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Contents

Oxidative Damage to DNA: Formation, Measurement, and Biological Significance

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Contents

1
Introduction

Deoxyribonucleic acid (DNA) appears to be a critical cellular target for the biological effects (cellular lethality, mutagenesis, carcinogenesis, and ageing) of oxidation processes mediated by various physical and chemical agents (Breimer 1990; Ames and Gold 1991; Sies 1991; Marnett and Burcham 1993; Clayson et al. 1994). These include, among others, ionizing radiation, solar light, photosensitizers, and several environmental carcinogens. The leakage of reactive oxygen species from endoplasmic reticulum and mitochondria represents another source of oxidative stress for the cells. It should be added that diet and psychological stress have been shown to induce oxidation processes within cellular DNA. Strand breaks, modified bases, abasic sites, and DNA–protein cross-links represent the four main classes of oxidative DNA damage (for recent reviews, see Knorre et al. 1993; Cadet 1994; Breen and Murphy 1995) which are at least partly characterized in model compounds. The exact biological role of the bulk of identified oxidative DNA lesions remains to be determined. However, relevant information is available on the mutagenicity of a few oxidized purine and pyrimidine bases. In addition, it is well established that oxidative DNA damage can be repaired by various enzymatic pathways (Lin and Sancar 1989; Kow et al. 1990; Boiteux 1993; Demple and Harrison 1994; Hatahet et al. 1994; Tchou et al. 1994; Hayakawa et al. 1995). More specifically, evidence has been provided that oxidized pyrimidine and purine bases may be removed mostly by base excision and to a lesser extent by nucleotide excision. Most of the information is inferred from studies on prokaryotic systems, but there is evidence that this should apply to animals and humans.

During the last decade, increasing interest has been devoted to the role of tumor suppression genes in the biological effects induced by oxidative stress. Evidence has been provided that mutations in the p53 tumor suppressor gene are involved in the majority of human cancers (Hollstein et al. 1991; Cho et al. 1994). A large body of information is now available on the putative role of p53 in either apoptosis or cell cycle arrest to allow repair of DNA damage (Kastan et al. 1992; Lowe et al. 1993). It was suggested that p53 may directly and indirectly stimulate nucleotide excision repair (NER) machinery through the Gadd45 gene, for example upon exposure of cells to ionizing radiation (Smith et al. 1994). However, it was recently reported by two independent groups that Gadd45 does not exert any detectable

stimulation on NER activity (Kazantsev and Sancar 1995; Kearsey et al. 1995). It was also shown that p21 protein, which is implicated in the G_1 cell arrest and cell death, is under the transcriptional control of p53 (El-Deiry et al. 1993).

   Emphasis has been placed in this review article on the description of the chemical structure and the mechanisms of formation of the main oxidation products of the purine and pyrimidine DNA bases as inferred from studies of models. In addition, the available accurate information on the mechanisms of formation of oxidative base damage to cellular DNA is provided together with a critical review of the various methods which have been used for such purposes. In the final section of the survey, the strategies which are currently used to gain insight into the biological role of oxidized DNA bases are reviewed. These mostly involve the synthesis of modified nucleotides and their insertion within oligonucleotides at specific sites. The related oligonucleotides can be further used for DNA replication and mutagenesis studies together as substrates for investigating the specificity of DNA repair enzymes.

2
Formation of Oxidative DNA Base Damage
Within Isolated DNA and Model Compounds

Various reactive oxygen species and oxidation processes may be involved in the induction of damage to DNA (Table 1). Among these, the hydroxyl radicals, the "ferryl" (Fe = O^{2+}) or the "perferryl" (Fe = O^{3+}) species generated by Fenton reactions (Chaudière 1994) and peroxynitrite are the most efficient, being able to react with both the base and sugar moieties at diffusion-controlled rates. On the other hand, singlet oxygen, ozone, and hydrogen peroxide react with DNA in a more specific way. It may be added that superoxide radical and its related conjugated forms do not exhibit any reactivity towards DNA components, at least in aqueous solutions (Cadet and Téoule 1978). One-electron oxidation of bases is an important process associated with the direct effect of ionizing radiation, type I photosensitization mechanism, high-intensity ultraviolet (UV) laser photochemistry and vacuum UV radiation (for reviews, see von Sonntag 1987a; Cadet and Vigny 1990; Becker and Sevilla 1993; Görner 1994).

   In the present survey, emphasis is placed on the structure assignment and the biological role of oxidative DNA base lesions. However, it should

Table 1. Reactive species and radicals involved in oxidative stress

Reactive species	Reactivity with DNA
Superoxide radical ($O_2 \cdot^-$)	Not detectable (reduction of ROO·)
Hydroperoxide radical (HO·2)	Not detectable
Hydroxyl radical (OH·)	Ixidizes bases and sugar moieties
Iron-oxo complex	Oxidizes bases and sugar moieties
Peroxinitrite (ONOO−)	Oxidizes bases and sugar moieties
Singlet oxygen (iO_2)	Oxidizes guanine
Ozone (O_3)	Oxidizes pyrimidine and purine bases
Hydrogen peroxide (H_2O_2)	Oxidizes adenine
Oxyl (RO·) and peroxyl (ROO·) radicals	Oxidize the sugar moieties
Purine and pyrimidine radical cations	Hydration and deprotonation

be recalled that DNA strand breakages represent a major class of oxidative damage to DNA. A large body of information is now available on the mechanism of hydroxyl radical-mediated formation of strand breaks in aerated aqueous solutions. In this respect, hydroxyl radicals are able to abstract hydrogen atoms within the sugar moiety with a preference at C4'. The resulting radicals are converted in most cases into strand breaks through mechanisms which are well documented (for comprehensive reviews, see von Sonntag 1987a; Breen and Murphy 1995). In particular, DNA cleavage involving the C4'-centred radical may be explained in terms of two mechanisms involving either the formation of a tetroxide from two neighboring peroxyl radicals (Schulte-Frohlinde and Bothe 1984; von Sonntag 1987a) or a 4'-hydroperoxide (Janicek et al. 1985) with subsequent Criegge rearrangement. The latter mechanism, which appears to be more likely, has been suggested to occur with iron-bleomycin (Giloni et al. 1981), a potent antitumoral antibiotic. It was shown that the reactions mediated at C4' by either OH· radical or iron-bleomycin lead to the formation of 3'-glycolate termini and base-propenal (Giloni et al. 1981; Henner et al. 1983a; Janicek et al. 1985). However, a more definitive elucidation of the putative mechanism proposed for the bleomycin-mediated cleavage of DNA, which is still open to debate (Burger et al. 1986; McGall et al. 1992), would require further studies. In this respect, an interesting piece of information dealing with the specific preparation of the related 3'-hydroxy-4'-hydroperoxide in a single-stranded oligonucleotide recently became available (Giese et al. 1995). This was achieved by photo-induced cleavage of the C–Se bond of a nucleotide after its incorporation in a DNA fragment (Giese et al. 1994). Interest-

ingly, it was shown that the 4'-hydroperoxide of thymidine is able to undergo a fast decomposition into thymine propenal with a concomitant release of glycolate according to a Grob fragmentation mechanism (Giese et al. 1995). In addition, it was suggested that the peroxyl radicals resulting from the initial action of OH· radicals on pyrimidine bases are involved in hydrogen abstraction reactions from the sugar moiety of polynucleotides and nucleic acids, causing chain breaks (Schulte-Frohlinde and Bothe 1984; Deeble and von Sonntag 1986; Jones and O'Neill 1990). However, this process, which may also involve radical cation and related neutral radicals from purine bases (Gut et al. 1993; Görner 1994), is at best of low efficiency.

2.1
Hydroxyl Radical-Mediated Oxidation Reactions

The mode of action of hydroxyl radicals on DNA constituents in aerated aqueous solution (indirect effect of ionizing radiation) has been extensively studied during the past two decades (von Sonntag 1987a). Most of the transient pyrimidine radicals formed (Fujita and Steenken 1981) and the resulting diamagnetic products have been characterized, especially in the case of thymine and thymidine (Téoule and Cadet 1978). On the other hand, information on the radical reactions of purine bases and nucleosides mediated by hydroxyl radicals in the presence of oxygen (O'Neill 1983; Cadet and Berger 1985) has been scarcer, at least until recently (Steenken 1989). It should be added that relevant structural information on OH· radical adducts to pyrimidine and purine nucleic acid components has been obtained from electron spin resonance/spin-trapping investigations (Hiraoka et al. 1990; Davies et al. 1995). The main hydroxyl radical-mediated oxidation reactions of the base moiety of the four major purine and pyrimidine bases of 2'-deoxyribonucleosides and isolated DNA in aerated aqueous solutions are reviewed in the present survey. These include the kinetic and mechanistic aspects of the transient radicals formed and the structural features of the resulting diamagnetic products.

2.1.1
Thymine

The main available information on the hydroxyl radical-mediated oxidation reactions of the thymine residue mostly relies on characterization of the bulk of the radiation-induced decomposition products of thymidine (1)

in aerated aqueous solutions. In particular, the structural assignment was achieved on the basis of extensive nuclear magnetic resonance (NMR) and mass spectrometry analyses. About 50% of the total decomposition products were identified as hydroperoxides that may be completely separated by high-performance liquid chromatography (HPLC) and detected specifically with a post-column derivatization method (Wagner et al. 1990a) These include the *cis* and *trans* diastereoisomers of 6-hydroperoxy-5-hydroxy-5,6-dihydrothymidine (8) and 5-hydroperoxy-6-hydroxy-5,6-dihydrothymidine (9) in addition to 5-(hydroperoxymethyl)-2'-deoxyuridine (10) (Wagner et al. 1987, 1994). In addition, the bulk of the stable decomposition thymidine products which arise from both the decomposition of the above hydroperoxides 8–10 and the fate of the related transient peroxyl radicals 5–7 have been isolated and characterized. These include (in decreasing order of quantitative importance): *N*-(2-deoxy-β-D-*erythro*-pentofuranosyl) formamide (11) > the four *cis* and *trans* diastereoisomers of 5,6-dihydroxy-5,6-dihydrothymidine (12) > the 5R* and 5S* forms of 1-(2-deoxy-β-D-*erythro*-pentofuranosyl)-5-hydroxy-5-methylhydantoin (13) > 5-(hydroxymethyl)-2'-deoxyuridine (15) > and 5-formyl-2'-deoxyuridine (16) (Cadet et al. 1975, 1979, 1981).

A comprehensive mechanism for the OH· radical-mediated decomposition of the pyrimidine ring of thymidine (1) (Fig. 1) may be proposed from both the pulse radiolysis results on the transient radicals (Fujita and Steenken 1981; Jovanovic and Simic 1986) and the quantitative balance of the resulting final diamagnetic compounds. The main reaction of the OH·radical with the thymine moiety of 1 is the addition at carbon C-5 (60%). This leads to the generation of the reducing radical 2 as inferred from its easy oxidation by tetranitromethane. It was also recently shown that dehydration of radical 2, prepared by photolysis from (5R,6S)-5-hydroxy-6-[*m*-(trifluoromethyl)benzoyl]-5,6-dihydrothymidine, into the radical cation 50 is at the most a minor process (Barvian et al. 1995). The carbon C-6 constitutes the second most important site (35%) for the reaction of the OH· radical. The resulting adduct, the 6-hydroxy-5,6-dihydrothymid-5-yl radical (3), exhibits oxidizing properties, since it is reduced by tetra-*N*-methyl-*p*-phenylenediamine. The latter reaction, which is a minor process (5%), consists in a hydrogen abstraction from the methyl group, giving rise to 4. In a subsequent step, the primary radicals 2–4 react with molecular oxygen at diffusion-controlled rates (Isildar et al. 1982), producing the corresponding peroxyl radicals (5–7).
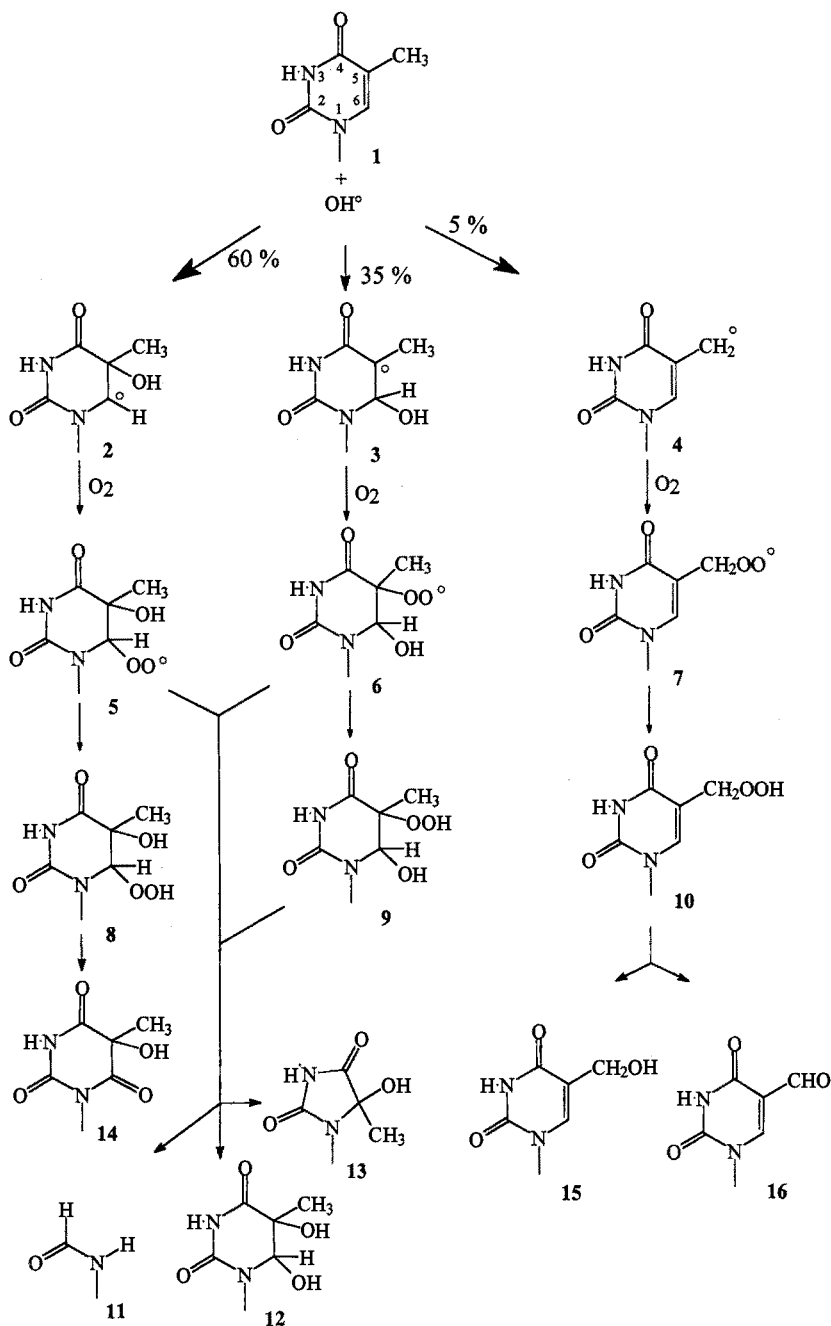
Fig. 1. Main OH·-mediated oxidation reactions of the base moiety of thymidine (1) in aerated aqueous solution

About half of the latter radicals are converted into hydroperoxides 8–10 after a key step of reduction by superoxide radicals (Wagner 1988; Wagner et al. 1990b). The half-lives of the hydroperoxides have been found to vary from a few days for **8** and **9** to 1 week for 10 in neutral aqueous solutions at 37°C. The hydrolytic decomposition of the thymidine hydroperoxides **8** and **9** is quite specific (Wagner et al. 1994). In this respect, 1-(2-deoxy-β-D-*erythro*-pentofuranosyl)-5-hydroxy-5-methylbarbituric acid (14) is the main decomposition product of **8** whereas the 5R* and 5S* diastereoisomers of 1-(2-deoxy-β-D-*erythro*-pentofuranosyl)-5-hydroxy-5-methylhydantoin (13) are predominantly generated from the thermal degradation of **9**. It should be added that a competitive dismutation reaction of peroxyl radicals 5–7 is likely to generate 11–13 and 15 through the transient formation of highly reactive oxyl radicals (von Sonntag 1987a). The latter radicals may abstract a hydrogen atom from a suitable donor to produce diols and/or undergo a β-scission reaction with subsequent formation of pyrimidine ring opening and rearrangement products, including 11 and 13.

In the last decade, relevant information on the OH·-mediated oxidation reactions of thymine was gained from radiation-induced decomposition studies of dinucleoside monophosphates and short oligonucleotides. Most of the work has involved isolation by HPLC of the main degradation products and their characterization by comparison of their ^{1}H NMR features with those of related radical oxidation products of thymidine. These include the *cis* and *trans* diastereoisomers of 5,6-dihydroxy-5,6-dihydrothymidine (12), N-(2-deoxy-β-D-*erythro*-pentofuranosyl) formamide (11), the 5R* and 5S* forms of 1-(2-deoxy-β-D-*erythro*-pentofuranosyl)-5-hydroxy-5-methyl-hydantoin (13) and the two oxidation products of the methyl group 15,16. The latter compounds have been also shown to be generated within isolated DNA upon exposure to OH· radicals in aerated aqueous solutions.

The measurement of the thymine oxidation products has required either acidic hydrolysis or enzymatic digestion of modified DNA, leading to the release of modified bases and nucleosides, respectively (Cadet and Weinfeld 1993). The formation of 5,6-dihydroxy-5,6-dihydrothymine (12a) and 5-hydroxy-5-methylhydantoin (13a) was observed in [^{14}CH$_3$]thymine DNA after exposure to ionizing radiation in aerated aqueous solution and subsequent mild acidic hydrolysis (Téoule et al. 1977; Téoule 1987). Similar information was gained from the gas chromatography-mass spectrometry (GC-MS) analysis of the formic acid hydrolysate of γ-irradiated DNA (Dizdaroglu and Bergtold 1986; Fuciarelli et al 1989). However, as will be

discussed later, the quantitative measurement of the latter assay suffers from severe drawbacks. Enzymatic digestion of oxidized DNA involving the use of DNases, phosphodiesterases, and phosphatases represents an interesting alternative. (5S,6R) and (5R,6S)-5,6-dihydroxy-5,6-dihydro-thymidine (12) were enzymatically released from [C^{3}H$_3$]thymine-DNA which was previously exposed to γ-radiation in aerated aqueous solution. The two thymidine glycols were separated by HPLC on a octadecylsilyl silicagel (ODS) column and then subjected to an acetylation reaction. The corresponding tri-O- and tetra-O-acylated derivatives were again separated by HPLC and their formation quantitated (Teebor et al. 1987). Interestingly, it was found that the two *cis* diastereoisomers are generated with the same radiochemical yield (G = 0.002), thereby showing the absence of stereospecificity in the formation of these oxidized nucleosides.

The same approach was used to detect and measure the radiation-induced formation of 5-(hydroxymethyl)-2'-deoxyuridine (15) within DNA in aerated aqueous solution (Frenkel et al 1985, 1991). It should be added that 5-formyl-2'-deoxyuridine (14) was also characterized in DNA following exposure to hydroxyl radicals (Kasai et al. 1990). However, the enzymatic approach may suffer from some limitations, in particular when highly modified nucleosides have lost their aromatic nature. This is the case for N-(2-deoxy-β-D-*erythro*-pentofuranosyl) formamide (11) which was found to resist hydrolysis of the vicinal phosphodiester bond by spleen phosphodiesterase and nuclease P1 (Cadet and Voituriez 1979; Maccubbin et al. 1991, 1992a,b; Budzinski et al. 1992). Indirect evidence for the radiation-induced formation of formylamine (11a) within DNA was provided by the measurement of pyruvamide in the irradiated aerated aqueous solution (Téoule et al. 1974).

2.1.2
Cytosine

As observed for the pyrimidine ring of thymidine (1), the main reaction of OH· radical with 2'-deoxycytidine (dCyd) (17) is the addition across the 5,6-ethylenic bond. The reducing 5-hydroxy-5,6-dihydro-2'-deoxycytidyl-6-yl (18) was found to be preferentially formed, as inferred from the results of pulsed radiolysis experiments using the redox titration method (Hazra and Steenken 1983). On the other hand, the addition of the OH· radical in position 6 occurred as a minor process (10%). In a subsequent step, as for 1, molecular oxygen reacts at diffusion controlled rates with 5-hydroxy-5,6-

dihydro-2'-deoxycytidyl-6-yl (19) and 6-hydroxy-5,6-dihydro-2'-deoxy-cytidyl-5-yl radicals (18), (Decarroz 1987), yielding the corresponding per-oxyl radicals 20,21. Attempts to isolate and characterize dCyd hydroxyhy-droperoxides 22,23 were unsuccessful (Wagner et al. 1990c). This may be accounted for by the instability of the latter hydroperoxides 22,23 and their ability to undergo intramolecular cyclization (see below).

