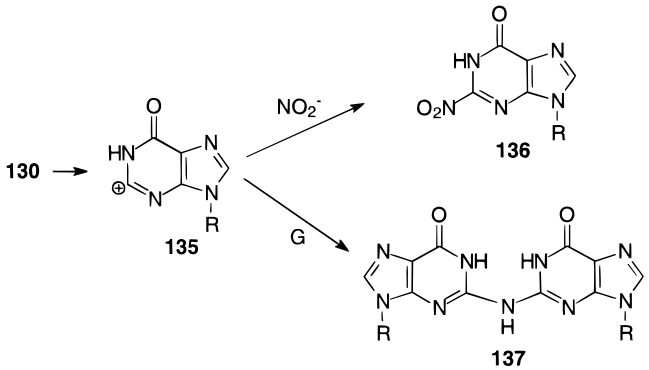

Figure 43. Other products from N^2-nitrosation of guanine include 2-nitroinosine (**136**) and cross-linked species (**137**).

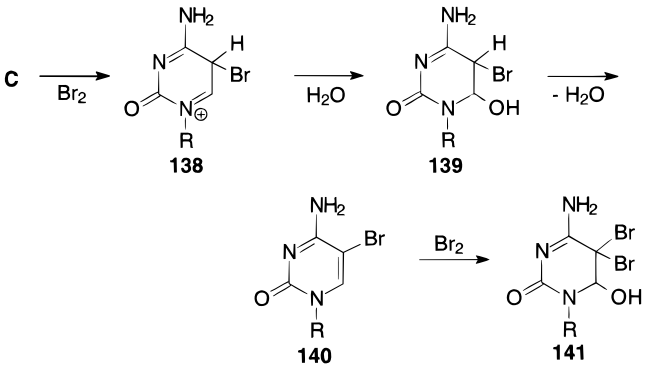

Figure 44. Proposed mechanism for bromination of cytosine.

The deglycosylation of some of the nitrous acid products of guanosine has been studied under acidic conditions.[416] 2′-Deoxyxanthosine is 49 times more labile than 2′-deoxyguanosine in a single-stranded oligonucleotide at 70 °C, pH 4. In contrast, the acid lability of 2′-deoxyoxanosine is nearly identical to that of 2′-deoxyguanosine. Similarly, modifications to guanine's N^2-amino group do not affect its hot piperidine lability, so none of the lesions discussed above are expected to be alkali-labile sites, with the possible exception of xanthosine, which has the greatest heat lability.[417]

C. Pyrimidine Reactions

While carbon electrophiles react exclusively at the heteroatoms of pyrimidines, radicals, halogens, and certain nucleophiles select the 5,6 double bond as the principal site of reaction. In this case, thymine and uracil have similar reactivity but both are less reactive than the electron-rich double bond of cytosine. Thus, halogenation occurs much more readily on C. In contrast, nitrogen electrophiles such as nitrous acid again select primary amino groups for reaction, as with guanine and adenine.

1. Additions to the 5,6 Double Bond

The major product of pyrimidine halogenation is the corresponding 5-halopyrimidine, although some over-reaction is possible in the case of bromine leading to a dibromoadduct. Figure 44 shows the likely mechanism of formation of 5-bromocytidine, **140**.[400] The intermediacy of bromohydrin **139** is established by NMR studies, as is the overbromination product **141**. An analogous pathway leads to 5-iodocytosine, although the reaction is pH dependent.[404] At higher pH's, dehydration and dehalogenation of the intermediate of type **139** are favored to regenerate starting material. Thus, higher yields of 5-iodocytosine are realized at pH 5 and below. Very little 5-iodoracil is formed at any pH, because of even greater base sensitivity.

5-Bromocytosine is a piperidine-labile site in DNA under the standard conditions of 90 °C for 30 min,[39] and 5-iodocytosine is likely labile as well.

2. Reactions of Cytosine N^4

Like guanine and adenine, the *exo*-amino group of cytosine is subject to deamination under various conditions.[86,400] C is the least reactive of the amino-containing nucleosides toward HONO, although the differences between G>A>C are relatively small.[418] Indeed, C is somewhat more reactive than A in calf thymus DNA compared to the nucleosides.[419] Deamination of C leads to a U residue that is mutagenic. Treatment of a single-stranded plasmid with NO also gives conversion of 2′-deoxycytidine to 2′-deoxyuridine,[420] although the details of the mechanistic pathway are not known. The deamination of C is a major event in the reaction of the nucleobase with electrophiles, but it does not lead to an alkali-labile lesion.