The formation of N-(2-deoxy-β-D-*erythro*-pentofuranosyl) formamide (11), the 5R and 5S forms of N1-(2-deoxy-β-D-*erythro*-pentofuranosyl)-5-hydroxyhydantoin (27) and the four *cis* and *trans* diastereoisomers of 5,6-dihydroxy-5,6-dihydro-2'-deoxyuridine (25) implies, at least partially, a mechanism similar to that proposed for the conversion of peroxyl radicals of thymidine 5,6. It should be noted that 25 results from the hydrolytic deamination of the corresponding 5,6-dihydroxy-5,6-dihydro-2'-deoxy-cytidine (24) (Polverelli and Téoule 1974). In addition, the latter nucleo-sides 24 may undergo a competitive dehydration reaction, giving rise to 5-hydroxy-2'-deoxycytidine (26) which is a specific oxidation product of dCyd (17).

Other oxidized nucleosides whose formation involves rearrangement mechanisms (Fig. 3) specific for 17, include the two *trans* diastereoisomers of N-(2-deoxy-β-D-*erythro* -pentofuranosyl)-1-carbamoyl-4,5-dihydro-2-oxoimidazolidine (30), the α and β furanosidic and pyranosidic anomers of N-(2-deoxy-β-D-*erythro*-pentosyl) biuret (31) and N-(2-deoxy-β-D-*erythro*-pentofuranosyl) N4-ureidocarboxylic acid (33) (Wagner et al 1990c; Cadet 1994). The formation of the latter three classes of modified nucleosides is explained by the transient formation of an endoperoxide 28 through the cyclization of 6-hydroperoxy-5-hydroxy-5,6-dihydro-2'-de-oxycytidine (23). This was inferred from the results of isotopic labeling experiments showing that an atom of [18]O was incorporated in the car-bamoyl group of the two imidazole type derivatives upon exposure of dCyd (17) to ionizing radiation in aqueous solution saturated with a stream of [18]O$_2$ (Decarroz 1987; Wagner 1988). The transient nucleoside 29 may give rise through cyclization to the two *trans* diastereoisomers of 30 which have been shown to interconvert between each other. The acyclic compound 29 may also undergo a competitive aldol-ketol isomerization, leading to nu-cleoside 32. The latter ketol is the likely precursor of the carboxylic deriva-tive of biuret nucleoside 33 through a series of reactions which involve cyclization and subsequent hydrolysis of the resulting intermediate (Fig. 3).
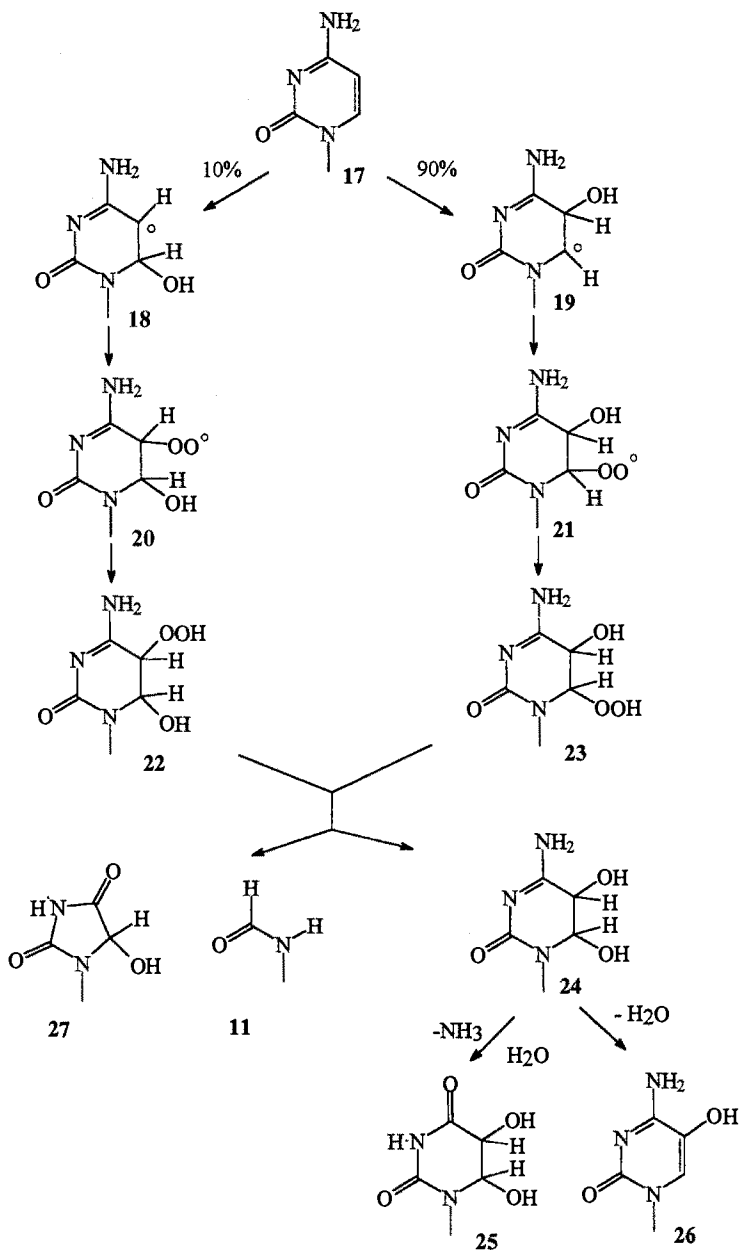
Fig. 2. Oxidation reactions of the base moiety of 2'-deoxycytidine (17) by hydroxyl radicals in aerated aqueous solution: pathways involving the thermal decomposition of 5(6)-hydroxy-6-(5)-hydroperoxides 22, 23
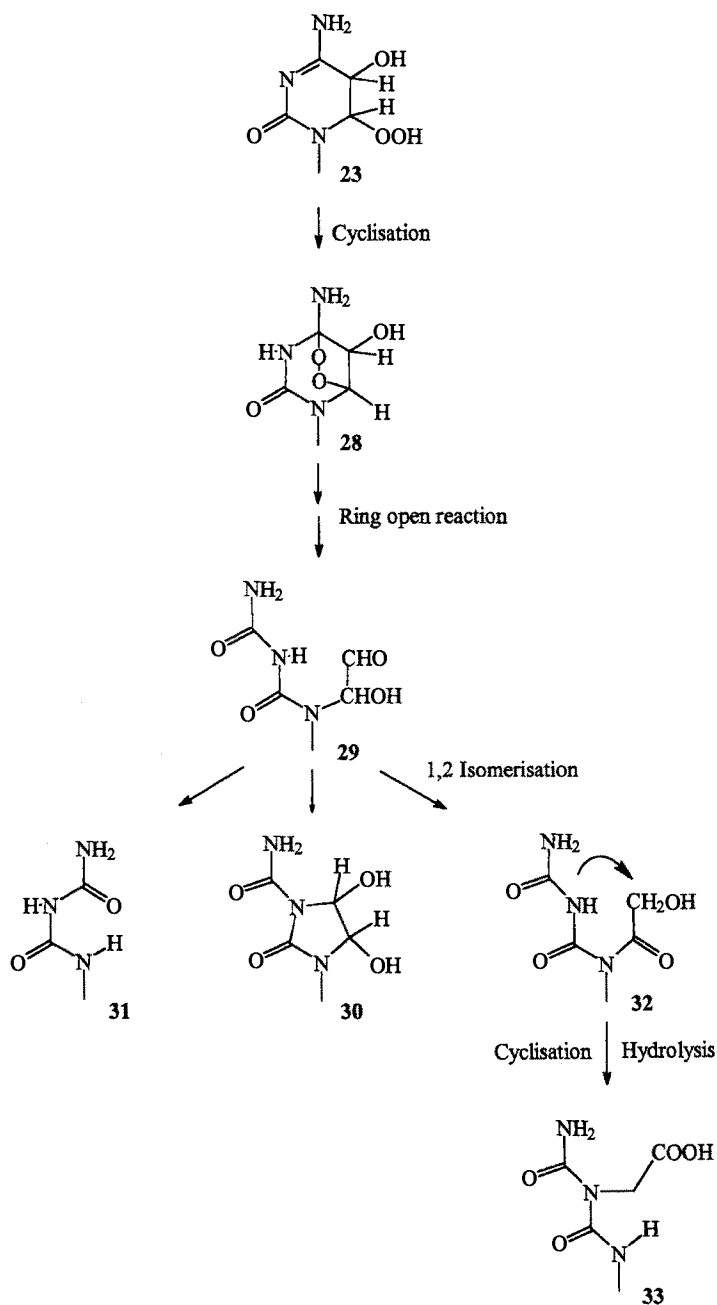
Fig. 3. Oxidation reactions of the base moiety of 2'-deoxycytidine (17) by hydroxyl radicals in aerated aqueous solution: pathways involving the intramolecular cyclization of 5-hydroxy-6-hydroperoxy-5,6-dihydro-2'-deoxycytidine (23)

The only available accurate information on the formation of OH·-mediated oxidation of cytosine bases within isolated DNA deals with the measurement of 5-hydroxy-2'-deoxycytidine 26 and 5-hydroxy-2'-deoxyuridine (Wagner et al. 1992). This was achieved by applying a HPLC-electrochemical detection assay to the enzymatic hydrolysate of oxidized DNA.

2.1.3
Adenine

The rate constants for the reaction of OH· radicals with purine nucleosides are higher than those observed with thymidine (1) and dCyd (17) (Steenken 1989). However, the latter reactive oxygen species are less effective in inducing the decomposition of 2'-deoxyadenosine (dAdo) (34) and 2'-deoxyguanosine (dGuo) (42) than that of their pyrimidine homologues. This may be explained by the occurrence of efficient restitution reactions that involve the initially generated purine radicals upon addition of OH· radicals. It has been shown that molecular oxygen reacts very slowly with most of the latter purinyl radicals and those which may derive from them (Willson 1970; Isildar et al. 1982), thereby enabling bimolecular radical reactions to take place. This contrasts with the chemistry of OH·-mediated formation of pyrimidine radicals, for which restitution reactions are only observed under anoxic conditions. 8-Oxo-7,8-dihydro-2'-deoxyadenosine (40) has been characterized as the main decomposition product of the reaction of hydroxyl radicals with dAdo (34) in aerated aqueous solution (Mariaggi and Téoule 1976). A reasonable mechanism for the formation of 40 (Fig. 4) involves initial addition of a hydroxyl radical to the C-8, giving rise to the reducing 8-hydroxy-7,8-dihydro-2'-deoxyadenos-7-yl radical (35) (Steenken 1989; Vieira and Steenken 1990). Oxidation of the latter intermediate 35 is expected to generate 40. On the other hand, the formamidopyrimidine nucleoside (41) which mostly exists in aqueous solution as a mixture of α and β pyranosidic isomers (Raoul et al. 1995), arises from the reduction of the same precursor 35. This explains why formamidopyrimidine nucleoside 41 is not generated, at least in detectable amounts, in aerated aqueous solution of 34 exposed to OH· radicals.

It has been proposed, on the basis of pulse radiolysis experiments (Viera and Steenken 1987), that OH· radicals also add significantly to the C-4 position of the adenine moiety, generating the radical 36. In a subsequent step, the latter intermediate is converted into the aminyl radical 37 through
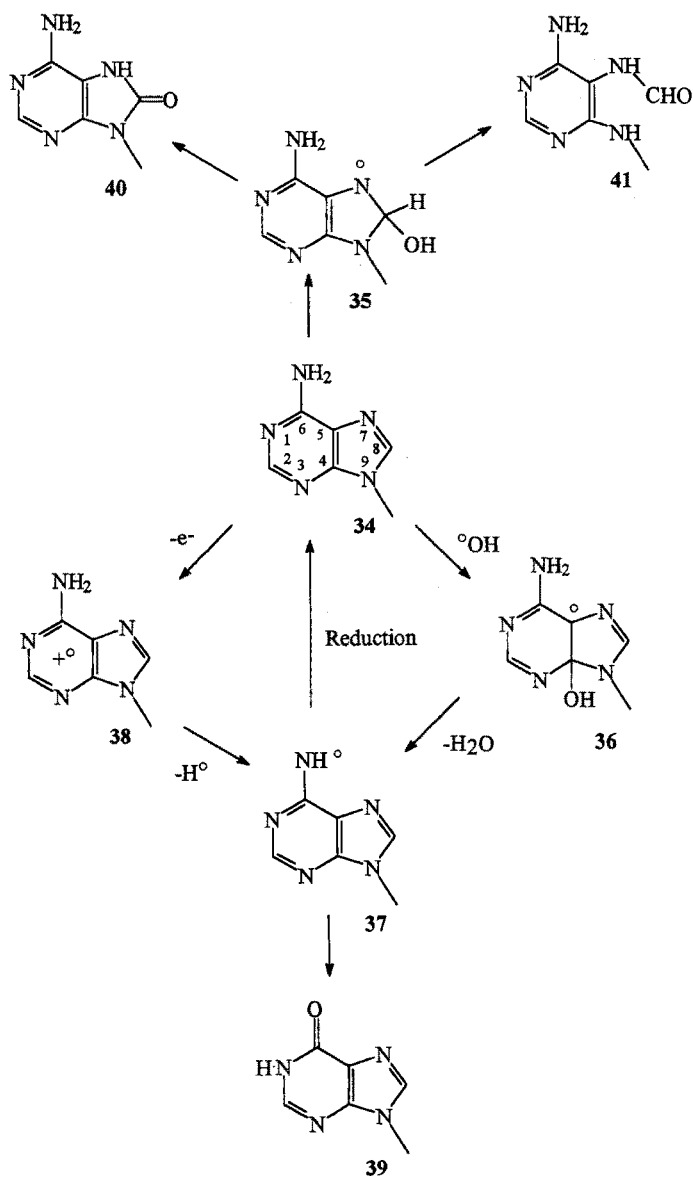
Fig. 4. Main decomposition pathways of 2'-deoxyadenosine (34) upon OH· radical and one-electron oxidation reactions in aerated aqueous solution

an efficient dehydration reaction (Steenken 1987). However, it should be noted that 2'-deoxyinosine (39), the main final transformation product of transient oxidising radical 37, is not generated by γ-irradiation of aerated aqueous solution of 34 (S. Raoul and J. Cadet, unpublished results). This may be explained by the occurrence of efficient restitution reactions between radicals 35 and 37.

There is still a paucity of information on the formation of modified adenine residues within DNA upon exposure to agents of oxidative stress. One of the major exceptions deals with the measurements by HPLC of radiolabeled 8-oxo-7,8-dihydroadenine (40a) and 4,6-diamino-5-formamidopyrimidne (41a) after exposure of [^{14}C]adenine-DNA to ionizing radiation in aerated aqueous solution (Bonicel et al. 1980). It shoud be also mentioned that the formation of 40 has been recently monitored in DNA using a HPLC assay (Berger et al. 1990) following exposure to hydroxyl radical and peroxynitrite (Douki and Cadet 1996).

2.1.4
Guanine

The first striking observation concerns the fact that 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxodGuo) (47) is only produced at trace levels in the reaction of hydroxyl radicals with dGuo (42) in aerated aqueous solutions. This contrasts with the efficient OH·-mediated formation of 8-oxo-7,8-dihydro-2'-deoxyadenosine (40), although the radical precursor 43 of 8-oxodGuo (47) is generated with a yield close to 25% (Steenken 1989). Again, this observation may be rationalized in terms of efficient restitution reactions involving both the reducing radical 43 and the oxidizing intermediate 45. The latter radical, which arises from the dehydration of the predominant OH· radical adduct at C-4 carbon 44 with a rate constant of $k=5 \times 10^3$ s^{-1} (Candeias and Steenken 1991), is the precursor of the two overwhelming OH· oxidation products of 42. The two modified nucleosides have been characterized as 2-amino-5-[(2-deoxy-β-D-erythro-pentofuranosyl)-amino]-4H-imidazol-4-one (49) and 2,2-diamino-4-[(2-deoxy-β-D-erythro-pentofuranosyl)amino]-5-(2H)-oxazolone (50) on the basis of extensive NMR and mass spectrometry measurements (Cadet et al. 1994a; Raoul et al. 1996). It should be added that the imidazolone 49 slowly converts into the oxazolone 50 in aqueous solution with a half-life of about 10 h at 20°C and at neutral pH. As already mentioned, the oxidizing purinyl radical 45 and, more likely, the related C-5 carbon centred tautomeric form (von Sonntag

1987a; Steenken 1989; Cadet et al 1994a) are the precursors of 49 and 50. Molecular oxygen was found to react with one of these intermediates, as inferred from the incorporation of one atom of ^{18}O in both 49 and 50 upon exposure of 42 to OH· radicals in aqueous solutions saturated with $^{18}O_2$ (Cadet et al. 1994a). It should be remembered that the addition of molecular oxygen to oxidizing 2'-deoxyguanosyl radicals is a slow reaction ($k \ll 107 \, M^{-1} \, s^{-1}$) that could not be measured by pulsed radiolysis (Steenken 1989). This also explains why several antioxidants, including ascorbic acid, serotonin, uric acid, and tetramethyl-p-phenylenediamine, are able to reduce oxidizing purine radicals *via* an electron transfer mechanism (O'Neill 1983; O'Neill and Chapman 1985; Simic 1988; Candeias and Steenken 1989). In particular, the formation of both the imidazolone 49 and oxazolone 50 was abolished upon exposure of dGuo (42) to OH· radicals in the presence of 1 mM ascorbic acid (Mouret et al. 1991a).

The formation of oxazolone 50 is still predominant within DNA exposed to OH· radicals in aerated aqueous solution. Interestingly, it should be noted that 8-oxodGuo (47) which was barely detected upon OH·-mediated oxidation of dGuo (42) is generated in significant amounts within DNA. The critical factors (e.g., presence of bound metals, sructural parameters) which are involved in the change of product distribution when moving from the nucleoside or short oligonucleotide to double-stranded DNA are not clear.

2.1.5
Clustered Base Damage

Increasing interest is currently being devoted to the determination of the biological role of bulky lesions induced in cellular DNA by various agents of oxidative stress. Locally multiple damage sites (LMDS), which may include double-strand DNA breaks and clustered base lesions, are likely to contribute significantly to the deleterious effects of ionizing radiation (Ward 1991, 1994, 1995; Brenner and Ward 1992). However, there is a still of paucity of information on such damage, whose formation may be explained by the generation of a high concentration of DNA radicals and related excited species along the track of ionizing radiation (Goodhead 1994). Evidence for the endogenous formation of bulky DNA lesions, so-called I (indigenous) compounds, has been inferred from their peculiar chromatographic behavior, as revealed by ^{32}P-postlabeling analysis. It was recently shown that the formation of the type II class of the latter modifi-

cations, which is mostly mediated by cellular oxidative processes, is increased by Fenton-type reagents in cells (Randerath et al. 1991; Chang et al. 1993) and by ferric nitriloacetate (Fe-NTA) on kidney DNA of male F344 rats (Randerath et al. 1995). Related bulky adducts were detected by a ^{32}P-postlabeling assay in the liver of patients with Wilson disease and primary hemochromatosis (Carmichael et al. 1995). However, these lesions were found to be different from the putative purine intrastrand adducts which were generated in isolated DNA upon incubation with Cu^+/Fe^{2+} and H_2O_2 (Carmichael et al. 1992). It has also been suggested that such oxidative DNA bulky lesions, whose structure still remains to be determined, are likely to be refractory to DNA repair processes in cancer-prone syndromes such as xeroderma pigmentosum (Lindahl; 1993, Satoh et al. 1993; Satoh and Lindahl 1994).

The first example of the OH·-mediated formation of a vicinal oxidative base lesion within a DNA fragment was recently provided by conducting a model study on 2'-deoxyguanosylyl-(3'-5')-thymidine (dGpT). X-irradiation of dGpT in aerated aqueous solution was found to give rise to a dinucleoside monophosphate bearing a clustered base damage as a primary radiation-induced decomposition product (Box et al. 1993). The guanine moiety has been converted to 8-oxo-7,8-dihydroguanine,(47a) whereas the thymine base has been transformed into a formylamine product. An identical decomposition product is formed in a dinucleoside monophosphate where the thymine base is replaced by a cytosine residue (Budzinski et al. 1993). Similar clustered base damage, but in an opposite sequence, has been isolated and characterized following exposure of dGpN dinucleoside monophophates, where G is a dGuo residue, whereas N may be either thymidine (1) or 2'-deoxycytidine (17) (Box et al. 1995). The exact mechanism of the formation of these two OH·-mediated vicinal base lesions, which were also found to occur within the tetranucleotide d(CpGpTpA) (Budzinski et al. 1995), is still open to debate. One possibility which is currently being investigated in our laboratory is that initial OH· addition to the pyrimidine base may, through the corresponding hydroxyhydroperoxide or the peroxyl radical precursor, lead to the radical oxidation of the vicinal guanine base. Attempts are also currently being made to insert such clustered damage in oligonucleotides in order to further investigate the biological properties of such bulky lesions (mutagenesis, repair).

2.2
Fenton Reactions and Other Metal-Mediated Oxidation Reactions

Fenton reactions appear to play a major role in the biological effects mediated by hydrogen peroxide within cells. However, the exact nature of the oxidizing species involved in the latter reactions is still a matter of debate (Yamazaki and Piette 1991; Wink et al. 1991a; Sawyer et al 1993). Free hydroxyl radicals and high-valency iron–oxo species may be generated in the reaction of H_2O_2 with ferrous iron, depending on the concentration of hydrogen peroxide, the pH, and the chemical nature of the chelating agents. Evidence was provided that hydroxyl radical is the reactive oxygen species of the reaction of $[Fe^{II}EDTA]^{-2}$ and H_2O_2 in the absence (Gilbert et al. 1988) and in the presence of ascorbate (Pogozelski et al. 1995). The generation of the main oxidizing species of the Udenfriend reaction requires oxygen instead of H_2O_2 (Ito et al. 1993a). The main oxidation product of the reaction of thymine with the latter reagent was identifed as N-formyl-N'-pyruvylurea. This is in agreement with previous results on the OH·-mediated decomposition of thymine (Téoule and Cadet 1978). On the other hand, iron(II)-bleomycin is expected to cleave DNA (Hecht 1986) by a mechanism involving an iron–oxo complex (Stubbe and Kozarich 1987). Other metals, including Cu(I), Co(II), Cr(V), and Ni(II), which exhibit carcinogenic activity (for a review, see Kasprzak 1991) are able to generate various reactive oxygen species in the presence of H_2O_2 (Yamamoto and Kawanishi 1989; Lefebvre and Pézerat 1992; Pézerat 1994; Shi et al. 1994a). In addition, several other redox systems such as Cu-1,10-phenanthroline complex (Yoon et al. 1990), metalloporphyrins in the presence of suitable oxygen donors (Meunier 1992) or peroxynitrite (Groves and Marla 1995), Fe(III) nitrilotriacetate (Inoue and Kawanishi 1987), and asbestos (Leanderson et al. 1988; Berger et al. 1993; Faux et al. 1994) have been shown to oxidize DNA. It was recently reported that a bithiazole derivative related to the reactive moiety present in bleomycin is capable to mediate guanine-specific DNA alkali-labile lesions in the presence of oxygen (Kane et al. 1995). An inner-sphere mechanism rather than a one-electron process has been suggested to be involved in the radical oxidation of the guanine residues.

Insights into the mechanism of Fenton reaction-mediated oxidation of the base moiety of purine 2'-deoxyribonucleosides were gained from the isolation of the main decomposition products of the reactions. Under these conditions, the formation of 8-oxodGuo 47 was found to be significantly

increased at the expense of the oxazolone 50 and imidazolone 49 nucleo-
sides (Mouret et al. 1991a). It was shown that the presence of Fe(II), which
is likely to reduce the oxidizing guanilyl radical 45, responsible for this
major change in the oxidation product distribution of dGuo (42). It should
be noted that an Udenfriend reaction has been used to prepare 47(Kasai
and Nishimura 1984). Interestingly, an opposite effect of Fe(II) on the
formation of 8-oxo-7,8-dihydro-2'-deoxyadenosine 40 was observed
(Mouret et al. 1991a). In this respect, 40 which is the main OH·-mediated
oxidation of dAdo (34) only generated in trace amounts in the Fenton
reaction. Oxidation of nucleosides 34 and 42 by several types of asbestos
led to similar observations (Berger et al. 1993). Differences in the ratio of
47 to 40 may be explained in terms of accessibility of Fe(II) in the minerals
(Cadet et al. 1994a). The presence of Fe(II) has been shown to promote the
formation of 8-oxodGuo (47) within DNA upon exposure to OH· radicals
in the presence of either ascorbate or 5-aminosalicylic acid (Fischer-Niel-
sen et al. 1992). However, such an enhancing effect was not observed when
glutathione was added to the solution. A significant increase in the level of
8-oxodGuo (47) was observed in liver DNA of mice fed with the polychlori-
nated biphenyl mixture Aroclor 1254 upon a single injection of iron (Faux
et al. 1992).

2.3
One-Electron Oxidation Reactions

One-electron oxidation of purine and pyrimidine nucleic acid bases leads
to the formation of the related radical cations. The increasing interest in
the determination of the chemical reactions of the latter highly reactive
intermediates is explained by their implication in several major physico-
chemical processes, including the direct effects of ionizing radiation (Bern-
hard 1981; Symons 1987), the type I photosensitization mechanism (Cadet
et al. 1983a; Cadet and Vigny 1990), and the mono- and biphotonic pho-
toionization mediated by high-intensity UV laser pulses (Nikogosyan 1990;
Bothe et al. 1990a; Angelov et al. 1991; Candeias and Steenken 1993). It is
also now well-documented that pyrimidine and purine radical cations can
be generated through the oxidizing reaction of inorganic radicals such as
Br_2^- (Willson et al. 1974), SO_4^- (von Sonntag 1987b; Schuchmann et al.
1987; von Sonntag et al. 1989; Bothe et al. 1990b; Deeble et al. 1990), and
Tl(II) (Jovanovic and Simic 1989) with DNA constituents. However, it was
shown that the use of inorganic radicals to generate one-electron oxidation

products of purine and pyrimidine nucleosides gave rise to the formation of secondary decomposition products, particularly in the case of $SO_4^{\cdot-}$ (Cadet et al. 1991) and $Br_2^{\cdot-}$ (Cadet et al. 1983b). It should be added that the bulk of the studies aimed at determining the mechanisms of the reactions of purine and pyrimidine radical cations in aqueous solutions have involved type I photosensitizers. In this respect, it should be mentioned that the use of frozen aqueous systems to study the direct effects of ionizing radiation presents a major limitation, since the role of oxygen cannot be studied in this system (Shaw and Cadet 1990). Two main competitive reactions, including hydration and deprotonation, are involved in the conversion of purine and pyrimidine radical cations in aqueous solution (see below). The relative importance of deprotonation over hydration in neutral aqueous solutions increases in the following order: thymidine (1) = dCyd (17) < dAdo (34) < dGuo (42), in proportion to the increasing value of the electron density of the corresponding bases. It should be added that the ability for photosensitizers to oxidize nucleosides *via* one-electron oxidation is directly related to the ionization potential of the DNA components with the following order of decreasing reactivity: dGuo (42) > dAdo (34) > dCyd (17)=thymidine (1).

2.3.1
Thymine

Upon exposure to UVA radiation, photoexcited 2-methyl-1,4-naphthoquinone (menadione) was found to induce the formation of the radical cation of thymidine 51 and 2'-deoxycytidine 52 with high efficiency (Fisher and Land 1983). It is worth mentioning that the distribution of the main stable products of the one-electron reaction of menadione with thymidine (1) in aerated aqueous solution (Cadet et al. 1986; Decarroz et al. 1986) is similar to that initiated by hydroxyl radicals. One of the main differences deals with a significant increase in the relative yield of 5-(hydroxymethyl)-2'-deoxyuridine (15) and of 5-formyl-2'-deoxyuridine (16), together with a greater formation of 5-hydroperoxymethyl-2'-deoxyuridine (10). In addition, another major feature which concerns unstable thymidine (dThd) photoproducts is the complete absence of the four *cis* and *trans* diastereoisomers of 6-hydroperoxy-5-hydroxy-5,6-dihydrothymidine (8) (Wagner et al. 1987). Further support for the specific formation of the four *cis* and *trans* diastereoisomers of 5-hydroperoxy-6-hydroxy-5,6-dihydrothymidine (9) was provided by the results of isotopic labeling experiments. These showed

the lack of incorporation of ^{18}O in position 6 of the *cis* and *trans* glycols of thymidine 12 upon exposure of dThd (1) to photoexcited menadione in $^{18}O_2$ saturated aqueous solution (Wagner et al. 1987). In addition, spin-trapping (Murali Krishna et al. 1987) of the menadione-photosensitized formation of pyrimidine radicals shows the presence of 3 and 4 associated with the complete lack of 2. The mechanism of the menadione-mediated photooxidation of thymidine (1) may be rationalized in terms of the initial formation of the pyrimidine radical cation 51. Predominant hydration of 51 in neutral aqueous solution specifically gives rise to 6-hydroxy-5,6-dihydrothymidyl-5-yl radical (3) with an estimated yield of 70%. In addition, competitive deprotonation of 51 which exclusively occurs at the methyl group in a 30% yield, leads to the transient formation of 5-methyl-(2'-deoxyuridyl) radical (4) (Fig. 5). The mechanisms of formation of the diamagnetic products, following the reaction of molecular oxygen with pyrimidine radicals 3 and 4 are identical to those described for hydroxyl radicals (see above).

Photoexcited menadione has been recently used to generate thymine oxidation products within isolated DNA (Bjelland et al. 1994, 1995). In particular, 5-formyluracil (16a) and 5-(hydroxymethyl)-uracil (15a) were found to be produced through the likely deprotonation reaction of the initially produced thymine radical cation 51a. However, it should be mentioned that double-stranded DNA has to be denatured in order to enable the one-electron oxidation mediated by photoexcited menadione to occur.
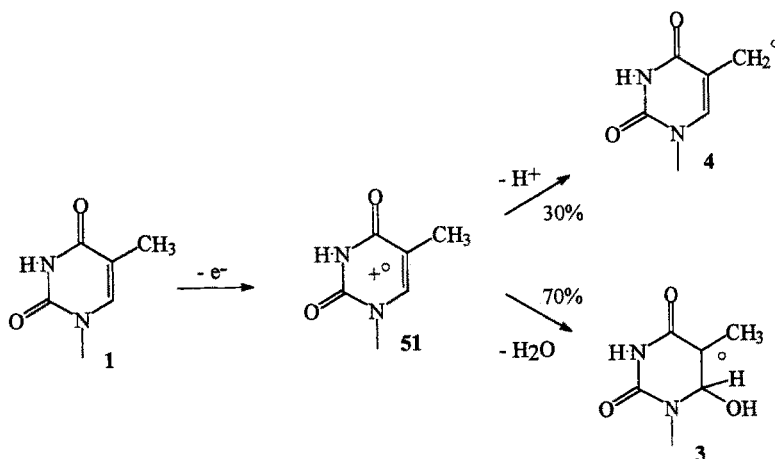
Fig. 5. Deprotonation and hydration of the pyrimidine radical cation of thymidine 51 in water

The formation of 5-formyluracil 16a was revealed by sequencing analysis, after hot piperidine treatment of deoxyoligonucleotides that had been photooxidized with a nitro derivative of lysine (Saito et al. 1995a).

2.3.2
Cytosine

The bulk of the menadione-photosensitized oxidation products of dCyd (17) are identical to those generated by the action of hydroxyl radicals (Wagner et al. 1990c), at least qualitatively, as observed for thymidine (1). The formation of the diamagnetic products may be explained in terms of initial generation of dCyd radical cation 52 through electron transfer to photoexcited menadione with subsequent hydration (83%) and deprotonation (17%) reactions. The former process was found to lead to a significant increase in the relative yield of the four *cis* and *trans* diastereoisomers of 5,6-dihydroxy-5,6-dihydro-2'-deoxyuridine (25) with respect to that observed in the OH·-mediated oxidation reactions of dCyd (17). Isotopic labeling experiments based on the incorporation of ^{18}O from molecular oxygen indicated that hydration of the radical cation occurs preferentially in position 6, giving rise to the oxidizing radical 6-hydroxy-5,6-dihydro-2'-deoxycytidyl-5-yl (18) (Wagner 1988). Two main sites of deprotonation (Fig. 6) within the pyrimidine nucleoside were inferred from the identification of specific diamagnetic photoproducts. One involves the exocyclic amino group in position 4 of the pyrimidine moiety. The resulting aminyl radical 53, or more likely the tautomeric N3-centred radical 54 may undergo deamination, leading to the specific formation of 2'-deoxyuridine (55) in a 12.5% yield (Decarroz et al. 1987). The second site of deprotonation involves the anomeric carbon of the sugar moiety. Addition of molecular oxygen to the resulting 1'-yl radical 56 leads to the formation of 2-deoxy-D-ribono-1,4-lactone (57) with the concomitant release of an equal quantity of cytosine (17a) (Decarroz et al. 1987). The latter process accounted for about 4.5% of the total photooxidation processes of the nucleoside (Decarroz et al. 1987).
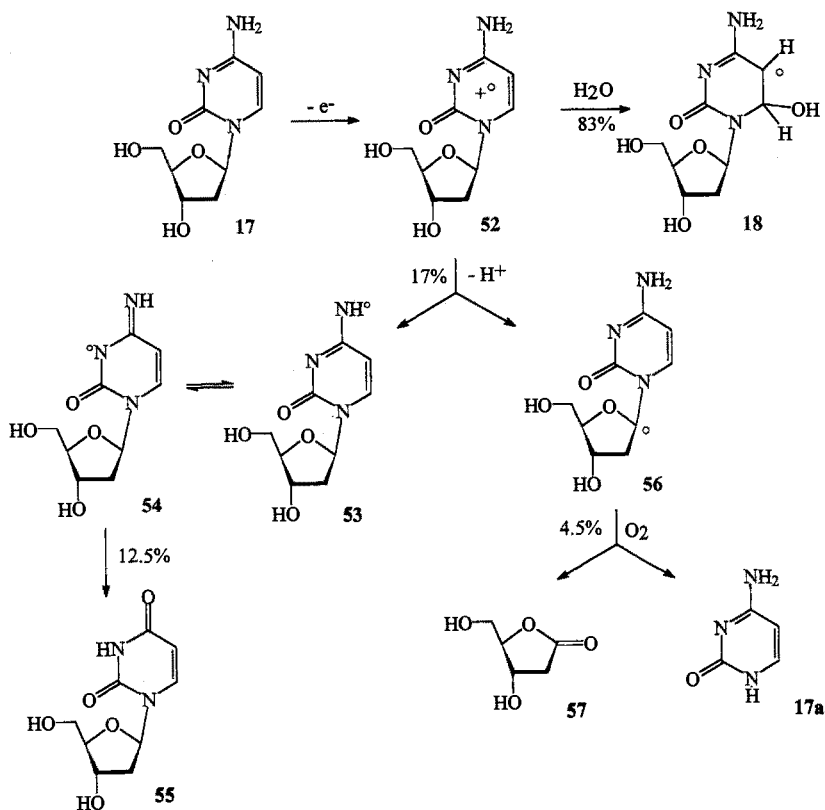
Fig. 6. Deprotonation and hydration reactions of the pyrimidine radical cation of 2'-deoxycytidine (52) in aerated aqueous solution

2.3.3
Adenine

Most common photosensitizers, including methylene blue, 3-carbethoxyp-soralen, hematoporphyrin derivatives, and proflavine, are not able to photooxidize dAdo (34) (Cadet et al. 1986). In contrast, riboflavin and benzophenone in their triplet state have been shown to oxidize dAdo (34) through initial electron transfer reaction, giving rise to the purine radical cation 38 (Fig. 4). The main final decomposition product of the photosensitized reaction in aerated aqueous solution has been identified as 2'-deoxyinosine (39). A likely mechanism for the formation of the latter nucleo-

side 39 is deprotonation of the dAdo radical cation 38 (Vieira and Steenken 1990; O'Neill et al. 1985; O'Neill and Davies 1987), leading to the oxidizing aminyl radical 37. The latter intermediate, or more likely the related N3-centred radical tactomer, is able to undergo deamination, giving rise to 39, resulting from the hydrolytic deamination of dAdo (34). Evidence was also provided for the occurrence to a smaller extent of a competitive hydration reaction of the radical cation 38. This leads to the formation of 8-oxo-7,8-dihydro-2'-deoxyadenosine (40) through the intermediary of the 8-hydroxy-7,8-dihydro-2'-deoxyadenos-7-yl radical (35) (Fig. 4).

2.3.4
Guanine

Most of the photodynamic agents which are used to oxidize dGuo (42) *via* one-electron reactions (type I mechanism) are able to generate singlet oxygen through an energy transfer mechanism (type II mechanism) (Cadet et al. 1983a, 1986). The latter process provides further complications, since dGuo (42) is an excellent substrate for $^1O_2$ (see below). However, several photosensitizers, including benzophenone and riboflavin, act primarily through charge transfer reaction with the substrate 42, giving rise almost quantitatively to the radical cation 46. Interestingly, the two main oxidation products of dGuo (42) which arise from the conversion of 46 in aerated aqueous solution are 2-amino-5-[(2-deoxy-β-D-*erythro*-pentofuranosyl)amino]-4*H*-imidazol-4-one (49) and 2,2-diamino-4-[(2-deoxy-β-D-*erythro*-pentofuranosyl)amino]-5-(2*H*)-oxazolone (50) (Cadet et al. 1994a; Raoul et al. 1996). The mechanism of formation of these modified nucleosides 49 and 50 (Fig. 7), which are also the predominant OH·-mediated oxidation products of 42, involves in the initial step deprotonation of the radical cation 46 as shown by pulsed radiolysis (Candeias and Steenken 1991). The resulting oxidizing oxyl radical 45 is identical to that generated through dehydration of the OH· radical adduct 44 in position 4 of the purine moiety of dGuo (42) (O'Neill 1983; Candeias and Steenken 1989; Fig. 7). The mechanism of formation of imidazolone 49, the precursor of oxazolone 50, was found to be rather complex. It involves the addition of both a molecule of oxygen and a molecule of water to a tautomeric isomer of 45, followed by a rearrangement of the purine ring. It should be noted that a decarboxylation reaction involving the C-6 carbon takes place during this multistage transformation of the guanine moiety. The competitive formation of (2S)-2,5'-anhydro-1-(2-deoxy-β-D-*erythro*-pentofuranosyl)-5-guanidinylidene-2-
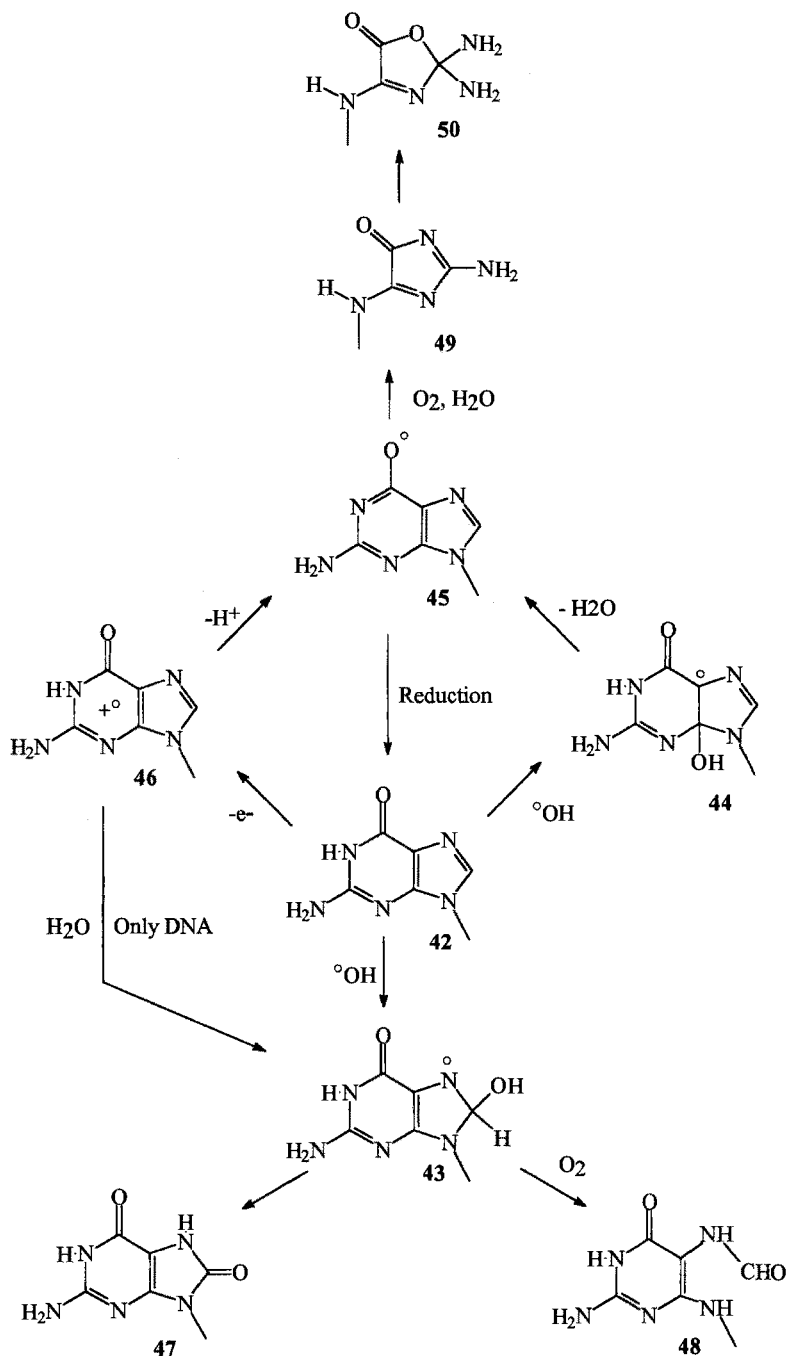
Fig. 7. Main decomposition pathways of 2'-deoxyguanosine (42) upon OH· radical and one-electron oxidation reactions in aerated aqueous solution

hydroxy -4-oxoimidazolidine (58) (Fig. 8; Buchko et al. 1993) provides further support for the implication of a nucleophilic reaction in the course of events leading to 49. This also constitutes an interesting model system to further investigate the photosensitized formation of DNA–protein cross-links (see below).