VI. Radical and Oxidative Alkylations

The alkylation of nucleobases by carbon electrophiles is an enormous field that includes the chemistry of nitrogen and sulfur mustards, the reactions of chemical agents used as probes of nucleic acid structure, and the action of natural products that modify DNA bases. Mention has already been made of the nitrogen mustards, especially in a historical context, but interest continues currently as these rather nonspecific G−N7 alkylating agents can be made more site- or cell-specific through bioconjugate formation.[421] A variety of carbon electrophiles act as structural probes to examine DNA or RNA folding through reaction with solvent-exposed nucleophilic sites on the bases. These include dimethyl sulfate (DMS), diethyl pyrocarbonate (DEPC), carbonyldiimidazole (CDI), and kethoxal. These alkylating agents have been thoroughly reviewed elsewhere in the context of structure probing studies.[13,201,422,423] Typically, their use involves a first chemical step of nucleobase modification followed by hot piperidine (or aniline for RNA) treatment for strand scission. (Alternatively, primer extension analysis is often used on large RNA substrates.) Similarly, the field of natural products that act as DNA alkylating agents (mitomycin, leinamycin, pyrrolobenzodiazepines, CC-1065, etc.) has been extensively reviewed.[424,425] Thus, this section of the review will cover only those carbon electrophiles (nonnatural products) that are either radical species or are generated via redox methods.

A. Alkylation Sites

The heteroatoms of the nucleobases present ample opportunity for reaction with carbon electrophiles. In the absence of DNA or RNA recognition elements that direct alkylating agents to specific sites, such as might be found in natural products, alkylating agents usually react with only a subset of the possible nucleophilic sites. The intrinsic reactivity of DNA alkylating agents has been nicely described by Rokita et al.[41] and is thoroughly reviewed by Singer and Grunberger.[426] Figure 45 summarizes the general characteristics of nucleobase alkylation based on type of alkylating agent. For example, reagents that react by an S_N2 mechanism (dialkyl sulfates, epoxides, aziridines, and alkyl halides and sulfonates) typically form G−N7 adducts in addition to A−N1 and N3 adducts. (Alkylation of A−N7 is much less common.)

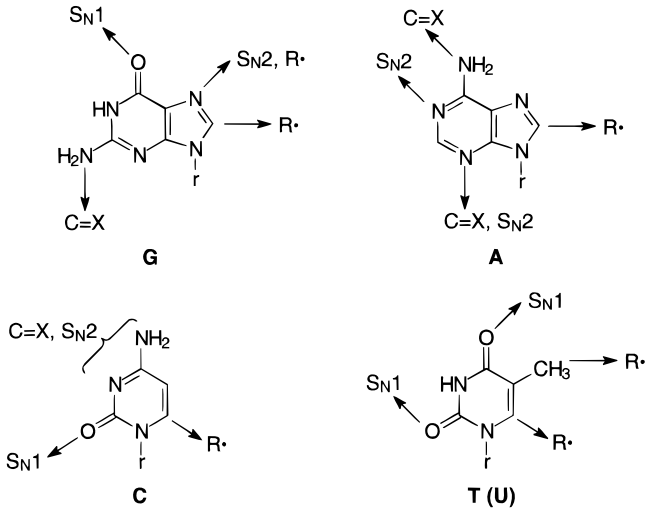

Figure 45. Summary of site specificity of electrophiles according to general type of mechanism.

Reagents that are more S_N1-like in their mechanism (alkylnitrosoureas and diazoalkanes) lead to a larger amount of O-alkylation at essentially all carbonyl oxygens. Electrophiles in which an sp^2-hybridized carbon is involved (imines, aldehydes, quinone methides, and conjugated systems) prefer to react at the *exo*-amino groups (G−N^2, A−N^6, and C−N^4) although some reaction is also seen at ring nitrogens, especially A−N3 and C−N3. Carbon radicals usually attack G−C8 (as does HO•) with less frequent reaction at A−C8, T−CH_3, and U−C6. Of these reaction sites, the major alkaline-labile lesions are alkylations at guanine N7 and C8.