Another comment deals with the lack of significant formation of 8-oxodGuo 47, the expected final product of the hydration of the guanine radical cation 46 upon either benzophenone- or riboflavin-mediated pho-
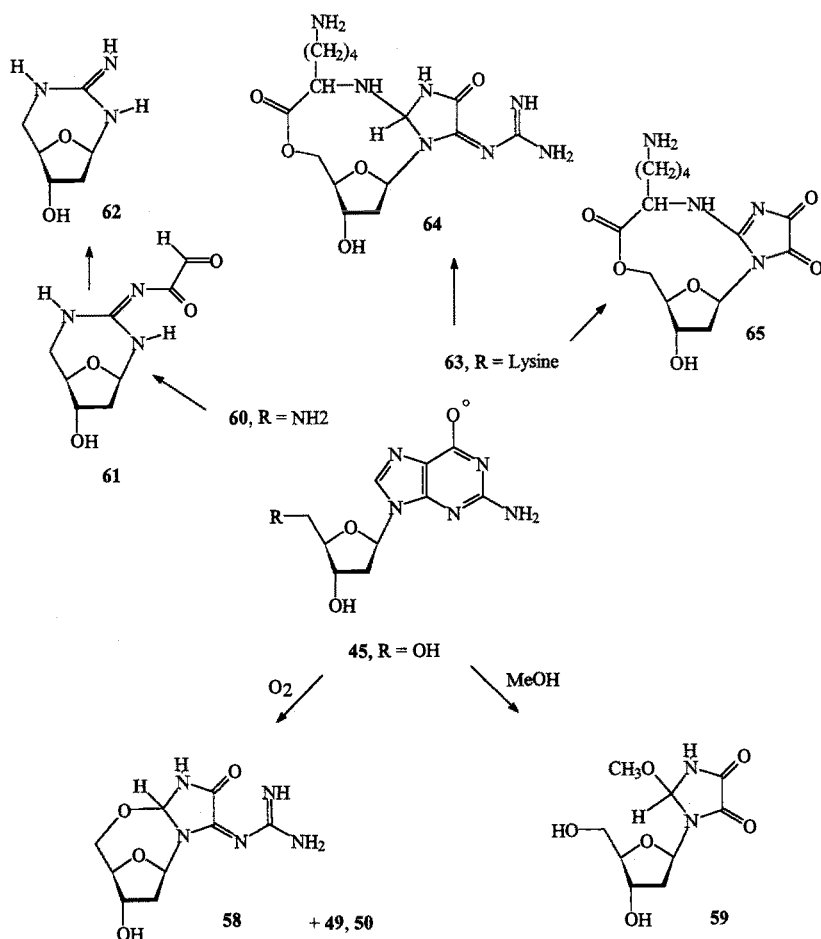
Fig. 8. Main adducts arising from nucleophilic addition and subsitution reactions of the oxidizing 2'-deoxyguanosyl radical 45. Model systems for DNA-protein cross-links

tosensitization of dGuo (42). This may be related to the low value of the
pKa of the radical cation 46,which was estimated to be 3.6. Interesting
information on the oxidizing properties of the oxyl radical 45 formed by
the deprotonation of the radical cation of guanine 46a in monocatenary
DNA of herring sperm was obtained by pulse radiolysis studies (Jovanovic
and Simic 1989). At neutral pH, the redox potential of radical 45a, whose
precursor 46a was generated by the reaction with thallic ion Tl(II), was
identical (E7 = 1.04 V) to that measured for the corresponding radical of
2'-deoxyguanosine 5'-monophosphate. Interestingly, the oxidizing radical
45a was quantitatively repaired by a number of electron donors, including
5-hydroxytryptamine, 5-hydroxytryptophan, tyrosine methyl ester hydro-
chloride, uric acid, ascorbic acid, and glycyl-tryptophan (Jovanovic and
Simic 1989).

The results concerning the chemical reactions of the guanine radical
cation 46a of dGuo and thymidylyl-(3'-5')-2'-deoxyguanosine (Buchko et
al. 1995a) cannot be completely transposed to double-stranded DNA. In
particular, the hydration reaction of 46a, which is completely absent within
dGuo 42 and short oligonucleotides, was found to be significant within
double-stranded DNA (Kasai et al. 1992). This was inferred from the
incorporation of one atom of ^{18}O from $H_2^{18}O$ in 8-oxo-7,8-dihydro-2'-de-
oxyguanosine (47), the final product of the reaction involving the transient
formation of radical 43 (Fig. 7). It should be added that deprotonation of
46, which is partly prevented within DNA (see above), gives rise (as ob-
served for dGuo 42 and dinucleoside monophosphates) to the formation
of both imidazolone 49 and oxazolone 50 compounds. The formation of
the latter oxidized nucleosides has been indirectly measured within DNA
by the release of guanidine, the quantitative alkali-induced decomposition
product of both 49 and 50 (Raoul et al. 1996). At the current stage of
available knowledge, it is possible to make a few general comments on the
radical reactions of the purine and pyrimidine bases of DNA and model
compounds. There are quite important similarities, at least in the qualita-
tive distribution of the decomposition products arising from OH· radicals
on one hand and one-electron oxidation on the other hand. This is particu-
larly true for the oxidation reactions involving guanine. In contrast, the
hydration reaction of the radical cation in the case of thymidine and dCyd
specifically led to the formation of the oxidizing radical (6-hydroxy-5,6-di-
hydro-6-yl), which was the minor addition product of the OH· radical. It
may be added that the distribution of oxidation products of pyrimidine
nucleosides is more complex than that of their purine homologues. How-

ever, the mechanisms of formation of the latter compounds, particularly for those involving dGuo (42), are much more complicated than that of dThyd (1) and dCyd (17). Another comment of importance concerns the observation of the major role played by the DNA structure in the orientation of radical oxidation of guanine (42a).

Interesting sequence effects on the photoionized formation of alkali-labile sites at guanine residues within deoxyoligonucleotides have been revealed by the use of electrophoretic analysis on denaturing polyacrylamide gels (Kovalsky et al. 1990; Ito et al. 1993b; Angelov et al. 1994; Melvin et al. 1995; Saito et al. 1995a). Most of the latter alkali-labile guanine lesions are likely to be the oxazolone damage 50 and its imidazolone precursor 49 as inferred from the indirect measurement of these modifications within DNA exposed to picosecond and nanosecond UV laser pulses (Angelov et al. 1994). In addition, the formation of 8-oxodGuo 47 whose conversion into piperidine-mediated DNA strand cleavage is of low efficiency (Chung et al. 1992), has been monitored in the latter photooxidized DNA. This was achieved by revealing alkali-labile sites generated by the removal of 47a after incubation of the oligonucleotides with the *Escherichia coli* formamidopyrimidine glycosylase (Boiteux 1993). One of the most striking sequence effects deals with the observation of a highly selective piperidine-induced cleavage at the 5'-side of adjacent guanine residues in duplex oligonucleotides that were photooxidized either with riboflavin (Ito et al. 1993a) or a lysine derivative possessing a naphthalimide chromophore (Saito et al. 1995a). This specific photoreaction was rationalized by the fact that guanines located 5' to a guanine residue constitute the most electron donating sites in duplex DNA. This was inferred from *ab initio* calculations of lowest ionization potentials of stacked nucleobases and flash photolysis studies (Saito et al. 1995b). Electron transfer has been suggested to occur within dinucleoside monophosphates and short oligonucleotides upon formation of pyrimidine and purine radical cations upon one-quantum photoionization by 193-nm laser pulses (Candeias and Steenken 1993; Melvin et al. 1995). However, more direct evidence is required to further assess whole migration in DNA aqueous solution at room temperature.

2.3.5
Guanine–Amino Acid Adducts

DNA–protein cross-links constitute an important class of damage pro-
duced by ionizing radiation and photoexcited sensitizers. However, there
is a paucity of information on the chemical structure of DNA–protein
cross-links generated under oxic conditions. One major exception deals
with the characterization of a tyrosine–thymine adduct (Margolis et al.
1988), which, however, was found to be significantly produced only in the
absence of oxygen. The observation of the occurrence of a nucleophilic
reaction in the radical oxidation of dGuo (42) (Buchko et al. 1993; Cadet et
al. 1994a) has allowed a working hypothesis to be proposed for the forma-
tion of at least one class of radically induced DNA cross-links. This is based
on the assumption that either the free hydroxyl group or the extra-amine
function which is not engaged in the peptide bond of certain amino acids
may react with guanine radicals produced by one-electron oxidation. In
this respect, methanol, which was used to mimic the hydroxyl group of
threonine and serine, was found to photoadd to the C-8 position of the
purine ring of dGuo (42) upon benzophenone photosensitization (Morin
and Cadet 1994), generating the two diastereoisomers of 59 (Fig. 8). The
reactivity of the free amino group toward guanine radicals was investigated
using 5'-amino-2',5'-dideoxyguanosine (60) as the substrate. An efficient
benzophenone-photosensitized intermolecular reaction leading to the ad-
ducts 61 and 62 was found to occur (Morin and Cadet 1995a). An aromatic
nucleophilic substitution reaction appears to be involved in the formation
of 61 and 62 whereas a nucleophilic addition reaction was proposed to
explain the photosensitized generation of the anhydronucleoside 58 via the
participation of the 5'-hydroxyl group as a less reactive nucleophile. A third
model system consisted in tethering a lysine residue through its carboxylic
group to the 5-hydroxyl group of dGuo (42). Interestingly, both reactions
are present in the benzophenone-mediated photoaddition reactions of the
lysine residue to position 8 of the guanine moiety of 63, as inferred from
the characterization of adducts 64 and 65 (Morin and Cadet 1995b).

2.4
Ozone-Mediated Oxidation of the Purine
and Pyrimidine Bases

Ozone, a pollutant of urban air produced by a series of complex photo-chemical reactions, is a highly toxic reactive oxygen species. The genotoxic effects of O3, including mutagenesis and carcinogenesis are now well established (for a review, see Victorin 1992). A significant increase in the level of 8-oxo-7,8-dihydro-2'-deoxyguanosine (47) has been observed in the chloroplast DNA of plants upon exposure to a steam of ozone (Floyd et al. 1989). However, oxidation of the guanine moiety is not the result of the direct action of ozone, since reaction of the latter reactive oxygen species with isolated DNA did not lead to any significant increase in the amount of 8-oxodGuo (47) in comparison to control experiments. It is likely that ozone may induce secondary oxidation reactions *via* the en-hanced formation of oxygen free radicals, at least at the cellular level. Recent model studies involving various DNA model compounds have shown that the oxidation reaction mediated by O3 is highly specific, involv-ing, for example, only the 5,6-ethylenic bond of pyrimidine nucleobases and nucleosides (Matsui et al. 1990a, b, 1991; Girault et al. 1993, 1994). The main O3 oxidation products of thymidine have been identified as *N*-(2-de-oxy-β-D-*erythro*-pent ofuranosyl) formamide (11), the 5R* and 5S* dia-stereoisomers of 1-(2-deoxy-β-D-*erythro*-pentofuranosyl)-5-hydroxy-5-methylhydantoin (13), *N*1-(2-deoxy-β-D-*erythro*-pentofuranosyl)-*N*1-ace-tylurea, and *N*1-(2-deoxy-β-D-*erythro*-pentofuranosyl)-*N*1-formylurea (Girault et al. 1994). Similarly, O3-mediated oxidation of dCyd (17) in aerated aqueous solution was found to give rise to *N*-(2-deoxy-β-D-*erythro*-pentofuranosyl)formamide (11) and the 5R* and 5S* diastereoi-somers of 1-(2-deoxy-β-D-*erythro*-pentofuranosyl)-5-hydroxyhydantoin (27) (Girault et al. 1993). The mechanism of formation of the latter dThd and dCyd oxidation products may be rationalized in terms of initial [2 + 4] cycloaddition of ozone with the 5,6-pyrimidine ethylenic bond. In a sub-sequent step, the hydrolytic decomposition of the resulting unstable molo-zonide (Kucz-kowski 1992) is likely to lead to the opening of the 5,6-bond *via* a Criegge intermediate. The *N*1-formyl-*N*3-pyruvylurea and *N*1-formyl-*N*3-glyoxylurea thus produced may undergo either hydrolysis or recycliza-tion. It should be noted that no release of the free base was observed in the O3 oxidation of dThd (1) or dCyd (17), ruling out the participation of any OH· radical under these oxidizing conditions. It should be added that ozone
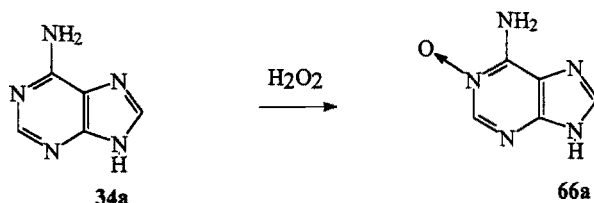
Fig. 9. Formation of adenine N-1-oxide (**66a**) by the reaction of hydrogen peroxide with adenine (**34a**).

was found to react with guanine, giving rise to the predominant formation of 2-amino-4,6-dihydroxy-1,3,5-triazine with a trace amount of parabanic acid (Matsui et al.1991).

2.5
Hydrogen Peroxide Oxidation of Adenine

Hydrogen peroxide, a side product of the dismutation reaction of super-oxide radical (or hydroperoxide radical), may react in a specific way with the adenine base in the absence of reduced transition metals (Fig. 9). Under non-Fenton reaction conditions, the unique product **66** of the reaction of H_2O_2 with dAdo (**34**) arises from the N-oxidation of the purine moiety at the N1 position, as inferred from exhaustive NMR analysis (Mouret et al. 1990). Interestingly, adenine N-1-oxide (**66a**) is formed in both isolated (Mouret et al. 1991b) and cellular (Mouret et al. 1990) DNA upon exposure to hydrogen peroxide. A sensitive HPLC-^{32}P-postlabeling assay (Mouret et al. 1990) was used to monitor the formation of adenine N-1-oxide within DNA.

2.6
Singlet Oxygen Oxidation of Guanine

Singlet oxygen ($^1O_2$), the lowest excited state of molecular oxygen ($^1\Delta g$, 94.2 kJ/mol), may be generated through energy transfer as the result of a type II photosensitization mechanism. It is likely that singlet oxygen produced by endogenous photosensitizers is a major contributor to the deleterious effects of UVA radiation and visible light on living systems. Singlet oxygen is generated by various chemical processes, including the thermal decomposition of endoperoxides (Di Mascio and Sies 1989) and dioxetanes

(Adam and Cilento 1983; Cilento et al. 1994), and the reaction of H_2O_2 with peroxynitrite (Di Mascio et al. 1994). Evidence was provided that several enzymatic reactions and chemiexcitation associated with lipid peroxidation may consitute biological sources of $^1O_2$. In addition, it was recently suggested that singlet oxygen may be generated through the reaction of oxygen with triplet excited purine and pyrimidine DNA components (Bishop et al. 1994). It is now well established that singlet oxygen is able to oxidize DNA with a much higher specificity than OH· radical, since only guanine is the substrate of the $^1O_2$ oxidation, at least at neutral pH. The two main stable oxidation products of the reaction of $^1O_2$ with dGuo (42) were identified as the 4R* and 4S* diastereoisomers of 4-hydroxy-8-oxo-4,8-dihydro-2'-deoxyguanosine (68) on the basis of extensive NMR and mass spectrometry measurements (Ravanat et al. 1992; Ravanat and Cadet 1995). Similar oxidation products were generated by the type II photooxidation reaction of 2'-deoxyguanosylyl-(3'-5')-thymidine (Buchko et al. 1992). It is likely that the oxidation product 68 arises from the thermal decomposition of the unstable endoperoxides 67 following initial [2+4] Diels-Alder cycloaddition of $^1O_2$ across the 4,8 purine carbons (Fig. 10).

Evidence for the transient formation of diastereoisomeric 67 was obtained from the characterization of related intermediates in the low-temperature (−78°C) photooxidation of 2',3',5'-O-(tert-butyldimethylsilyl)-8-methylguanosine in CH_2Cl_2 using tetraphenylporphyrin as the photosensitizer (Sheu and Foote 1993). Structural assignment of the unstable endoperoxides was achieved by ^1H and ^{13}C NMR. Additional confirmation of the incorporation of a molecular oxygen in the latter oxidized nucleosides was provided by low-temperature, fast atom bombardment mass spectrrometry analysis. In addition, a relatively small amount of 8-oxodGuo (47) is produced at a steady level of 0.8% in the Zn disulfonated aluminium phthalocyanine-mediated photooxidation of dGuo (42) (Ravanat et al. 1992). It has been recently suggested that 47 may arise from the rearrangement of the initially generated endoperoxides 67 into 8-hydroperoxy-2'-deoxyguanosine 69 (Sheu and Foote 1995a). Interestingly, 47 was found to be two orders of magnitude more reactive with $^1O_2$ than its dGuo (42) precursor (Sheu and Foote 1995b). The main stable singlet oxygen oxidation products were identified as N-(2-deoxy-β-D-erythro-pentofuranosyl)cyanuric acid (71), and 4-hydroxy-8-oxo-4,8-dihydro-2'-deoxyguanosine (68) together with the type I photosensitization oxidation products 49 and 50 (Buchko et al. 1995b; Raoul and Cadet 1996). A reasonable mechanism for the formation of 68 and 71 would involve the initial
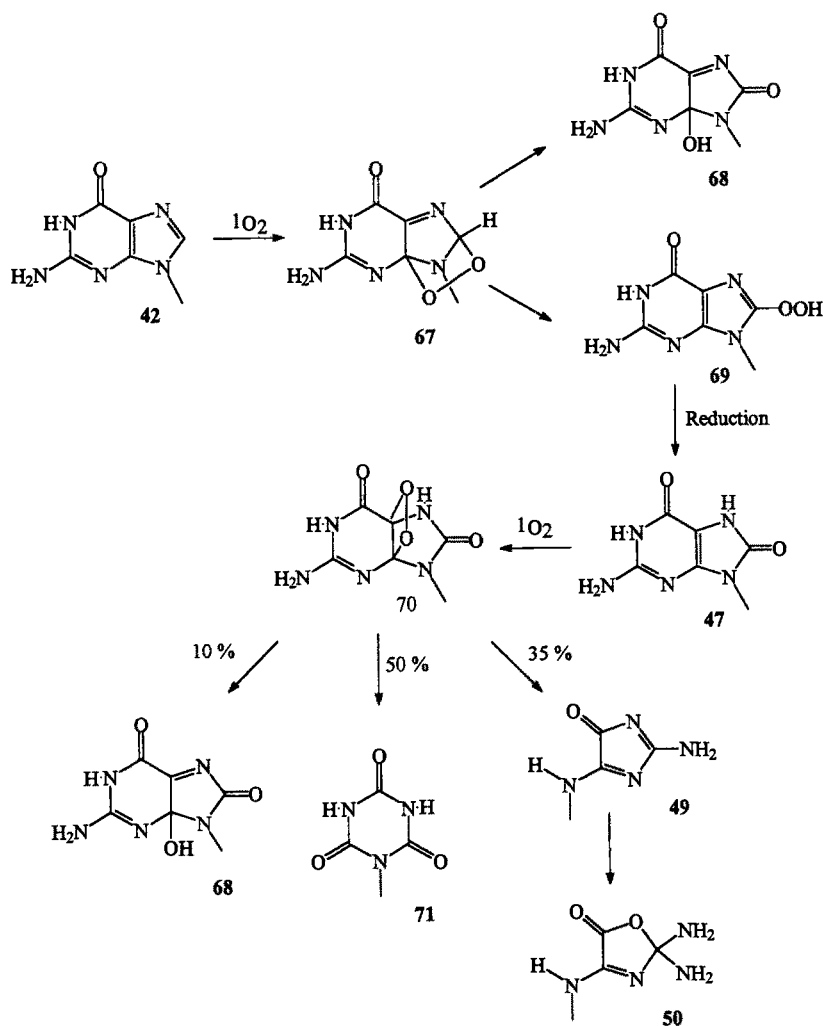
Fig. 10. Main oxidation reaction of 2'-deoxyguanosine (42) and 8-oxo-7,8-dihydro-2'-deoxyguanosine (47) by singlet oxygen in water

formation of the dioxetane 70 through $^1O_2$ addition across the 4,5-purine bond. In subsequent steps, 70 may either rearrange into the 4-hydroperoxide or undergo a 1,2-cleavage (Sheu and Foote 1995c). Again, DNA structure appears to play a major role in the orientation of the oxidation reaction of dGuo (42) mediated by singlet oxygen. Attempts to search for

the photosensitized formation of 4-hydroxy-8-oxo-4,8-dihydroguanine (67a) within isolated DNA by using a sensitive GC-MS assay were unsuccessful (Cadet et al. 1994a). On the other hand, 8-oxodGuo (47) was found to be the main $^1O_2$ oxidation product of DNA upon exposure to either a chemical source of singlet oxygen or excited photosensitizers (Müller et al. 1990; Schneider et al. 1990; Devasagayam et al. 1991).

2.7
Reactions of NO· Derivatives with DNA

The biological role of the free radical nitric oxide (NO·) has been widely studied in recent years. NO· is a major bioregulator involved in numerous physiological processes, including smooth muscle relaxation, inhibition of platelet aggregation, and neurotransmission. NO· is also involved in macrophage-mediated cell killing (Liew et al. 1990). In addition to these endogenous productions, NO· is also provided by environmental sources, including cigarette smoke, automobile exhaust, and gas cooking. The mutagenicity of NO· is now well established. Mutations and chromosome aberrations have been observed in the lung cells of rats exposed to NO· (Isomura et al. 1984). NO· was also found to induce mutations in *Salmonella typhimurium* (Arroyo et al. 1992) and human lymphoblast cells (Nguyen et al. 1992). NO· is only weakly reactive with biomolecules (for a review, see Stamler et al. 1992). Addition on metals of metalloproteins, such as hemoglobin, (Doyle and Hoekstra 1981) and to the tyrosine radical of the active site of ribonucleotide reductase (Lepoivre et al. 1991) are among the few reactions reported. NO·-mediated damage to biomolecules, and in particular DNA, is thus likely to involve activated species of NO·. The likely candidates for the induction of DNA lesions reviewed below include deaminating derivatives, peroxynitrite, and nitrogen dioxide.

2.7.1
NO·-Mediated Deamination of Nucleobases

Mutations observed in *Salmonella typhimurium* cells treated with NO· are mainly G:C → A:T transitions (Wink et al. 1991b). Similar results were obtained with plasmids incubated in aerobic solutions of NO· or NO· donors prior to their transfection and replication in human and *E. coli* cells (Routledge et al. 1993, 1994). Based on observations made with nucleosides and isolated DNA (Wink et al. 1991a; Nguyen et al. 1992), deamination of

cytosine was proposed to account for these results. The NO·-mediated deamination of nucleobases is dependent on the presence of oxygen, indicating that N_2O_3 is involved (Nguyen et al. 1992). This is in agreement with the known ability of acidic solution of nitrite to induce cytosine deamination, via the nitrosation of the exocyclic amino groups by N_2O_3. Moreover, the latter compound has been shown to be the *N*-nitrosating agent of morpholine in aerated solution of NO· (Lewis et al. 1995). However, the involvement of the deamination of cytosine and 5-methylcytosine in NO· mutagenicity has recently been questioned. Similar mutation frequency was observed in plasmids exposed to NO· subsequently transfected in either *E. coli* mutant cells deficient in uracil-DNA-glycosylase or in the wild-type cells (Schmutte et al. 1994). It should be added that deamination is not specific for NO·, since it also occurs spontaneously in DNA (Wang et al. 1982). Another possible pathway for the mutagenicity of N_2O_3 is the endogenous formation of nitrosamines, which are powerful DNA-alkylating agents (Bartsch et al. 1990).

2.7.2
Peroxynitrite

Peroxynitrite anion (OONO–) is the product of the reaction between NO and superoxide anion (O_2·–) (Blough and Zafiriou 1985):

$$NO· + O_2·^{-} \rightarrow -O-O-N = O$$
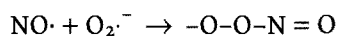
The endogenous production of both NO· and superoxide anion in cells suggests the biological relevance of this reaction. This was confirmed by the observation of the release of peroxynitrite by both activated macrophages (Ischiropoulos et al. 1992a) and neutrophils (Carreras et al. 1994) *in vitro*. In addition, 3-nitrotyrosine, the main reaction product of peroxynitrite and tyrosine, was detected *in vivo* (Kaur and Halliwell 1994; Salman-Tabcheh et al. 1995). Peroxynitrite exhibits bactericidal activities (Zhu et al. 1992) and is more toxic than NO· towards *E. coli* (Brunelli et al. 1995). The pKa of the peroxynitrite anion is around 6.8, which indicates that it would predominantly exist under its protonated form in cells. The half-life of peroxynitrous acid (HOONO), which is 7 s at 0°C and 1 s at 37°C, should be long enough to allow the molecule to diffuse inside the cell and react with DNA. The decomposition pathway of HOONO, leading to the formation of nitric acid, remains open to debate. Several experiments based on

model systems, including oxidation of ribose, formaldehyde, and di-methylsulfoxide (DMSO), suggested the formation of hydroxyl radicals (Beckman et al. 1990; Yang et al. 1992). However, thermodynamic consid-erations (Koppenol et al. 1992) and the absence of electron spin resonance signals corresponding to OH· during decomposition of peroxynitrous acid (Shi et al. 1994b) seem to rule out this possibility. Evidence is growing that an excited form of peroxynitrite exhibiting a *trans* configuration is the actual reactive species (Tsai et al. 1994; Squadrito et al. 1995).

Peroxynitrite may react with biomolecules according to two main path-ways. A first possibility is the induction of oxidation reactions, as observed for small molecules, including methionine (Pryor et al. 1994), α-tocopherol (Hogg et al. 1994), and oxyhemoglobin (Schmidt et al. 1994). The oxidative properties of peroxynitrite have also been observed with lipids (Radi et al. 1991; Rubbo et al. 1994) and proteins (King et al. 1992). Studies using isolated DNA showed that peroxynitrite, like OH· radicals, induces oli-gonucleotide strand breaks (King et al. 1992; Salgo et al. 1995). This reaction can be catalyzed by manganese porphyrin (Groves and Marla 1995). As far as base modifications are concerned, we observed that purine nucleosides are oxidized with a product distribution similar to that observed after exposure to ionizing radiation in aerated aqueous solution. The main oxidation products of dAdo (34) and dGuo (42) were 8-oxo-7,8-dihydro-2'-deoxyadenosine (40) and 2,2-diamino-4-[(2-deoxy-β-D-*erythro*-pento-furanosyl) amino]-5-(2H)-oxazolone (50), respectively (Douki and Cadet 1996). The yield of 8-oxo-7,8-dihydro-2'-deoxyguanosine (47) was very low. The formation of singlet oxygen through the reaction of peroxynitrite with hydrogen peroxide (Di Mascio et al. 1994) was confirmed in this study. The specific addition products of $^1O_2$ to dGuo (42), the $^4R*$ and $^4S*$ dias-tereoisomers of 4-hydroxy-8-oxo-4,8-dihydro-2'-deoxyguanosine (68), were observed, *albeit* in very low yield, in samples treated with a mixture of HOONO and H_2O_2. The results obtained with nucleosides suggesting that peroxynitrite exhibit a reactivity similar to OH·, could not be extended to isolated DNA (Douki and Cadet 1996). Peroxynitrite induced the forma-tion of similar amount of 40 and 50 with very little 47 whereas the latter compound is the major OH·-mediated oxidation product of the guanine moeity of DNA. This shows that peroxynitrite does not damage DNA with a OH·-like reactivity. However, the actual reactive species remains to be identified in order to better assess the role of peroxynitrite in biochemical processes such as the oxidation of DNA in activated macrophages (de Rojas-Walker et al. 1995).

Other possible types of reaction of peroxynitrite with biomolecules include addition of nitrogen-containing groups. Nitration of phenylalanine and tyrosine have been reported (van der Vliet et al. 1994). The formation of nitrotyrosine in the active site of superoxide dismutase was also observed upon peroxynitrite treatment (Ischiropoulos et al. 1992b). Moreover, a series of nitrogen derivatives of fatty acids were suggested to be involved in the mechanism of action of peroxynitrite on lipids (Rubbo et al. 1994). Similar reactions can take place with DNA nucleobases. The main product of the reaction between guanine (42a) and peroxynitrite (Fig. 11) is 8-nitroguanine (72a) (Shigenaga et al. 1994; Yermilov et al. 1995a). The development of a sensitive HPLC-EC assay for 72a allowed its detection in isolated DNA exposed to peroxynitrite (Yermilov et al. 1995b). Peroxynitrous acid was found to undergo an homolytic addition reaction on the purine ring of dGuo (42). The resulting adduct was characterized as 5-hydroxy-4-nitrosoxy-4,5-dihydro-2'-deoxyguanosine (73) on the basis of its chemical properties and its UV, NMR, and mass spectroscopy features (Douki et al. 1996b). The development of an assay for the latter guanine derivative in DNA would be of interest to assess its biological relevance.

In spite of these results showing the high reactivity of peroxynitrite towards DNA, evidence is still lacking for its presence *in vivo* and its involvement in NO· mutagenicity. Contradictory results can be found in the literature. For instance, in spite of the reported cytotoxicity of peroxynitrite, NO· has been shown to protect against the lethal effects of superoxide
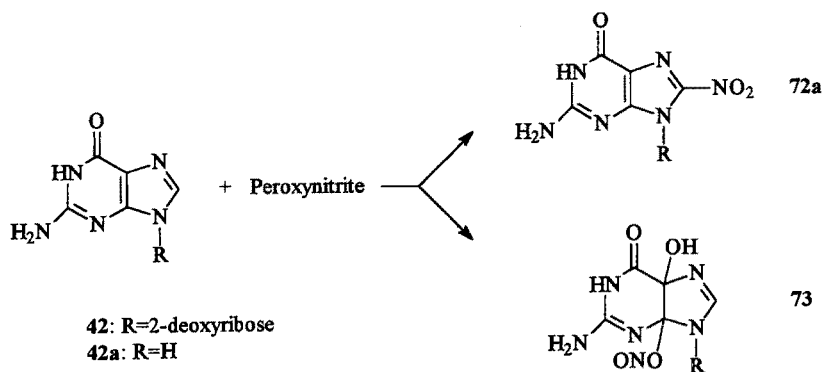
Fig. 11. Main products of the reaction of peroxynitrite with guanine (42a) and 2'-deoxyguanosine (42): 8-nitroguanine (72a) and 5-hydroxy-4-nitosoxy-4,5-dihydro-2'-deoxyguanosine (73)

anion generated by the xanthine–xanthine oxidase system (Wink et al. 1993a).

2.7.3
Nitrogen Dioxide

The mutagenicity of $NO_5 \cdot$ requires the presence of oxygen and is inhibited by antioxidants (Arroyo et al. 1992). The main product of the reaction of $NO \cdot$ with oxygen is nitrite with nitrogen dioxide (NO_2) as intermediate (Lewis and Deen 1994). The latter compound might be one of the species involved in the mutagenicity of $NO \cdot$. The mutagenic properties of NO_2 have been widely studied (for a review, see Victorin 1994), mainly because of its implication in environmental pollution. NO_2 is mutagenic in *drosophilia* (Inoue et al. 1981) and rats (Isomura et al. 1984). This was confirmed by *in vitro* studies in *Salmonella typhimurium* (Isomura et al. 1984). The possible involvement of NO_2 in $NO \cdot$ mutagenicity is suggested by the observation that NO_2 is more mutagenic in lung cells of rats than its precursor (Isomura et al. 1984) and more cytotoxic to *Salmonella typhimurium* (Arroyo et al. 1992). As for $NO \cdot$, the biochemical mechanism of the mutagenicity of NO_2 is poorly understood. According to Wink et al. (1993b), it does not involve nitrosation. In addition to strand breaks (Görsdorf et al. 1990), nitration might be another pathway, as observed with proteins (Kikugawa et al. 1994).

2.8
Reactions Mediated by Lipid Hydroperoxides
and Related Decomposition Products

Lipid peroxidation is a major consequence of oxidative stress for cells. Reactive compounds are produced during this process and can diffuse through the cell and damage DNA. Lipid peroxidation is initiated by the formation of lipid radicals, which are converted into peroxyl radicals in the propagation phase. In the termination phase, oxidized lipids give rise to chemically stable compounds, including alkanes, alkenes, and carbonyl derivatives (Vaca et al. 1988). A first possible source of mutagens includes the lipid peroxyl radicals, which might diffuse into the nucleus and reach DNA. In addition, emphasis has been placed in recent years on the mutagenic properties of the final products of lipid peroxidation (Marnett et al. 1985; Esterbauer et al. 1991; Eckl et al. 1993), mainly malondialdehyde
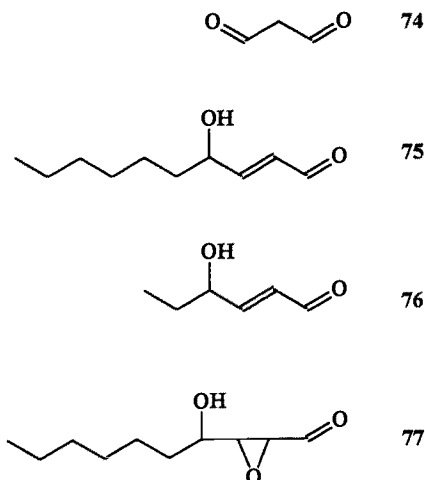
Fig. 12. Structure of the breakdown products of lipid peroxidation that are able to react with DNA bases: malondialdehyde (74), 4-hydroxynonenal (75), 4-hydroxy-hexenal (76), and $1,N^2$ and 3,4-epoxy-4-hydroxynonanal (77)

(74) and 4-hydroxynonenal (75) (Fig. 12). The ability of the two latter reactive aldehydes to form exocyclic adducts to nucleobases within DNA will be extensively discussed, together with the possibility of induction of DNA-protein cross-links.

2.8.1
Direct Effect of Lipid Radicals

The diffusion of the radicals produced during the two first phases of lipid peroxidation inside the cell may be considered as a possible cause of DNA modification. Model experiments have shown that peroxidized lipids are able to induce oligonucleotide strand breaks in isolated DNA (Morita et al. 1983; Inouye 1984; Nakayama et al. 1986). Only few data on the biological relevance of this process are available. It has been recently shown that DNA extracted from organs of rats treated with lipid hydroperoxides exhibited a high level of damage identified as malondialdehyde adducts (see below; Wang and Liehr 1995a). However, the formation of such compounds does not provide support for direct oxidation of DNA by lipid hydroperoxides. It is likely that lipid radicals and peroxyl derivatives are too reactive and exhibit too short a half-life to reach DNA.

2.8.2 Malondialdehyde Pyrimidopurine Adduct

Malondialdehyde 74 is an highly reactive molecule, mainly toward nucleophilic groups. In this respect, amino groups of cytosine (17a), adenine, (34a) and guanine (42a) are major sites of addition reactions in DNA. The adducts of bases or nucleosides with one or two malondialdehyde molecules have been isolated and characterized (Seto et al. 1983; Basu et al. 1988; Stone et al. 1990a,b). 2'-Deoxyguanosine (42), the most reactive nucleoside, is converted into an exocyclic pyrimido purine derivative (78) (Fig. 13). The formation of this compound in biological samples has been a matter of controversy. Hadley and Draper (1990) first reported the isolation of 78a in urine by using a technique based on the quantitation of 74 after acidic hydrolysis of the HPLC fraction corresponding to the retention time of the adduct. The detection method was based on the use of the nonspecific thiobarbituric assay, and thus the obtained results may be largely due to artifacts. Using an online version of the latter assay, Seto and Ohkubo (1991) were able to detect the presence of the guanosine derivative of 78 in urine. Measurement of 78 in DNA extracted from tissues has also been a matter of debate. Taking advantage of the fluorescence of 78, it was suggested that the latter adduct is generated in sevenfold higher yield than 8-oxo-7,8-dihydro-2'-deoxyguanosine (47) in liver DNA of rats (Agarwal and Draper 1992). More recently, it was reported that 78 accumulates in rat liver DNA with age (Draper et al. 1995). However, the amounts of 78 found in rat liver DNA in the latter studies are much higher that those determined by Marnett and coworkers, who have developed several accurate assays. The detection limit of assays for 78a involving the coupling of HPLC to either electrochemical detection (Goda and Marnett 1991) or mass spectrometry through a thermospray interface (Jajoo et al. 1992) was too high to allow the detection of the product in biological samples.

   Adduct 78a was finally detected in human liver using gas chromatography coupled to mass spectrometry with electron capture detection (Chaudhary et al. 1994). The steady-state level is very low, ranging from five to 11 adducts per 10^7 bases in human liver DNA. ^{32}P-postlabeling has also been used for the detection of 78 in mice (Vaca et al. 1992) and human tissues (Vaca et al. 1995). The values obtained in the latter study for human white blood cells and breast tissue DNA are in the range of two to three adducts per 10^7 nucleotides (Vaca et al. 1995), in agreement with the GC-MS data of human liver studies (Chaudhary et al. 1994). ^{32}P-postlabeling also allowed the observation of 78 in rodents kidney and liver DNA (Wang and Liehr

1995a,b) The presence of 78 in cellular DNA is of importance, since experiments using plasmid treated with malondialdehyde prior to their transfection in *E. coli* are replicated with a high mutation rate, indicating a likely high mutagenicity of the malondialdehyde adducts (Benamira et al. 1995).

2.8.3
1,N^2-Propano Adducts of α,β-Unsaturated Aldehydes

Among the unsaturated aldehydes produced during lipid peroxidation, 4-hydroxynonenal 75 (Fig. 12) has been the most widely studied. It is produced by decomposition of ω-6 polyunsaturated acids, such as linolenic, linoleic, and arachnoid acids (Esterbauer et al. 1991). It has been identified as the major unsaturated aldehyde produced during lipid peroxidation in rat hepatocytes, together with 4-hydroxy-2,6-dienal and 4-hydroxyhexenal (76) which are produced in smaller amounts (Poli et al. 1985). 75 is detected in most organs of rat in the nmol/g range (Orada et al. 1986; van Kuijk et al. 1986). Its concentration in human plasma has been reported to be between 0.28 and 0.68 nmol/g (Esterbauer et al. 1991). 75 has been found to be mutagenic in rodent cells (Cajelli et al. 1987) and is able to induce SOS response in *Salmonella typhimurium* (Benamira and Marnett 1992). The exact molecular mechanism of the interaction between 75 and DNA is not clearly established, even though fragmentation and sister-chromatid exchange have been observed *in vitro* in rodent cells (Brambilla et al. 1986). Incubation of dGuo (Winter et al. 1986) and isolated DNA (Douki and Ames 1994) with 75 leads to the formation of 1,N^2-propano exocyclic adducts to dGuo 19 (Fig. 13). Similar results are observed with 76. 1,N^2-Propano adducts are involved in the mutagenicity of other unsaturated aldehydes, including the widely studied acrolein and crotonaldehyde (Chung et al. 1984, 1989; Foiles et al. 1989). Interestingly, the 1,N^2-propano adducts of the two latter compounds have been detected in low amounts in the DNA extracted from human liver (Nath and Chung 1994). Only one attempt to detect 79a in DNA of biological samples has been reported (Douki and Ames 1994). DNA was extracted form rat liver and analyzed by HPLC coupled with electrochemical detection. No adducts were detected, probably because of a lack of sensitivity of the assay, which allowed the detection of one lesion per 10^5 guanine molecules. It is likely that the amount of unsaturated aldehydes reaching the nucleus is very low because of their high reactivity. In particular, these compounds have been shown to react efficiently with nucleophilic groups of protein (Uchida et al. 1994;

78

79

80

81

82
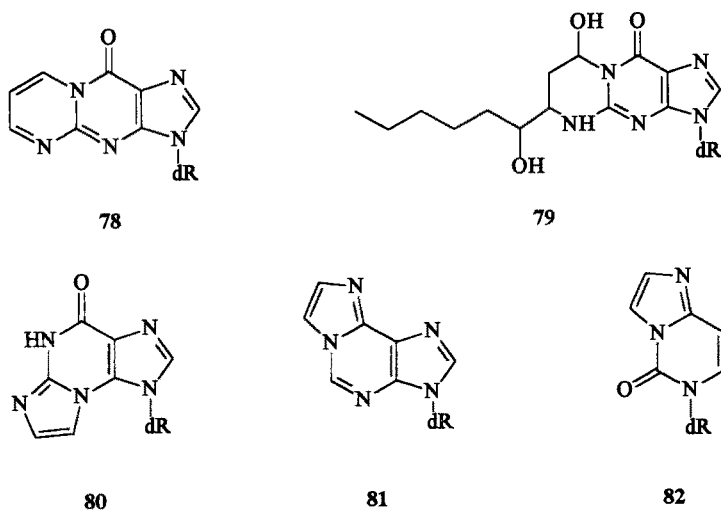
Fig. 13. Exocyclic adducts of reactive breakdown products of lipid peroxides with 2'-deoxyribonucleosides: $1,N^2$-pyrimido-2'-deoxyguanosine (78), $1,N^2$-propano-2'-deoxyguanosine derivative (79), $N^2,3$-etheno-2'-deoxyguanosine (80) and $1,N^6$-etheno-2'-deoxyadenosine (81) and $3,N^4$-etheno-2'-deoxycytidine (82)

Toyokuni et al. 1994) and thiol-containing compounds such as glutathione (de Toranzo and Castro 1994). More sensitive techniques are needed to clearly establish the involvement of 79 in the mutagenesis mediated by lipid peroxidation.

2.8.4
Ethenobases

Sodum and Chung (1988, 1991) have reported the formation of $1,N^2$-etheno-2'-deoxyguanosine (80) and $1,N^6$-etheno-2'-deoxyadenosine (81) (Fig. 13) in samples of nucleosides and isolated DNA treated with 2,3-epoxy-4-hydroxynonanal 77 (Fig. 12), the epoxide derivative of 4-hydroxynonenal. 80 and $3,N^4$-etheno-2'-deoxycytidine (82) are also produced by treatment of the corresponding nucleosides and nucleotides by oxidized rat liver microsomes and oxidized arachidonic acid (El Ghissassi et al. 1995). A sensitive ^{32}P-postlabeling assay has been developed to detect ethenobases in living systems. A level of approximately one $1,N^6$-ethenoadenine molecule per 10^8 Ade and two $3,N^4$-ethenocytosine per 10^8 Cyt were measured in

human liver DNA (Nair et al. 1995). The question of the origin of these products remains open, since they are known to be produced by a variety of chemicals provided by sources other than lipid peroxidation (Kusmierek and Singer 1982; Leithauser et al. 1990; Swenberg et al. 1992; Foiles et al. 1993). For instance, **80** and **82** were detected in a much higher amounts in cirrhotic than in other human liver DNA (Nair et al. 1995). This may be explained by the presence of ethylcarbamate, a product known to generate ethenobases, in alcoholic drinks (Zimmerli and Schaltter 1991). In addition, ethenobases have been proposed to be involved in the mutagenicity of 4-hydroxynonenal on the basis of the metabolic conversion of ethylenic compounds into epoxides by cytochrome P450 (Sodum and Chung 1991). The biological relevance of this pathway remains to be established since several studies have shown that 4-hydroxynonenals is mainly metabolized as glutathione adduct, 4-hydroxy-nonenoic acid and 1,4-dihydroxy-nonene (Esterbauer et al. 1985; Ålin et al. 1985; Ullrich et al. 1994; Grune et al. 1994). The two latter products and **75** are also excreted in urine as mercapturic acid conjugates (Alary et al. 1995). Taken together, these observations indicate that the lipid peroxidation-mediated formation of ethenobases remains to be strictly established *in vivo*.