B. Carbon Radicals

1. Methyl Radical

The methyl radical has been generated in a variety of ways as summarized in Figure 46. Hydrazines have been shown to be animal carcinogens, and this biological activity might be correlated to the formation of alkyl radicals from oxidative decomposition of alkylhydrazines. Thus, a variety of methylhydrazines has been shown to produce •CH_3 by treatment with hydrogen peroxide and a catalyst (horseradish peroxidase or ferricyanide[311]) or by metabolic action of methylhydrazines in vivo.[427,428] Reactions with •CH_3 so produced led to in vitro formation of 7-methyl- and 8-methylguanine in calf thymus DNA, and in vivo studies showed both 8-methyl- and O^6-methylguanine as products when methylhydrazines were administered to rats. The same nucleobase products in addition to N3-methyladenine were identified in DNA hydrolysates when methyl radical was generated from *tert*-butyl hydroperoxide in the presence of iron(II).[429,430]

Methyl radical can also be generated by photolysis of *tert*-butyl peracetate. When this reaction was studied with nucleosides, guanine methylation predominated at low pH, but above pH 4, methylation of cytosine at N3 and N^4 predominated over guanine products.[431,432] In addition, alkylation of adenine at C8 and N^6 was observed as well as some attack at uracil's C5. Curiously, the methylpyrimidine adducts

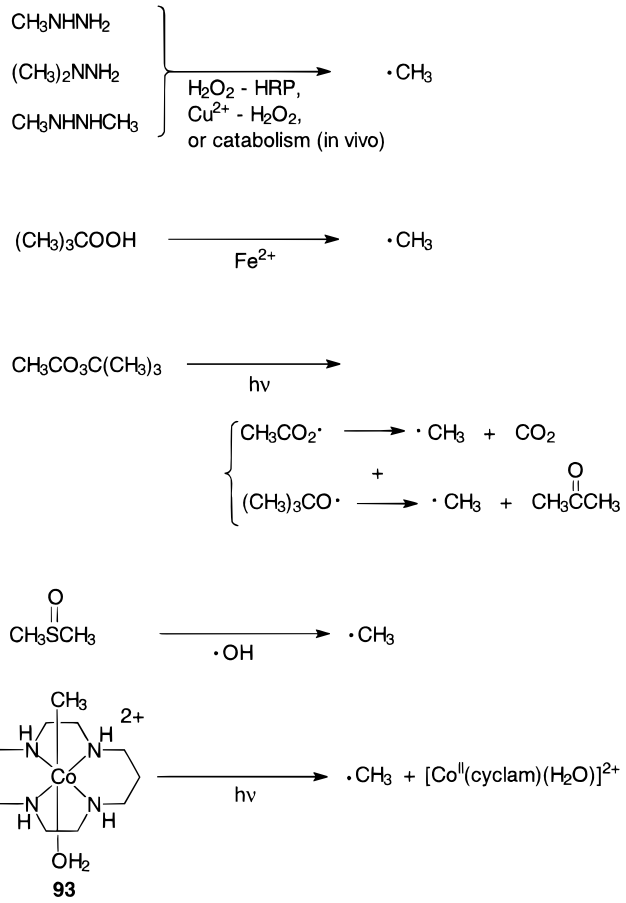

Figure 46. Methods of generation of methyl radical.

have not been detected in DNA studies despite the fact that they were reported to predominate in nucleoside studies.

Generation of methyl radical from reaction of dimethyl sulfoxide with hydroxyl radical (from Fenton conditions) was shown to modify bases in calf liver RNA, and the predominant products were again methylpurines, specifically 8-methyl-G, 8-methyl-A, and 2-methyl-A.[433] There is also a suggestion that plasmid DNA damage results from formation of •CH_3 by photolysis of an alkylcobalt complex, **93**.

Other alkyl radicals have scarcely been investigated with DNA. 2-Phenylethylhydrazine (phenelzine), a clinical antidepressant was shown to generate the 2-phenylethyl radical upon reaction with oxyhemoglobin or ferricyanide by analogy to studies reporting on methylhydrazines.[434] In studies with DNA, piperidine-labile guanine chemistry was observed by gel electrophoresis suggesting that N7 or C8 adducts are formed.