2.8.5
DNA–Protein Cross-links

Both malondialdehyde **74** and 4-hydroxynonenal **75** are highly reactive compounds. The low level of the malondialdehyde adduct **78** detected within DNA and the lack of a similar lesion for 4-hydroxynonenal (**79**) indicate that these two unsaturated aldehydes react in a very low yield with nucleobases *in vivo*. One possible explanation is the difficulty for these molecules to reach the double DNA helix, which is protected by histones and other nuclear proteins. In that regard, the formation of DNA-protein cross-links is an interesting possibility to account for the mutagenicity of **74** and **75**, both exhibiting two reactive sites. As a matter of fact, malondial-dehyde has been reported to induce this type of lesions (Summerfield and Tappel 1984a,b). There is no equivalent data for 4-hydroxynonenal, but it has been shown that unsaturated aldehydes are very efficient for inducing DNA-protein cross-links (Kuykendall and Bogdanffy 1992).

3
Measurement of Oxidative DNA Base Damage

Considerable efforts have been directed during the last decade toward the development of analytical methods for the detection of oxidized DNA bases. It should be remembered that the assays have to be highly sensitive, since they have to be able to detect at least one modification in 10^5 normal bases in a few micrograms of DNA. Two different general approaches have been developed. One involves the detection of the modified base in intact DNA using either immunological methods or specific repair enzymes (enzymatic incision at the site of the lesion leads to the formation of a strand break, which is subsequently quantitated by using various assays). The second approach requires quantitative hydrolysis of the DNA macromolecule to monomeric units, which are then separated and measured quantitatively. DNA may be hydrolyzed either chemically (acid hydrolysis), leading to the release of the free base, or enzymatically (using DNAse, exonucleases and phosphatase). In the latter case, nucleosides, nucleotides, or short oligonucleotides may be obtained. The separation of the monomer units is then achieved using HPLC, GC, and capillary electrophoresis. In the final step, DNA modifications are detected online using various sensitive methods including mass spectrometry, fluorescence, radioactivity, and amperometry.

3.1
Methods Using Whole DNA

Various approaches have been used for the development of assays aimed at measuring oxidized nucleobases in either whole DNA or after hydrolysis of the latter polymer.

3.1.1
Immunological Assays

Antibodies (Ab) have been widely used to detect DNA modifications induced by various chemical and physical agents. Two major immunological techniques, the radioimmunological assay (RIA) and the competitive enzyme-linked immunosorbent assay (ELISA), have been established. Both methods may use either monoclonal or polyclonal antibodies. The RIA assay is based on the ability of the lesion contained into the sample to

inhibit the binding of the antigen (i.e., the modified base or nucleoside) to the antibodies. The ELISA method, which is more sensitive, was developed later. This technique requires the attachment of appropriate antigens (usually a protein carrying the modified DNA base or nucleoside) to the wells of polyvinyl chloride microtiter plates, followed by incubation with either polyclonal or monoclonal antibodies. The recognition of the damage can be made either on intact DNA and/or after hydrolysis of the biopolymer, depending on the specificity of the antibodies. If the DNA sample containing the modification to be detected is recognized by the antibodies, specific binding to the well would decline. A second antibody (with a covalently bound enzyme) that recognizes the primary antibody is used to determine the efficiency of binding of the first antibody, usually by the means of a colorimetric or fluorimetric reaction (the extent of the enzyme-mediated reaction is proportional to the amount of enzyme bound through the interaction of the secondary antibody to the primary antibody). Immunological assays have been applied to the measurement of several oxidized DNA bases. Initially, a RIA method was used to detect thymine glycol (12a) within DNA using a rabbit antiserum (West et al. 1982). Shortly thereafter, a monoclonal antibody was raised against 12a (Leadon and Hanawalt 1983). More recently, monoclonal antibodies against 8-oxo-7,8-dihydroguanine (47a) became available (Park et al. 1992), and the ELISA method was used to monitor the formation of 47a (8-oxo-7,8-dihydroguanine) within DNA (Yin et al. 1995). The sensitivity of the assay was reported to be close to one modification per 10^6 DNA bases. In addition, a sensitive, noncompetitive solid-phase immunoassay was developed for the quantitation of 8-oxo-7,8-dihydroguanine (47a) (Musarrat and Wani 1994). The latter method, named immunoslot blot (ISB), is based on the ability for the monoclonal antibody to bind DNA immobilized on filters, the binding capacity being proportional to the amount of DNA modifications recognised by the antibody. It should be noted that antibodies can also be used to prepurify oxidized bases or nucleosides from either DNA constituents or biological fluid by immunoaffinity chromatography. The first step involves binding of the antibody to a Sepharose gel. The affinity of the antibody is then used to selectively retain oxidized bases and nucleosides such as 47a and 47 from complex mixtures (Degan et al. 1991).

3.1.2
Enzymatic Assays

The recognition of oxidative DNA damage by purified repair endonucleases is exploited to quantify various types of modifications (Allan and Garner 1994). The repair endonucleases, such as the Fpg protein (Boiteux et al. 1992) and the endonuclease III (Dizdaroglu et al. 1993), incise DNA at the site of the modification, generating either a strand break or an alkalilabile site. The breaks are then detected using sensitive methods including the alkaline elution assay for nuclear DNA and the relaxation assay for circular DNA. In addition, the number of frank strand breaks which may be directly generated by the damaging agent is determined in the absence of DNA repair enzymes. The sensitivity of the assay is close to one modification per 10^7 DNA bases. It should be added that single strand breaks and several types of base modifications may be determined in parallel (Epe et al. 1993; Epe 1995). However, the assay may suffer from a lack of specificity. The major limitation is due to the relatively broad, and sometimes not completely established, substrate specificity of the repair enzymes. On the other hand, the assay allows the determination of DNA damage profiles, which are likely to provide important information on the mechanism of action of the damaging agent. For example, the effect of UV light in L1210 mouse leukemia cells was found to arise largely from the reaction of DNA with singlet oxygen. This was inferred from the observation that the profile of single strand breaks induced by different repair endonucleases (e.g., Fpg protein, endonuclease III) was similar to that obtained after singlet oxygen-mediated DNA oxidation (Pflaum et al. 1994; Epe 1995).

3.2
Assays Requiring DNA Hydrolysis

This approach requires the quantitative hydrolysis of DNA prior to the detection of the modified DNA bases. Hydrolysis can be performed enzymatically (using nucleases and phosphatases) that release the nucleosides. A second approach involving acidic hydrolysis (chlorhydric and formic acids, fluorhydric acid stabilized in pyridine) leads to the release of the corresponding free bases (Téoule et al. 1977; Polverelli et al. 1990; Cadet and Weinfeld 1993; Frenkel and Klein 1993). The compound of interest then has to be separated from the largely unmodified nucleosides or nucleobases. Separation may be achieved using different analytical methods, including

HPLC, GC, and capillary electrophoresis. The separated lesions are then detected online by more or less specific methods such as radioactivity, fluorescence, amperometry, and mass spectrometry. Some of the measurements requires prederivatization, biochemical postlabeling, or postcolumn reaction of the sample (Cadet and Weinfeld 1993).

3.2.1
DNA Isolation and Hydrolysis

In the first step of the assay, DNA has to be extracted, isolated, and then hydrolyzed. The methods used for this purpose have to minimize artifactual DNA oxidation during the workup (for reviews, see Frenkel and Klein 1993; Cadet and Weinfeld 1993). For example, DNA isolation using phenol/chloroform extraction has been shown to induce significant oxidation of guanine (42a) to 8-oxo-7,8-dihydroguanine (47a) (Claycamp 1992; Floyd et al. 1990). However, other authors (Harris et al. 1994) have reported that DNA extracted from biological samples with freshly distilled phenol did not give rise to higher levels of 47a by comparison with other methods. Further efforts need to be made to determine the biological content of oxidized bases in pro- and eukaryotic cells. It should be noted that the different available methods to isolate and hydrolyze DNA have not been validated using DNA known to contain a small amount of oxidized bases, since such a modified DNA is not available. One possibility to obtain further information on the accuracy of DNA extraction methods involves a direct comparison of the techniques using identical biological samples.

3.2.2
Fluorescent and Radioactive Detection Assays

The formation of 5-hydroxymethyl-2'-deoxyuridine (15) in DNA following exposure to γ-irradiation has been monitored by an assay involving HPLC separation and a detector of radioactivity (Frenkel et al. 1985). The method required radioactive prelabeling of the thymine bases in DNA. However, this approach is not suitable for the determination of oxidized DNA bases due to the occurrence of self-radiolysis associated with the use of ^{3}H- and ^{14}C-radiolabeled bases (for a review, see Cadet and Berger 1985). Thus postlabeling of the sample to be analyzed is an appropriate alternative when radioactivity is used for the detection of the DNA damage (for a review, see Cadet et al. 1992). A highly sensitive postlabeling assay based on the

incorporation of ^{32}P into nucleotide was initially devised by Randerath et al. (1981) to measure carcinogen-DNA adducts. More recently, the method has been extended to the measurement of several types of oxidized base damage to DNA (for reviews, see Cadet et al. 1992; Cadet and Weinfeld 1993). Typically, oxidized DNA is first digested to nucleoside 3'-monophosphates or dinucleoside monophosphates, which are subsequently radiolabeled enzymatically using a polynucleotide kinase and [32P]adenosine triphosphate ([32P]ATP) as a source of radiolabeled phosphate. A variety of techniques such as HPLC or two-dimensional thin-layer chromatography (TLC) are then used to separate the radiolabeled product. This assay is very sensitive, since [32P]ATP is commercially available with a highly specific activity (about 3000 Ci/mmol).

The strategy that has been developed for the measurement of oxidized DNA bases by either ^{32}P-postlabeling requires prepurification or enrichment of the targeted compound prior to enzymatic labeling. One of the reasons for this is to prevent the formation of radioactive background due to self-radiolysis from normal nucleoside 3'-monophosphates. It should be added that the removal of the largely normal nucleotides facilitates the phosphorylation of oxidized DNA substrates. For this purpose, three approaches were used. The first one involves chromatographic enrichment of the 3'-phosphate ester of 2'-deoxyadenosine N-1-oxide (66) (Mouret et al. 1990, 1991b), 5-(hydroxymethyl)-2'-deoxyuridine (15) and the *cis* diastereoisomers of 5,6-dihydroxy-5,6-dihydrothymidine (12) (Weinfeld and Soderlind 1991) prior to labeling. A second possibility, which was employed for the measurement of 8-oxo-7,8-dihydro-2'-deoxyguanosine (47) is based on the higher stability of the N-glydosidic bond of the related 3'-nucleotide with respect to that of 2'-deoxyguanosine 3-monophosphate (3'-dGMP) (Lutgerink et al. 1992). The third method is based on the resistance of formylamine nucleoside (11) to the exonuclease activity of nuclease P1 (Maccubbin et al. 1992b). The latter damage is released as dinucleoside monophosphates, whereas the bulk of the normal and oxidized nucleosides is converted into nucleosides which are not substrates for the polynucleotide kinases.

Chemical derivatization was also used for the detection of modified DNA nucleosides through the esterification of the alcoholic functions of the bases and the sugar moieties with radioactive acetic anhydride (Frenkel et al. 1991). The assay has been applied to the measurement of 8-oxo-7,8-dihydro-2'-deoxyguanosine (47), 5-(hydroxymethyl)-2'-deoxyuridine (15) and the *cis* diastereoisomers of 5,6-dihydroxy-5,6-dihydrothymidine (12).

Fluorophore labeling of 2'-deoxyribonucleoside monophosphates was also considered (Sharma et al. 1990b). After enzymatic digestion of DNA to nucleotides, the compound of interest is converted into a fluorescent derivative through the formation of a phosphoramidate (Sharma et al. 1990a). The limit of detection is close to one modification per 10^6 DNA bases using about 100 µg DNA, which makes the assay difficult to use for biological applications.

3.2.3
Other Chromatographic Methods

HPLC separation associated with amperometric detection (HPLC-EC) represents a sensitive and quantitative method for measuring several oxidized bases and nucleosides. The assay is widely used for the quantitative determination of 8-oxodGuo (47) in DNA samples. Initially reported by Floyd et al. (1986) using an amperometric detection, the method has been continually improved (Adachi et al. 1995) and extended to other oxidized DNA components that exhibit low oxidation potentials. These include 8-oxo-7,8-dihydroadenine (40a) and their nucleosides (Berger et al. 1990) together with 5-hydroxyuracil, 5-hydroxycytosine (26a) and the related nucleosides (Wagner et al. 1992). It may be added that formamidopyrimidine derivatives of adenine (41a) and guanine (48a) are also electroactive compounds that can be detected by the HPLC-EC assay. The sensitivity of the method is close to one modification per 5×10^5 normal DNA bases. However, the assay requires at least 25 µg DNA to accurately quantify DNA modifications in cellular DNA (Douki et al. 1996b).

The measurement of thymidine hydroperoxides may be also carried out in a specific way. This is achieved by a postcolumn reaction since the hydroperoxides are able to oxidize the ferrous sulphate xylenol orange reagent, allowing a colorimetric detection with a sensitivity in the picomole range. An example of the application of the assay is the separation and detection of the five hydroperoxides of thymidine induced by menadione photooxidation (Wagner et al. 1994). However, the method is not enough sensitive to be used for monitoring the formation of thymidine hydroperoxides in cellular DNA. For this purpose, better alternatives would involve either postcolumn chemiluminescence (Zhang et al. 1995) or electrochemical detection in the reduction mode (Korytowski et al. 1995).

During the last decade, a major development among the methods aimed at measuring oxidized DNA bases occurred by the association of mass

spectrometry used as a detector with chromatographic separation methods. The high resolutive capabilities of GC were coupled with accurate mass spectrometry using either electron ionization (EI) (GC/SIM) or electron capture negative ion (ECNI). Both methods require derivatization of the oxidized bases and nucleosides which are not enough volatile to be analyzed directly. In the original approach described by Dizdaroglu (1984), the bulk of the nucleobases (obtained after formic acid hydrolysis of DNA) were converted into the corresponding trimethylsilyl derivatives. The detection was achieved online by electron impact. The sensitivity of the GC-MS assay in the selected ion monitoring mode (SIM) (Dizdaroglu 1985, 1991) is close to that obtained with HPLC-EC. In addition, it was recently shown that the limit of detection of *tert*-butyldimethylsilyl derivatives of modified nucleobases, such as 5-(hydroxymethyl)-uracil (15a), 5-formyluracil (16a), 5-hydroxycytosine (26a) and 5-hydroxyuracil is about fourfold lower than that of the corresponding trimethylsilylated compounds (Faure et al. 1993; Incardona et al. 1995). The other approach using GC/ECNI involves offline alkylation of oxidized nucleosides to their highly electrophoric pentafluorobenzyl derivatives after initial isolation by either HPLC or solid-phase extraction (Teixeira et al. 1993, 1995). The use of this procedure allows an efficient and sensitive measurement of 8-oxo-7,8-dihydro-2'-deoxyguanosine (47) and 5-(hydroxymethyl)-2'-deoxyuridine (15) at the femtomole level.

Mass spectrometry has been also used online with HPLC separation methods. Thermospray MS associated with reversed-phase HPLC has been applied to the analysis of radiation-induced thymine and thymidine decomposition products. These include, in particular, 5-hydroxy-5,6-dihydrothymine, the *cis* isomer of 5,6-dihydroxy-5,6-dihydrothymine (12a) and the related 2'-deoxyribonucleosides (Berger et al. 1992). The sensitivity of the detection is, at best (for 5-hydroxy-5,6-dihydrothymine), of the order of one modification per 10^5 DNA bases in a sample size of 20 µg DNA. HPLC-MS detection assays were also used to measure OH·-mediated decomposition products of adenine nucleosides, including 8-oxo-7,8-dihydroadenosine (40a) and 8,5'-cycloadenosine (Alexander et al. 1987). This was achieved by utilizing HPLC/tandem mass spectrometry with an atmospheric pressure chemical ionization (APCI) source. Similar approaches were used to monitor the formation of oxidized guanine compounds, including 8-oxo-7,8-dihydroguanine (47a) (Hayakawa et al. 1991) and guanine–malondialdehyde (MDA) adducts (Jajoo et al. 1992). Further devel-

opment is expected in the field with the availability of electrospray as a versatile ionization source.

Mass spectrometric detection provides accurate structural information on base and nucleoside damage. In addition, this method allows precise quantitative measurement when isotopically labeled compounds are used as internal standards. Thus isotopically labeled oxidized DNA bases and nucleosides were synthesized (Stadler et al. 1994; Incardona et al. 1995; Scheller et al. 1995) and used for isotope dilution mass spectrometry analysis (Dizdaroglu 1993; Faure et al. 1993; Hamberg and Zhang 1995; Teixeira et al. 1995).

4
Oxidative Base Damage to DNA in Cells and Organisms

4.1
Cellular DNA Damage: Comparison of the Available Data

The bulk of the available information on the formation of oxidative base damage to cellular DNA has mostly been obtained by using GC-MS and HPLC-EC assays. However, comparison of the available data on the levels of oxidative base damage in cellular DNA reveals striking differences depending largely on the method which was used for the measurement. The best examples of these conflicting results are provided by the results concerning the yields of 8-oxo-7,8-dihydroguanine (47a) and its related 2'-deoxyribonucleoside 47 in DNA exposed to similar conditions of oxidative stress (for a review, see Halliwell and Dizdaroglu 1992). The values obtained by the GC-MS assay are generally one order of magnitude higher than those determined by the HPLC-EC detection method. For example, the background level of 8-oxoGua (47a) in commercial calf thymus DNA as determined by HPLC-EC detection was reported to range between eight and 70 modifications per 10^6 DNA bases. On the other hand, 159–318 8-oxoGua (47a) residues per 10^6 DNA bases were measured by using the GC-MS technique (Halliwell and Dizdaroglu 1992). The observed discrepancies may, in part, be attributed to the DNA hydrolysis method used. Enzymatic digestion is generally used for the HPLC-EC assays, whereas acid hydrolysis is preferred for the GC-MS method (Floyd et al. 1990). It was suggested that the GC-MS assay may lead to some overestimation of the results, whereas the HPLC-EC measurement provides underestimated

data, as remarked in a recent review article (Halliwell and Dizdaroglu 1992). Relevant information was recently obtained from a direct comparison of the results obtained for the measurement of 8-oxo-7,8-dihydroguanine (47a) by the GC-MS and HPLC-EC assays using identical hydrolysis conditions (Ravanat et al. 1995). It was found that the derivatization reaction used prior to the GC-MS analysis induces an artifactual oxidation of guanine (42a) that generates significant amount of 47a. These observations were independently confirmed by Hamberg and Zhang (1995), who showed that the temperature used for the trimethylsilylation of the DNA samples was critical for promoting oxidation of guanine. A method for the derivatization of DNA samples that minimizes the artifactual formation of 47a has been developed (Hamberg and Zhang 1995). Another possibility to prevent the formation of artifactual background of 47a from the large amount of guanine present in the bulk of DNA hydrolysate is to prepurify 8(8-oxo6-a)oxoGua (47a) initially produced. This may be achieved either by immunoaffinity chromatography or HPLC prior to derivatization. Under these conditions, the yields of 47a obtained by either GC-MS analysis or HPLC-EC measurement are similar (Ravanat et al. 1995). The artifactual formation of 47a during GC-MS workup may explain conflicting conclusions drawn from results obtained using the two latter methods. Thus Nagashima et al. (1995) have recently shown by HPLC-EC analysis that the levels of 8-oxoGua (47a) in breast cancer cells are not different from those in surrounding normal tissues, as inferred from HPLC-EC analysis. In addition, the values are about ten times lower than those obtained by CG-MS measurement (Malins and Haimanot 1991).

Interestingly, it was recently shown that not only guanine (42a) may be oxidized during derivatization. This also applies to thymine (1a), cytosine (17a) and adenine (34a) which undergo partial oxidation during trimethylsilylation (Douki et al. 1996b). In particular, 5-formyluracil (16a) and 5-(hydroxymethyl)-uracil (15a) were found to be generated in significant yields during the derivatization of thymine (1a). In addition, derivatization of 17 and 34 gives rise to 5-hydroxycytosine (26a) and 8-oxo-7,8-dihydroadenine (40a) respectively (Douki et al. 1996b). This probably explains why the steady-state level of 5-hydroxycytosine (26a) and 5-hydroxyuracil, as determined by Wagner et al. (1992) using a HPLC-EC assay, is eightfold lower than that obtained by GC-MS (Aruoma et al. 1991). From these observations, it is clear that the previous data obtained by CG-MS analysis have to be considered with caution. Work is in progress in our laboratory aimed at determining the level of electroactive oxidized bases such as

8-oxo-7,8-dihydroadenine (40a), 5-hydroxycytosine (26a), 5-hydroxy-uracil, and formamidopyrimidines by using both the HPLC-EC method and the improved CG-MS assay.

Comparison of the values of 8-oxo-7,8-dihydroguanine (47a) obtained by HPLC-EC measurement are also partly inconsistent with other methods, including immunoassays and DNA repair method. Thus data inferred from immunoaffinity-monoclonal antibody-based ELISA and HPLC-EC assays correlated quite well. In contrast, the levels of 47a determined by ELISA are approximately sixfold higher than those obtained by HPLC-EC (Musarrat and Wani 1994; Yin et al. 1995). On the other hand, values as low as 5×10^7 8-oxoGua (47a) per DNA base pairs were obtained for the control level of 8-oxoGua (47a) from the assay involving the combined use of Fpg DNA repair protein (Boiteux 1993) and the alkaline elution technique (Pflaum et al. 1994). Further work is required to resolve these discrepencies.

4.2
Noninvasive Assays

Another possibility to determine *in vivo* DNA oxidation is to measure oxidized bases and nucleosides in biological fluids (Pryor and Godber 1991). When DNA is damaged, base-specific repair glycosylases may excise the oxidized base; which are then transported through the blood and excreted in urine. In addition, other enzymes excise DNA lesions as short oligonucleotides (Lin and Sancar 1989; Kow et al. 1990) that may be enzymatically converted to nucleosides before excretion in urine. Therefore, the level of the modified bases and nucleosides might reflect the general "oxidative stress" status of individuals. Thus several authors have developed analytical techniques to use the amount of oxidized bases and nucleosides in urine as biological markers of oxidative DNA damage (Shigenaga et al. 1989; Simic 1992).

The analytical techniques that have been developed for this purpose involve the initial prepurification of the modified base from urine. The compounds of interest are then separated and detected using a sensitive assay. Thus the level of thymine glycol (12a) and its corresponding nucleosides, 12 have been determined in human and rat urine (Cathcart et al. 1984). It was found that both 12 and 12a were present in human and rat urine, and it was suggested that these modifications arise at least partly from repair of oxidatively damaged DNA. 8-Oxo-7,8-dihydro-2'-deoxyguanosine (47) has been also detected in human urine (Shigenaga et al. 1989, 1990).

8-OxodGuo (47) was prepurified using a series of solid-phase extractions and analyzed by HPLC with electrochemical detection (Shigenaga et al. 1989, 1990). More recently, the same group has used monoclonal antibodies (Park et al. 1992) for the prepurification step. This allows the determination not only of the modified 2'-deoxyribonucleoside, but also of the free base and the related ribonucleoside. However, the HPLC-EC detection method associated with various prepurification techniques has so far been used for several interesting applications (Loft et al. 1995; Suzuki et al. 1995; Tagesson et al. 1995). Using both methods, changes in the level of urinary excreted 8-oxoGua (47a) was observed. An increase was detected after exposure to reactive oxygen species (Suzuki et al. 1995). The level of 8-oxo-7,8-dihydroguanine (47a) in the urine of smokers was found to be about two times higher than that of nonsmokers (Suzuki et al. 1995). In contrast, a decrease in 47a content was observed in the urine of humans who were fed with potential antioxidants such as Brussels sprouts (Verhagen et al. 1995). A method was also developed for the determination of 5-(hydroxymethyl)-uracil (15a) in human urine involving the use of HPLC for prepurification and GC-MS isotope dilution assay for quantification (Faure et al. 1993). It was shown that the yield of 15a increases in the urine of cancer patients who have received adriamycin (doxorubicin hydrochloride) treatment (Faure et al. 1996).

However, there is still a pending question regarding the origin or origins of the oxidized bases and nucleosides in urine. As already mentioned, it is likely that the release of urinary oxidized bases and nucleosides involves, at least partly, DNA damage processes within cells. However, alternative sources, independent of DNA repair, cannot be ruled out. Thus the level of thymine glycol (12a) (Cathcart et al. 1984) and 8-oxo-7,8-dihydroguanine (47a) (Park et al. 1992) in urine of rat fed with a nucleic acid-free diet (only glucose and water) was found to be 40% and 90% lower, respectively, than that of rat fed a normal diet. This is indicative of at least a partial dietary origin for 12a and 47a. In contrast, the level of the corresponding nucleosides is not affected by the diet. However, it should be remembered that the release of nucleosides is not mediated by the base excision-repair pathway, which is probably the most efficient repair mechanism for most of the oxidized nucleobases. As postulated recently, oxidized nucleosides might derive from degradation of DNA within dead cells. This involves nonspecific nucleases and phosphatases, whereas oxidation may occur during passage through the kidney (Lindahl 1993). It is clear that efforts have to be made to further validate the application of these noninvasive assays. In this

respect, it appears quite important to measure several types of oxidative base damage, including both the base and the nucleoside, under well-established conditions of oxidative stress.

5
Biological Significance of Oxidative Base Damage

The determination of the biological role of oxidative DNA damage mainly requires indirect approaches that are largely based on the use of modified oligonucleotides. In addition, an important parameter deals with the assessment of the repair capacity of the DNA lesions, including the measurement of the specificity and the kinetics of excision of the oxidized bases, nucleotides, or sugar moieties (for recent reviews, see Boiteux 1993; Tchou and Grollmann 1995). In addition, the DNA repair machinery which copes with oxidative DNA damage is completed by the MutT protein (Maki and Sekiguchi 1992; Hayakawa et al. 1995). The latter specific repair protein has been shown to convert 8-oxo-7,8-dihydro-2'-deoxyguanosine 5'-triphosphate into the corresponding nucleoside 5'-monophosphate, preventing misincorporation of the former nucleoside triphosphate into DNA from the nucleotide pool.

5.1
Synthesis of Modified Oligonucleotides
and Conformational Studies

At least three main approaches have been used to prepare site-specific modified oligonucleotides. The most usual method includes the chemical site-specific insertion of a modified nucleotide in a defined sequence of oligonucleotides. This requires the synthesis of the related synthon with the appropriate protecting groups, which have to be compatible with solid-phase oligonucleotide synthesis methods. However, in most cases mild conditions of deprotection are used in order to prevent decomposition of the oxidized nucleotide during the deprotection steps, particurlarly those involving alkaline treatment. The chemical insertion of several oxidized nucleosides into DNA fragments has been achieved. These include 8-oxo-7,8-dihydro-2'-deoxyguanosine (47) (Kuchino et al. 1987; Bodepudi et al. 1991; Moryia et al. 1991; Shibutani et al. 1991; Tchou et al. 1991; Klein et al. 1992; Reddy et al. 1994), 8-oxo-7,8-dihydro-2'-deoxyadenosine (40)

(Guy et al. 1988; Bodepudi et al. 1992; Shibutani et al. 1993), N-(2-deoxy-β-D-erythro-pentofuranosyl)formamide (11) (Guy et al. 1991; Shida et al. 1993, 1994; Baillet and Behr 1995), the 5R* and 5S* diastereoisomers of 1-(2-deoxy-β-D-erythro-pentofuranosyl)-5-hydroxy-5-methylhydantoin (13) (Guy et al. 1993), 5-(hydroxymethyl)-2'-deoxyuridine (15) (Sowers and Beardsley 1993), and 5-formyl-2'-deoxyuridine (16) (Ono et al. 1994; Berthod et al. 1996). Interestingly, 3-(2-deoxy-β-D-erythro-pentofuranosyl)-pyrimido[1, 2-a] purin-10(3H)-one (78), the main malondialdehyde adduct to 2'-deoxyguanosine (42) has been site-specifically incorporated into oligonucleotides (Reddy and Marnett 1995) using 2-(acetoxymethyl) benzoyl protecting groups. This has avoided the use of alkaline conditions during synthesis. In addition, $1,N^2$-propano-2'-deoxyguanosine, a more stable analogue of the latter adduct, has been inserted into short DNA fragments (Marinelli et al. 1990; Weisenseel et al. 1995) using the convential solid-state methodology. A second approach, which may represent an alternative to chemical methods for the insertion of unstable nucleotides into oligonucleotides, is based on the use of specific enzymes with appropriate substrates. RNA ligase was found to be able to insert 8-oxo-7,8-dihydro-2'-deoxyguanosine (47) as its 3',5'-diposphate ester in short DNA fragments (Wood et al. 1990). More recently, a less time-consuming enzymatic method has been developed for the incorporation of several oxidized nucleosides as their 5'-triphosphate derivatives. Thus 5,6-dihydroxy-5,6-dihydrothymidine (12), 5-hydroxy-2'-deoxycytidine (26), 5-hydroxy-2'-deoxyuridine, 8-oxo-7,8-dihydro-2'-deoxyadenosine (40) and 8-oxo-7,8-dihydro-2'-deoxyguanosine (47) have been successfully site-specifically incorporated into short oligonucleotides (Hatahet et al. 1993) that were extented using the phage T4 DNA ligase (Hatahet et al. 1993). A third approach, which was mostly used for the introduction of the alkali-labile thymidine glycol, (12) was based on the specific oxidation of the unique thymine residue within the sequence of oligonucleotides by either osmium tetraoxide (Clark and Beardsley 1987; Basu et al. 1989) or potassium permanganate (Kao et al. 1993).

Relevant information on the conformational changes induced by the presence of a modified nucleoside within oligonucleotides has been inferred from detailed ^{1}H NMR analysis. The DNA duplexes containing the cis diastereoisomers of 5,6-dihydroxy-5,6-dihydrothymidine (12) are close to the normal B structure with, however, a large localized conformational change in the vicinity of the lesion (Kao et al. 1993). In addition, the thermal stability of the duplex DNA was found to be significantly decreased. Only

significant structural changes were also observed at the site of 8-oxo-7,8-dihydro-2'-deoxyadenosine (40) within duplex oligonucleotides (Guschlbauer et al. 1991). The latter modified purine nucleoside was found to mostly exist in the 8-keto form with an *anti* orientation within the oligonucleotides. Similarly, only significant conformational changes were observed in the vicinity of 8-oxo-7,8-dihydro-2'-deoxyguanosine (47) in a self-complementary dodecanucleotide. In particular, 8-oxodGuo (47) was found to be base-paired to cytosine in a Watson-Crick hydrogen bond arrangement within a B-form structure (Oda et al. 1991). Similarly, 8-oxodGuo (47) forms a stable pair opposite dAdo (34) in a duplex DNA (Kouchakdjian et al. 1991). It was also shown that the oxidized purine nucleoside is mostly in a 6,8-diketo form with a preferential *syn* orientation in both types of duplexes. Further confirmation of the lack of sigificant changes in the overall conformation of duplex oligonucleotides containing 8-oxodGuo (47) with different opposite bases was provided by a circular dichroism study (Plum et al. 1995). However, it was shown that the presence of 47 influences the thermal and thermodynamic features of the duplexes. 5-(Hydroxymethyl)uracil (15a) may mispair with guanine (42a) through either a partly ionized Watson-Crick structure or a wobble base pair (Mellac et al. 1993).

Interesting information on the conformational properties of modified oligonucleotides containing 8-oxopurine bases was inferred from several X-ray cristallography studies. In particular, similarity in the structure of 8-oxo-7,8-dihydroguanine (47a)–adenine mispair was observed in aqueous solution (Kouchadjian et al. 1991) and in the solid state (Brown et al. 1993; McAuley-Hecht et al. 1994). In a recent X-ray study, it was shown that 8-oxo-7,8-dihydroguanine (47a) forms a Watson-Crick base pair with opposite cytosine (Lipscomb et al. 1995). However, in contrast to what was observed in the liquid phase, the oxidized guanine moiety exhibits a preferential *anti* orientation. It should be added that an unusual guanine (*anti*)-8-oxo-7,8-dihydroadenine (40a) (syn) wobble conformation was noticed in the X-ray structure of a synthetic dodecamer (Leonard et al. 1992).

Restrained molecular dynamics studies based on the use of nuclear Overhauser effect data have provided complementary structural information on duplex DNA containing 5,6-dihydroxy-5,6-dihydrothymidine (12) (Kao et al 1993) and 1,N^2-propano-2'-deoxyguanosine (Weisenseel et al. 1995). Forthcoming theoretical investigations of modified oligonucleotides should involve the combined utilization of molecular dynamics and *ab*

initio approaches. This should be the case for the *cis* diastereoisomers of 5,6-dihydroxy-5,6-dihydrothymidine, (12) for which detailed conformational features obtained from *an ibitio* calculations are now available (Jolibois et al. 1996).

5.2
DNA Replication and Mutagenesis

Thymidine glycol 12 has been shown to be an efficient blocking lesion in several replication studies involving DNA polymerases (Ide et al 1985; Basu et al. 1989; Rouet and Essigmann 1985; Clark and Beardsley 1986, 1987; Basu and Essigmann 1988; Evans et al. 1993). However, the latter lesion 12 may be bypassed in a peculiar sequence context such as 5'-cytosine-thymine glycol 12a-purine-3' (Hayes and Leclerc 1986). 5-(Hydroxymethyl)uracil (15a) does not appear to be strongly mutagenic, since replication investigations have shown that, in most cases, the correct adenine base is incorporated opposite 15a (Mellac et al. 1993). Two main oxidized cytosine lesions, including 5-hydroxycytosine (26a) and 5-hydroxuracil, were found to be efficiently bypassed in *in vitro* DNA polymerase studies (Purmal et al. 1994). However, both 5-hydroxycytosine (26a) and 5-hydroxyuracil may be considered as premutagenic lesions, as inferred from the observation of misincorporation of adenine opposite the lesions, depending on the sequence context.This is particularly true for 5-hydroxyuracil. The incorporation of guanine opposite 8-oxo-7,8-dihydroadenine (40a) was found to occur with a very low efficiency on a short DNA template in the course of *in vitro* translesional synthesis (Shibutani et al. 1993). A lack of mutation was noticed upon transfection into *E. coli* cells of a single-strand phage DNA containing a single 8-oxo-7,8-dihydroadenine (40a) residue. On the other hand, the presence of 8-oxo-7,8-dihydroguanine (47a) may lead to 0.3% of G→T transversions (Wood et al. 1990). The latter observation is in agreement with similar results obtained in plasmid replication studies using a single-strand gap (Moriya et al. 1991) and a double-stranded phage vector (Cheng et al. 1992). Similarly, 8-oxodGuo (47) to T targeted tranversions were also observed in mammalian cells with the oxidized guanine positioned at a unique site in episomal DNA (Moriya 1993; Le Page et al. 1995). The extrachromosomal DNA used for the latter replication study consisted of a fragment of human Ha-*ras* bearing a single 47a that was subsequently inserted into the shuttle plasmid. These results are consistent with translesional studies which have shown that cytosine or adenine might

be incorporated opposite 8-oxodGuo (47) of the template (Shibutani et al. 1991).

Apurinic sites are likely mutagenic lesions, since preferential incorporation of adenine opposite the damage was observed using synthetic DNA as the templates (Kunkel et al. 1983; Shager and Strauss 1983). It may be added that 3'-glycolate end, a major OH·-mediated oxidation product of the sugar moiety of DNA is a blocking lesion (Henner et al. 1983b).

6
Conclusions

The present review illustrates the complexity of the oxidation reactions of DNA. Further efforts should be made to investigate in greater depth the formation of complex oxidative DNA lesions, including clustered base damage and DNA-protein cross-links. It is also clear that there is still a need for accurate and sensitive methods to detect oxidative damage within cellular DNA. Further developments should involve both molecular biology techniques such as the polymerase chain reaction (PCR) approach and highly resolutive analytical methods such as capillary electrophoresis. In particular, the association of the PCR technique with monoclonal antibodies against DNA damage should provide a much higher sensitivity than the currently available immunological assays. The capillary gel electrophoresis technique coupled with either fluorescence or mass spectrometry (Tseng et al. 1994; Norwood et al. 1993) offers interesting potential. In this respect the availability of electrospray mass spectrometers with an ion trap device is particularly relevant for both structural characterization of the damage and improvement in the sensitivity of detection. Another sensitive method for the measurement of oxidative DNA damage in single cells, the so-called comet assay, has already been used in interesting applications (Fairbairn et al. 1995; Ashby et al. 1995; Nocentini 1995). It should be added that noninvasive assays are particularly relevant for epidemiological studies. However, validation of the methods will require further investigation. Finally, there is still a considerable need for information on the biological role of oxidative DNA damage.

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Metabolism of Cyclic ADP-Ribose: A New Role for NAD$^+$ Glycohydrolases

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Contents

1
Introduction

The existence of enzymatic activities that cleave the nicotinamide-ribose bond in NAD^+ has been known for more than 50 years (Handler and Klein 1942). This pathway was regarded as a catabolic route of NAD^+, whose important role as a coenzyme in biological oxidation-reduction reactions was well recognized. However, the amount of energy required for the resynthesis of NAD^+ from ADP-ribose and nicotinamide questioned its continuous formation and degradation to be a reasonable physiological process. The discovery of ADP-ribosylation (Chambon et al. 1963) and the realization of its biological role has changed the view of the reaction catalyzed by NAD^+ glycohydrolases (NADases, EC 3.2.2.5 and 6). As compared to ADP-ribosyl transferases, NADases would transfer ADP-ribose onto water rather than protein, possibly forming a metabolite with biological function. It has been suggested that, as a subsequent reaction, free ADP-ribose could specifically modify proteins via a nonenzymatic mechanism (reviewed in Jacobson et al. 1994; Richter and Kass 1991). Another aspect of potential physiological significance of NAD^+ glycohydrolases was realized following the demonstration that a cell differentiation marker, CD38, possesses NADase activity (Gelman et al. 1993; Howard et al. 1993; Kontani et al. 1993; Summerhill et al. 1993; Takasawa et al. 1993a; Zocchi et al. 1993).

Recent studies have led to the discovery of cyclic ADP-ribose (Clapper et al. 1987; Lee et al. 1989), a potent intracellular Ca^{2+}-mobilizing metabolite. Cyclic ADP-ribose (cADPR) is synthesized from NAD^+ by enzymes previously known as NAD^+ glycohydrolases. Some ADP-ribosyl cyclases have been shown to be bifunctional in that they also catalyze the hydrolysis of cyclic ADP-ribose to ADP-ribose (H. Kim et al. 1993a; Howard et al. 1993; Takasawa et al. 1993a; Zocchi et al. 1993; Muller-Steffner et al. 1994). Therefore, recent developments in this field let appear NAD^+ glycohydrolases to function as key regulators of intracellular calcium signalling.

2
NAD$^+$ Glycohydrolases

NAD$^+$ glycohydrolases catalyze the hydrolysis of the β-*N*-glycosidic link-age between the nicotinamide and the ADP-ribose moieties of NAD$^+$ (Fig. 1, reaction 1). For most eucaryotic enzymes the reaction mechanism appears to include a stabilized protein-ADP-ribose intermediate permit-ting a pyridine base exchange (transglycosidation). This feature of NADases has been utilized to synthesize several analogs of NAD$^+$ contain-ing pyridine bases such as nicotinic acid or 3-acetyl pyridine (Kaplan and
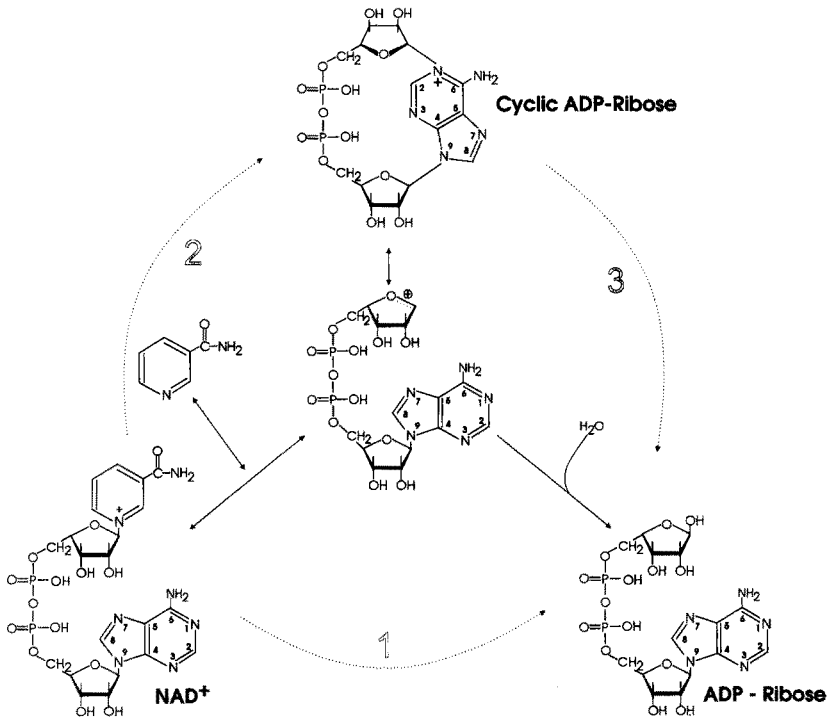
Fig. 1. Proposed catalytic mechanism of NAD$^+$ glycohydrolases and cADP-ribosyl synthases/hydrolases. The reactions proceed via an oxocarbenium ion intermediate (*center*). The NAD$^+$ glycohydrolase reaction (1), the cyclase reaction (2), and the cADPR hydrolase reaction (3) are indicated. The reversible reaction from NAD$^+$ to the oxocar-benium ion intermediate represents the transglycosidation reaction if another pyridine base (e.g., nicotinic acid) is present

Ciotti 1954; Zatman et al. 1954; Schuber et al. 1976; Anderson and Anderson 1984). The ability of several of these enzymes to synthesize cyclic ADP-ribose will be discussed in the subsequent section.