2. Benzo[a]pyrene Radical

Alkylation of nucleobases by oxidative metabolites of benzo[*a*]pyrene, namely the diol epoxides, is well established.[435] On the other hand, benzo[*a*]pyrene itself can form adducts with guanine via the intermediacy of one-electron oxidized species.[436] Thus, formation of the pyrene radical cation by either electrochemical or enzymatic (horseradish peroxidase) methods leads to a G−N7 adduct as well as the C8-substituted guanine adduct **142** (Figure 47) formed

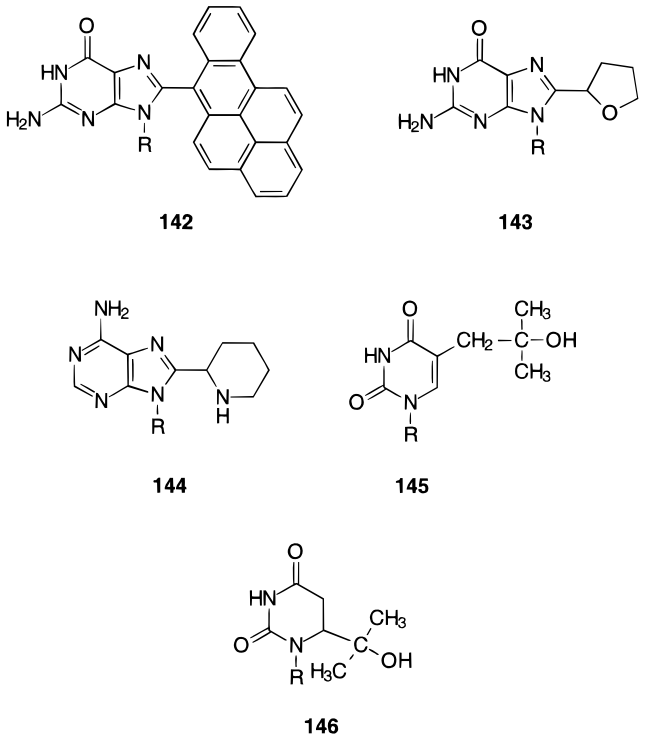

Figure 47. Carbon radical adducts to nucleobases.

in about a 2:1 ratio. Both adducts are formed via covalent linkage to C6 of benzo[*a*]pyrene. Isolation and characterization of these adducts by DNA digestion followed by HPLC analysis showed that about 30% of the C8 adduct **142** had undergone deglycosylation, even without alkaline treatment, underscoring the lability of C8 alkylated guanine lesions.

3. α-Alkoxy, α-Hydroxy, and α-Amino Radicals

Carbon radicals can be formed at the α position of alcohols,[437−439] ethers,[440] and amines[441] through a radical chain mechanism initiated by peroxides and ultraviolet light or by γ rays. In the presence of DNA nucleobases, the principle adducts formed are at guanine C8 and adenine C8. Two examples, **143** and **144**, are depicted in Figure 47. Competition studies showed that pyrimidine adducts constituted minor amounts of the total base lesions.[439,442] When isolated, thymidine adducts were found to be bound via the methyl group (e.g., **145**) while uracil adducts were linked via C6 (e.g., **146**).[442] As mentioned, the G−N7 and G−C8 adducts are expected to be alkali-labile lesions. Adducts to thymine's methyl groups should be stable, while the uracil C6 adduct should be alkali labile by virtue of the sp^3 hybridization of the 5−6 bond. Unfortunately, data are not available for these adducts concerning alkali lability.

4. Phenol Radicals

Phenols undergo facile one-electron oxidation, especially at higher pH or in the presence of transition metals that facilitate phenolate anion formation. The corresponding radicals form covalent adducts with nucleobases by alkylation of the ortho or para positions of the aromatic ring. One would expect C8 of purines to be a major reaction site, based on the radical reactions described above. However, studies of oxidative DNA protein cross-links have led only to the isolation of pyrimidine adducts of tyrosine, and whether purine adducts are formed remains to be seen. In studies with hydroxyl radical-induced cross-linking in calf thymus nucleohistone, a tyrosine adduct to cytosine C6 (**147**, Figure 48) was isolated by acidic hydrolysis followed by derivatization for GC−MS analysis.[443] In a similar experiment using Fe^{3+}- or Cu^{2+}-catalyzed oxidation of nucleohistone with H_2O_2, a thymine−tyrosine adduct was characterized in which the 5-methyl substituent was the site of alkylation with the ortho position of the tyrosyl radical (**148**).[444] Neither of these pyrimidine adducts would be expected to be alkali-labile lesions in DNA. It is not known whether purine adducts were formed under these reaction conditions, and if formed, whether they would be stable to the acidic workup.

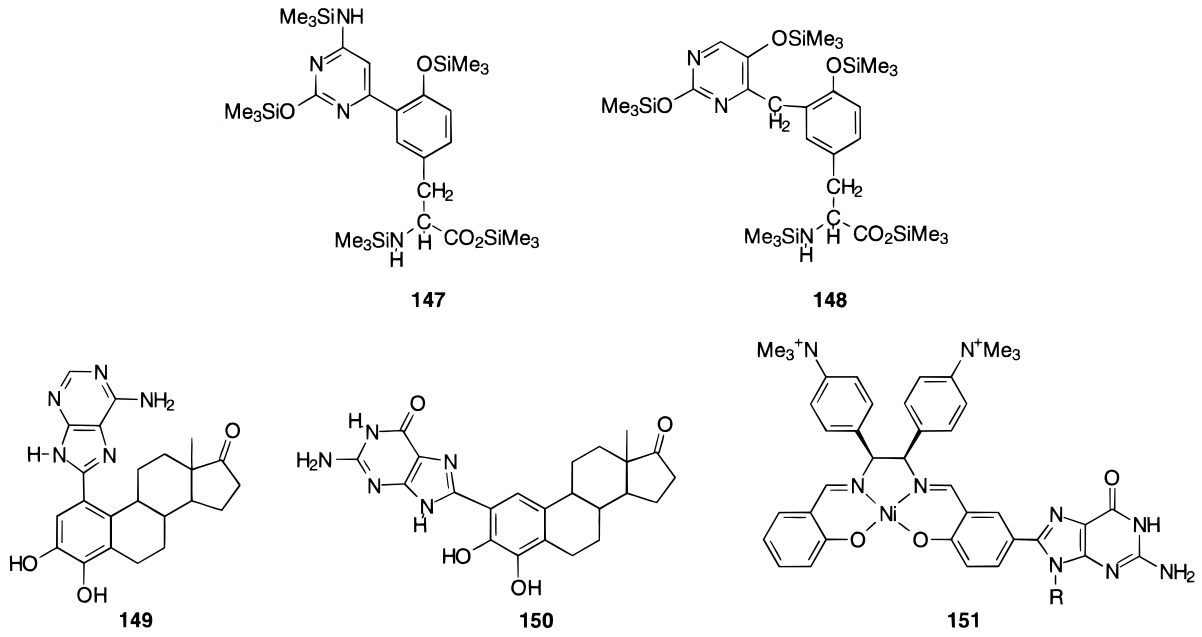

Figure 48. Phenol radical adducts to nucleobases.

Phenol radicals can also be generated by one-electron reduction of quinones, a common constituent of DNA-targeted drugs. The resulting semiquinone radical can participate in redox cycling with other active oxygen species. Alternatively, adducts can form to nucleobases. The particular case of 3,4-estrone-*o*-quinone has been recently studied since this metabolite of estradiol has been implicated in estrogen-linked carcinogenesis.[445,446] Alkali-labile strand scission of DNA was observed in a human breast cancer cell line exposed to the quinone. To investigate the possibility of adduct formation more thoroughly, studies were carried out with the nucleobases[447,448] and deoxyribonucleosides individually,[449] and the adducts characterized. Comparative amounts of the different adducts are not available, but all nucleobases were found to be reactive with the semiquinone radical formed by dithionite reduction of the quinone. The site of reaction of the quinone was either C1 (para to the 4-hydroxy position, e.g., **149** in Figure 48) or C2 (ortho to the 3-hydroxyl group, e.g., **150**). The nucleobase sites of cross-linking were found to be C8 and N^6 of A and similarly, C8 and N^2 of G. For pyrimidines, C was reactive at N^4 and T at N1 and N3. The reactions carried out on nucleosides further demonstrated that deglycosylation was easily effected for the purine C8 and the T−N3 adducts. Thus, the purine products are of two types—the alkali-labile C8 adduct and the non-alkali-labile adduct to the *exo*-amino group.

A phenol radical adduct to guanine is also the suspected lesion responsible for formation of a high molecular weight species in nickel−salen-catalyzed oxidation of DNA (see **50** in Figure 15) as previously discussed (sections III.D.3 and 4).[94] Oxidative conditions are thought to form a nickel-ligated phenol radical with unpaired electron density in the ortho and para positions. Two types of guanine adducts are formed—one piperidine labile and proposed to be the C8 adduct **151**, and the other nonpiperidine labile, possibly the N^2 adduct by analogy to the estrone system described above.

C. Redox-Activated Carbon Electrophiles

A number of natural products form reactive species upon redox activation, particularly in the presence of thiols. As already mentioned, the natural products chemistry has been reviewed elsewhere.[424,425] In this section we present two other classes of carbon electrophiles, quinone methides and peptide imines, that can be formed as a result of reductive or oxidative activation, and that subsequently lead to DNA alkylation.

1. Quinone Methides

Quinone methides can be generated as transient intermediates during reduction of quinone antibiotics such as mitomycin C, adriamycin, and daunomycin.[424] When bound to DNA, nucleophilic sites on the bases form covalent adducts with the activated drugs. This behavior has also been observed in synthetic analogues capable of quinone methide or semiquinone formation. Figure 49 shows two examples of this type of chemistry. In the first, a naphthoquinone derivative **152** was successfully reduced using chemical reagents ($NaBH_4$, $Na_2S_2O_6$, or β-nicotinamide mononucleotide) or enzymatic activation (cytochrome *c* reductase/NADH, xanthine oxidase/xanthine, or glutathione reductase/NADPH).[450−452] The resulting hydroquinone **153** rapidly eliminates mesylate to generate the quinone methide **154**, leading to a DNA adduct. The sites of adduct formation included both guanine and cytosine. Typically *exo*-amino groups are known to react with quinone methides, and guanine N^2 appears to be a major reaction site in this reaction. However, cytosine was shown by NMR studies to be alkylated at N3.[41] None of these products are piperidine-labile lesions, and so their existence was determined by other methods.

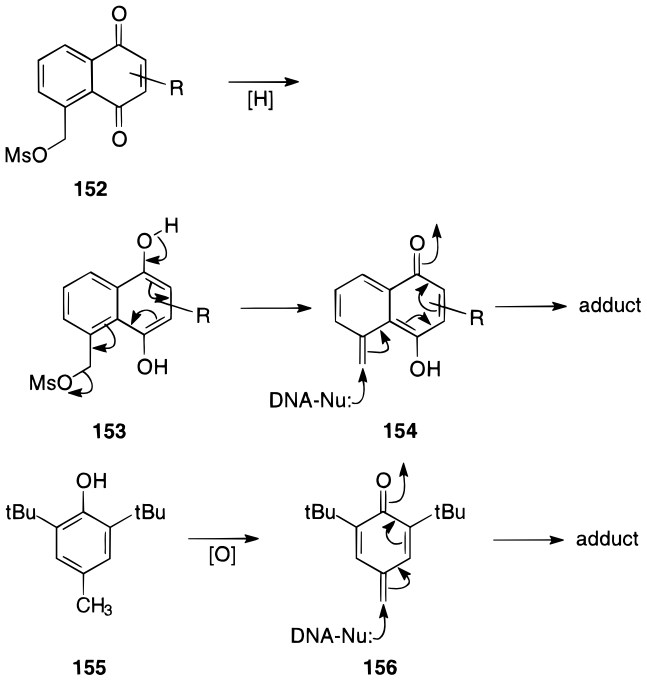

Figure 49. Proposed mechanism for the reaction of quinone methides with DNA.

Quinone methides can also be generated by oxidative procedures as illustrated for 2,6-di-*tert*-butyl-4-methylphenol (BHT), **155** (Figure 49).[453] Oxidation with AgO leads to quinone methide **156**, which then forms adducts with DNA. The order of reactivity of nucleophilic sites was determine by digestion and HPLC analysis of calf thymus DNA to be G−N^2 > A−N^6 > G−N7 > G−N1. Thus, some of the quinone methide chemistry leads to strand scission after alkaline workup (namely, the G−N7 adducts), but much of it does not give DNA cleavage.

2. Peptide Imines

Covalent cross-linking of proteins with DNA is a significant cause of cytotoxicity. Tyrosine is commonly involved since one-electron oxidation to form a tyrosyl radical leads to a species capable of reaction with nucleobases, as described in section VI.B.4. Alternatively, oxidation of the peptide backbone to form *N*-acylimines leads to covalent adduct formation with DNA nucleophiles because of the potent electrophilicity of this species.[454] This has been demon-

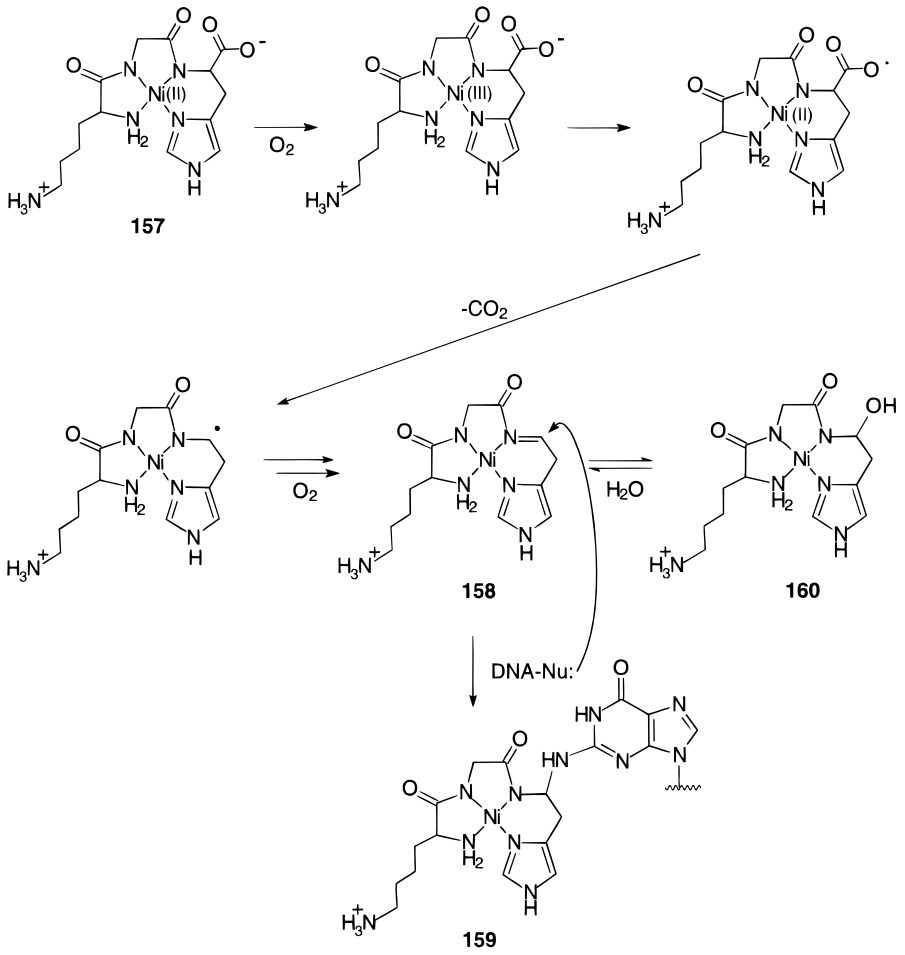

Figure 50. Reaction scheme for the formation of a peptide−DNA cross-link from the interaction of a Ni(II) tripeptide complex **157**, O_2 and DNA.

strated for tripeptide complexes of nickel(II) (e.g., **157**, Figure 50)[455] in which the metal ion promotes oxidative decarboxylation of the C-terminus in the presence of dioxygen.[294,295,456] A mechanism is proposed in which the *N*-acylimine (**158**) so generated is subject to attack by a nucleobase of DNA in competition with solvent water providing both the adduct **159** as well as the hydrated species **160**. The sites of adduct formation were shown to be G ≫ T ≈ A > C.[457] The majority of cross-linked species did not lead to strand scission upon piperidine treatment, but heating reversed some of the adduct formation. These traits are characteristic of G−N^2 alkylation, as is expected for sp^2-hybridized electrophiles. Indeed, imine-containing natural products such as the pyrrolo[1,4]benzodiazepines dehydrate to produce conjugated imines that undergo nucleophilic attack by G−N^2.[458] In principle, peptide imines may be formed under oxidative conditions at any site in the peptide backbone via radical hydroxylation of C_α followed by dehydration. However, examples of this chemistry have yet to be uncovered.

VII. Other Base Modifications Leading to Strand Scission

Two other major classes of nucleobase modification need to be mentioned here because they lead to

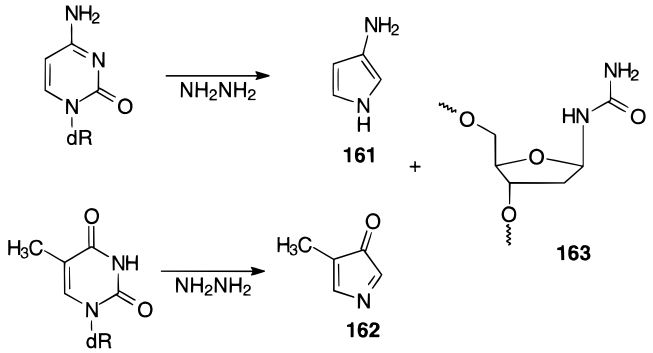

Figure 51. Maxam−Gilbert reactions of cytosine and thymine with hydrazine.

alkali-labile lesions, despite the fact that they are not oxidative in nature. These are (i) pyrimidine reduction and (ii) reactions of pyrimidines with nucleophiles. Ionizing radiation generates solvated electrons, effectively H•, in addition to HO•.[17] The 5,6 double bond of pyrimidines is subject to reduction under these conditions, and essentially any conversion of C5 and C6 to sp^3 centers leads to alkali lability.[459] A second set of reactions concerns nucleophilic attack at pyrimidines' 5,6 double bond. In fact, the strong nucleophile hydrazine, in conjunction with hot piperidine treatment, is used for Maxam−Gilbert sequencing.[12] In this reaction, the difference between cytosine and thymine can be detected by thymine's inability to undergo reaction with H_2NNH_2 under

conditions of high salt, whereas cytosine is reactive regardless of salt concentration. The hydrazine reactions with C and T generate aminopyrazole (**161**) and pyrazol-2-one (**162**), respectively, in addition to a *N*-ribosylurea residue (**163**) that can undergo deglycosylation and β-elimination upon piperidine treatment (Figure 51).

VIII. Conclusions

The goal of this review is to alert the community of nucleic acid chemists to the multitude of nucleobase modifications that may ultimately lead to formation of an abasic site. The abasic site is, in turn, a trigger for strand scission under alkaline conditions or in the presence of lyases. A great deal of attention has focused on direct strand scission events, usually occurring at the ribose or phosphodiester group, despite the fact that nucleobases, by virtue of being electron-rich heterocycles, are intrinsically very reactive. As a result, a large fraction of the total reaction of DNA or RNA with a particular reagent may be missed entirely if one examines only direct strand scission monitored by gel electrophoresis. A combination of studies including direct strand scission, alkali or enzyme-labile strand scission, primer extension analysis, and product determination will give the complete reaction profile for a nucleic acid-targeted reagent. Nucleobase-targeted reagents are inherently more site-specific than those directed at sugar or phosphate functionalities, and they may even be sequence specific to a certain extent as demonstrated by the sequence preference of one-electron guanine oxidation. Although guanine is the most commonly modified of the bases due to its susceptibility to both alkylation and to one-electron oxidation, base-specific reactions also exist for thymine (dihydroxylation) and for cytosine (bromination) that can be carried out under mild, near-physiological conditions. Adenine-specific reagents that operate under such conditions are a future challenge. Finally, it should be emphasized that careful characterization of the alkali lability of most nucleobase modifications is still wanting.

IX. Abbreviations

AAPH	2,2′-azobis(2-amidinopropane) hydrochloride
5-BrdU	5-bromo-2′-deoxyuridine
BSTFA	*N*,*O*-bis(trimethylsilyl)trifluoroacetamide
CDI	carbonyldiimidazole
DEPC	diethyl pyrocarbonate
Dien	diethylenetriamine
DMF	dimethylformamide
DMS	dimethyl sulfate
DTT	dithiothreitol
dU	2′-deoxyuridine
FAPy-A	4,6-diamino-5-formamidinopyrimidine
FAPy-G	2,6-diamino-5-formamidino-4-hydroxypyrimidine
HAA	3-hydroxyanthranilic acid
HRP	horseradish peroxidase
MMPP	magnesium monoperoxyphthalate
8-OxoA	7,8-dihydro-8-oxoadenine
8-OxoG	7,8-dihydro-8-oxoguanine
PAA	peracetic acid
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
Ssb	single-strand breaks
Tacn	1,4,7-triazacyclononane
Trien	triethylenetetraamine

X. Acknowledgments

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