The occurrence, characteristics, and assays of NAD$^+$ glycohydrolases have been comprehensively reviewed by Price and Pekala (1987). Here, only some major aspects regarding the NAD$^+$ glycohydrolase reaction will be covered.

2.1
Occurrence

NAD$^+$ glycohydrolases have been detected in a variety of species including procaryotes and eucaryotes. While all known NADases of procaryotic origin are soluble proteins (Price and Pekala 1987), the majority of these enzymes from mammalian tissues are membrane-bound. Naturally occurring soluble forms of mammalian NADases have been found only in bovine seminal fluid (Yuan and Anderson 1971) and bovine brain (Yamauchi and Tanuma 1994). The NADases of other nonmammalian eucaryotes, for example, *Neurospora crassa* or *Bungarus fasciatus*, are soluble proteins (Price and Pekala 1987). The NAD$^+$ glycohydrolase from *Aplysia californica* is soluble. However, this enzyme appears to function almost exclusively as ADP-ribosyl cyclase (Hellmich and Strumwasser 1991; Lee and Aarhus 1991).

Mammalian NADases have been isolated from a variety of tissues, for example, from erythrocytes (Pekala and Anderson 1978; Kim et al. 1993; Zocchi et al. 1993), spleen (Swislocki et al. 1967; Schuber and Travo 1976), liver (Swislocki et al. 1967; DiAugustine et al. 1978; Moser et al. 1983; J. Zhang et al. 1995), testes (Yuan and Anderson 1971), kidney and brain (Swislocki et al. 1967; Yamauchi and Tanuma 1994). The subcellular localization of many of these enzymes has so far not been conclusive. Enzyme activity has been detected in plasma membrane and microsomal fractions (Price and Pekala 1987). NADase activity appears also to be associated with mitochondrial membranes (Lötscher et al. 1980; Hilz et al. 1984; Masmoudi and Mandel 1987; Ziegler et al. 1996a). The NADase activity of erythrocytes has been found on the outer surface of the plasma membrane (U.-H. Kim et al. 1993; Lee et al. 1993a).

2.2
Structure

Although NAD$^+$ glycohydrolases have been known for a long time, little information is available as to the primary structure of these enzymes. In fact, considering NADases from eucaryotes, cDNA and deduced amino acid sequences have been reported only for those from *Aplysia* (Glick et al. 1991; Nata et al. 1995), as well as CD38 (Jackson and Bell 1990; Harada et al. 1993; Koguma et al. 1994) and BST-1 (Kaisho et al. 1994; Dong et al. 1994; Itoh et al. 1994; Furuya et al. 1995) from human and rodent species. These enzymes have been of major interest due to their ability to form cyclic ADP-ribose. The RT6 T cell differentiation markers have also been shown to possess NADase activity (Takada et al. 1994; Haag et al. 1995).

Several bacterial toxins exerting NADase activity have been reported. These toxins catalyze a monoADP-ribosyl transfer reaction leading to modification of highly specific target proteins in the host cell and the subsequent pathological response (Moss and Vaughan 1988). Their NADase activity occurs apparently only in the absence of the acceptor protein.

The amino acid composition of NADases from rat liver microsomes (DiAugustine et al. 1978), bovine seminal fluid (Yuan et al. 1972), *Neurospora crassa* (Everse et al. 1975), *Streptococcus pyogenes* (Grushoff et al. 1975), and *Bacillus subtilis* (Everse et al. 1975) has been compared (Price and Pekala 1987). It is striking that both the *Neurospora* and the *Bacillus* enzymes do not contain any cysteine or tryptophan residues. As will be discussed in a later section, cysteine residues appear to be essential for the metabolism of cyclic ADP-ribose by NAD$^+$ glycohydrolases.

Estimates of molecular masses have been derived primarily from SDS-PAGE or gel filtration analyses. Since a number of NADases contain significant amounts of carbohydrates, these data may not be easily compared with respect to potential similarity. For example, the degree of glycosylation was determined in the NADases from *Bacillus subtilis* and *Neurospora crassa*. In both cases the sugars contributed substantially to the dry weight, namely about 55% and about 80%, respectively (Everse and Kaplan 1968; Everse et al. 1975). In another report the carbohydrate content of the *Neurospora* enzyme was estimated to be about 20% (Menegus and Pace 1981). The human CD38 migrated in SDS-PAGEs according to an apparent molecular mass of 46 000, while the calculated mass of the polypeptide chain amounts only to about 34 000 (Jackson and Bell 1990). The esti-

Table 1. Properties of some NAD$^+$ glycohydrolases

Enzyme source	Cellular location	Mol. weight (kDa)	V$_{max}$ (U/mg)	K$_m$ (μM)	pH optimum	Effect of thiol addition	Glyco-sylation	Cyclase Activity	References
Human CD38	Plasma membrane, outer surface	46	89.0	14.8	6.5	Inactivation	Yes	Yes	Jackson and Bell 1990; Kontani et al. 1993; Howard et al. 1993; Zocchi et al. 1993; 1995; Summerhill et al. 1993; Takasawa et al. 1993a; Tohgo et al. 1994; Graeff et al. 1994a, b; Malavasi et al. 1994; Inageda et al. 1995
Neurospora crassa	Conidia	33	51 000	500	NR	NR	Yes	No	Graeff et al. 1994b; Menegus and Pace 1981; Everse et al. 1975
Calf spleen	Microsomes Plasma membrane	30	180	60	9.0, broad	NR	Yes	Yes	Schuber and Travo 1976; Schuber et al. 1980; Muller-Steffner et al. 1993, 1994
Bovine liver	Mitochondria	32	17	10.0[a]	8.5	Inactivation	NR	Yes	J. Zhang et al. 1995; Ziegler et al. 1996a, b
Pig brain	Microsomes	26	127	65	broad	Inactivation	NR	NR	Price and Pekala 1987; Cayama et al. 1973
Bungarus fasciatus	Extracellular	62	1 380	14	7.5, broad	NR	Yes	Yes	Yost and Anderson 1981; 1982; H. Kim et al. 1993a
Streptococcus pyogenes	Extracellular	55	890	0.5	7.5	NR	NR	Yes	Karasawa et al. 1995; Grushoff et al. 1975

NR, Not reported.
[a] With ε-NAD$^+$ as substrate.

mated molecular masses of some known NADases are summarized in Table 1.

Some of the enzyme preparations from mammalian tissues have been obtained using crude pancreatic lipase (steapsin) for solubilization. As apparently both lipolytic as well as proteolytic activities are required for the solubilization by steapsin (Green and Bodansky 1965; Swislocki et al. 1967; Bock et al. 1971; Schuber and Travo 1976), the original size of the membrane-bound form has remained unknown. In two instances the original size was compared to the steapsin-solubilized form. While the apparent molecular mass of the enzyme from calf spleen microsomes was reduced from about 30 000 (Muller-Steffner et al. 1993) to about 24 000 (Schuber and Travo 1976), the NADase isolated from bovine liver mitochondria exhibited an apparent molecular mass of about 28 000 after the steapsin treatment as compared to its original size of about 30 000 (Ziegler et al. 1996a). Considering these variabilities, direct comparison of NADases from different sources regarding their size has only limited value unless molecular data is available.

So far little is known about the tertiary and quaternary structure of NADases. The available evidence suggests that the active form of these enzymes consists of a single polypeptide chain.

A number of NADases is sensitive to reducing agents such as dithiothreitol (Cayama et al. 1973; Kontani et al. 1993; Zocchi et al. 1995; Ziegler et al. 1996b) suggesting the presence of an essential disulfide bridge. It is unlikely that such a disulfide bridge would link two subunits, because the mobility of these enzymes on SDS-PAGEs is virtually unaffected by reducing agents (Jackson and Bell 1990; Ziegler et al. 1996a).

Models have been presented for the structure of CD38 (Malavasi et al. 1994; Jacobson et al. 1995a) considering the mode of anchoring within the membrane and the presence of disulfide bridges in the extracellular domain.

2.3
Kinetic Properties

Studies using either detergents or steapsin for solubilization of membrane-bound enzymes from calf spleen (Schuber and Travo 1976) or bovine liver (Ziegler et al. 1996a) indicated that the steapsin treatment did not affect the catalytic properties. Therefore, values obtained from steapsin-solubilized enzymes are likely to represent those of the intact forms. Table 1 lists kinetic

characteristics of some NADases. Both K_m and V_{max} values vary widely among these enzymes. Most mammalian NADases exhibit K_m values around or below 100 μM. In general, enzymes from lower organisms exhibit a significantly lower substrate affinity (Price and Pekala 1987).

Product inhibition has been reported for most of the known mammalian NADases. While ADP-ribose inhibits competetively, the inhibition by nicotinamide is of the noncompetitive type. Only the enzyme from bovine erythrocytes has been detected to be competetively inhibited by nicotinamide and noncompetetively by ADP-ribose (Pekala and Anderson 1978).

NADases exhibit a high specificity towards the β-configuration of NAD$^+$. None of these enzymes would cleave α-NAD$^+$. NADP$^+$ serves as a substrate, but less efficiently. A number of analogs of NAD$^+$ have been utilized for the characterization of NADases. Of them the most valuable has been the fluorescent analog 1,N^6-etheno-NAD$^+$ (ε-NAD$^+$). The fluorescence enhancement after separation of the quenching nicotinamide moiety from the modified fluorescent adenine (Barrio et al. 1972) has been widely used as a simple continous assay of NADase activity. The known NADases appear to tolerate modifications in the adenine as well as in the nicotinamide moieties to varying extent (Price and Pekala 1987).

The NADase from calf spleen microsomes is one of the most thoroughly studied mammalian NADases. It was recently found that this enzyme also catalyzes the hydrolysis of cyclic ADP-ribose (Muller-Steffner et al. 1994). Calf spleen NADase catalyzed the hydrolysis of a number of NAD$^+$ analogs (Schuber et al. 1978; 1979), as well as a transglycosidation reaction using 3-acetylpyridine as pyridine base (Schuber et al. 1976). These properties were consistent with a kinetic mechanism best described by an ordered ping-pong bi-bi model. From the observation that the reaction products of the transglycosidation retained the configuration it was concluded that the reaction proceeded via an intermediary complex of the enzyme with ADP-ribose. The available evidence suggests this complex to be a stable, solvent-equilibrated oxocarbenium ion (Bull et al. 1978; Schuber et al. 1979; Tarnus et al. 1988; Oppenheimer 1994). Therefore, hydrolysis and transglycosidation may be regarded as competition between water and a pyridine base for this complex (Fig. 1).

3
ADP-Ribosyl Cyclases (Cyclic ADP-Ribose Synthases)

Only a decade ago it was discovered by Lee and coworkers (Clapper et al. 1987) that a metabolite of NAD^+ greatly stimulated the release of Ca^{2+} from sea urchin egg microsomes. It was found that the pathway of Ca^{2+} mobilization was independent but as potent as that of inositol 1,4,5-trisphosphate (IP_3). This metabolite was in subsequent studies shown to be a cyclic analog of ADP-ribose and, therefore, termed cyclic ADP-ribose. Since then it has received enormous attention and it is now beyond doubt that cyclic ADP-ribose represents a key regulator of the calcium homeostasis in a variety of cells.

3.1
Structure of Cyclic ADP-Ribose

Structural studies of cyclic ADP-ribose have revealed an unprecedented form of nucleotide. Figure 1 presents the structure of this metabolite. The enzymatic synthesis of cyclic ADP-ribose includes the cleavage of the nicotinamide-ribose bond followed by the cyclization of the anomeric carbon of the ribose to the N^1 nitrogen of the adenine ring (Fig. 1, reaction 2). This structure has been confirmed using several approaches including mass spectrometry (Lee et al. 1989), NMR (Lee et al. 1989; Wada et al. 1995), and UV-spectroscopy (H. Kim et al. 1993b). Subsequently, the crystal structure of cyclic ADP-ribose has been obtained demonstrating in addition the retention of the β-configuration (Lee et al. 1994a). This aspect has been established also using NMR techniques (Jacobson et al. 1995b).

As a consequence of the cyclization linkage to the N^1 atom, the adenine ring becomes positively charged at physiological pH (Fig. 1). The pK_a of the protonated group is about 8.3 (Jacobson et al. 1995b). The question arises as to whether this feature may be of importance for the physiological function of cyclic ADP-ribose. Both circular dichroism and nuclear magnetic resonance properties of cyclic ADP-ribose indicated a pH-dependent existence of at least two distinct conformations (Jacobson et al. 1995b).

3.2
NAD$^+$ Glycohydrolases as ADP-Ribosyl Cyclases

The first enzyme shown to catalyze the formation of cyclic ADP-ribose from NAD$^+$ was isolated from ovotestis of the mollusc *Aplysia californica* (Hellmich and Strumwasser 1991; Lee and Aarhus 1991). At that time it was referred to as NAD$^+$ glycohydrolase, because it appeared to generate ADP-ribose and nicotinamide from NAD$^+$. However, later on it was demonstrated that this enzyme functions virtually exclusively as ADP-ribosyl cyclase, forming cADPR, and did not appear to produce any free ADP-ribose (Lee and Aarhus 1991). In the mean time, a number of enzymes, previously known as NAD$^+$ glycohydrolases, have been demonstrated to possess cyclase activity (Table 1). Recently, ADP-ribosyl cyclase activity has been reported for a procaryotic enzyme (Karasawa et al. 1995). Besides the *Aplysia* enzyme, an ectoenzyme of red blood cells and lymphocytes, CD38, has been extensively characterized in various mammals (Malavasi et al. 1994; Lund et al. 1995). The NAD$^+$ glycohydrolase and ADP-ribosyl cyclase isolated from canine spleen (H. Kim et al. 1993a) would appear to be the canine homolog of CD38 (Jacobson et al. 1995a). The gene encoding the protein BST-1 has been cloned from a bone marrow stromal cell line (Kaisho et al. 1994). This protein is homologous to CD38 and the *Aplysia* enzyme and was shown to possess ADP-ribosyl cyclase activity (Hirata et al. 1994; Table 2). Other NADases capable of synthesizing cyclic ADP-ribose have been detected in brain from dog, chicken, and salamander as well as in sea urchin eggs (Lee 1994). The NADase isolated from bovine liver mitochondria did also exhibit cyclase activity (Ziegler et al. 1996b).

It should be noted that some NAD$^+$ glycohydrolases do not appear to have the ability to produce cyclic ADP-ribose. For example, the NADase from *Neurospora crassa* did not catalyze the formation of the cyclic metabolite (Graeff et al. 1994b; Fig. 2). On the other hand, as pointed out above, the cyclase from *Aplysia* is apparently unable to catalyze a conventional NAD$^+$ glycohydrolase reaction yielding ADP-ribose. In any case, formation of cyclic ADP-ribose would appear to be accomplished by a class of enzymes that is related to NAD$^+$ glycohydrolases, since on a molecular basis the *Aplysia* enzyme is highly similar to CD38 (States et al. 1992), which is capable of catalyzing both reactions.

ADP-ribosyl cyclase activity has been detected in a great variety of cells and tissues (Rusinko and Lee 1989). The occurrence of this type of enzyme in molluscs, snake venom, sea urchins, mammals, and other organisms

suggests that it is widespread and may serve a fundamental function, for example, in calcium signalling.

However, the problem remains as to the function of "classical" NADases, that is, those that do not catalyze the cyclization of ADPR.

3.3
Structure and Properties of ADP-Ribosyl Cyclases

The first cDNA of an ADP-ribosyl cyclase was cloned and sequenced by Glick et al. (1991) from the mollusc *Aplysia californica*. The deduced amino acid sequence showed no significant similarity to any known protein. Only following comparison to the human leukocyte marker CD38 (Jackson and Bell 1990) it was obvious that ADP-ribosyl cyclases may represent a protein family of their own (States et al. 1992). As mentioned before, several homologs of CD38 have been cloned and sequenced from other mammals.

The molecular mass of the enzyme from *Aplysia* as calculated from the amino acid sequence amounts to about 30 000 (Glick et al. 1991) which is in good agreement with its mobility on SDS-PAGE (Hellmich and Strumwasser 1991; Lee and Aarhus 1991; Fig. 2). On the other hand, while the human CD38 has a calculated molecular mass of about 34 000, its mobility on SDS-PAGE corresponds to a molecular mass of about 46 000 (Jackson and Bell 1990). It is likely that this apparent discrepancy is due to the carbohydrate content of this ectoenzyme. Its primary structure contains four potential glycosylation sites (Table 2).

As opposed to the cytosolic ADP-ribosyl cyclase from *Aplysia*, CD38 is a typical type II membrane glycoprotein. It contains a short cytoplasmic domain and a single transmembrane helix. The majority of the peptide chain, including the catalytic site, is located at the external surface. This location of the catalytic center has raised the problem regarding the potential role of CD38 in intracellular calcium signalling. The possibility is being discussed that this enzyme may exert its catalytic function within the cell following internalization by endocytosis. In such a case, at least a transient occurrence of an intracellular form of CD38, perhaps, even solubilized by specific cleavage from the membrane, should be found.

A similar situation has to be considered for BST-1, a membrane-bound protein with structural and functional similarities to CD38 (Kaisho et al. 1994). BST-1 appears, however, to be attached to the membrane via a GPI-anchor (Kaisho et al. 1994).

Table 2. Characteristics of ADP-ribosyl cyclases from *Aplysia californica*, human CD38, and human BST-1

	Aplysia Cyclase	CD38	BST-1
M$_r$, calculated	29 500	34 000	30 200
M$_r$, SDS-PAGE	29 000	46 000	43 000
Potential glycosylation sites	None	4	4
Amino acid identity with CD38	30%	100%	33%
Subcellular localization	Cytosol, granules	Cell surface	Cell surface
Mode of anchoring in the membrane	–	Transmembrane protein	GPI
Occurrence	Ovotestis	Leukocytes, erythrocytes, pancreatic islet cells	Bone marrow stromal cells, pancreatic islet cells, and others

3.4
Cyclic ADP-Ribose Hydrolase Activity

Since cyclic ADP-ribose acts as a potent Ca^{2+}-mobilizing agent, the require-
ment for an efficient way of inactivation of this metabolite is obvious.
Indeed, an enzymatic activity catalyzing the hydrolysis of cADPR to ADPR
(Fig. 1, reaction 3) has been detected in a variety of animal tissues (Lee and
Aarhus 1993). Surprisingly, only ADP-ribosyl cyclases have been shown to
catalyze the reversal of the cyclization liberating free ADP-ribose (H. Kim
et al. 1993a; Howard et al. 1993; Takasawa et al. 1993a; Zocchi et al. 1993;
Muller-Steffner et al. 1994), which is biologically inactive. Therefore, regu-
lation of the level of cyclic ADP-ribose would lie exclusively with the
ADP-ribosyl cyclases. However, the enzyme from *Aplysia* does not itself
hydrolyze cyclic ADP-ribose (Lee and Aarhus 1991) raising the possibility
of the existence of a separate hydrolase activity or even an alternative
degradation pathway. In addition, if cyclic ADP-ribose were to be a second
messenger, it would appear unlikely that its formation and degradation
depended on an equilibrium maintained by a single class of enzymes. In
this respect the possibility that ADP-ribosyl cyclase may be regulated via a
cyclic GMP-dependent kinase receives further importance. It has been
demonstrated that cGMP stimulated the formation of cyclic ADP-ribose in

sea urchin eggs (Galione et al. 1993a). In addition, the amino acid sequences of both CD38 and the cyclase from *Aplysia* contain potential phosphorylation sites for cGMP-dependent kinase (Lee et al. 1994b). Therefore, the cyclase or hydrolase activity of these enzymes may be switched by phosphorylation. However, so far no direct experimental evidence for such a regulation has been presented.

3.5
Reaction Mechanism of the Bifunctional ADP-Ribosyl Cyclase/ Cyclic ADP-Ribose Hydrolase Enzymes

The possibility that ADP-ribosyl cyclases themselves may hydrolyze cyclic ADP-ribose to ADP-ribose was first demonstrated by Kim et al. (1993a) using an enzyme isolated from canine spleen as well as from *Bungarus fasciatus*.

The NAD^+ glycohydrolase activity associated with ADP-ribosyl cyclases may possibly be ascribed to the sequential occurrence of synthesis (from NAD^+) and hydrolysis of cyclic ADP-ribose. Assuming the existence of a stable enzyme-ADP-ribosyl intermediate, hydrolysis of NAD^+ could also be regarded as a competition of water with the N^1 atom of the adenine ring (acceptor in the cyclase reaction) for the anomeric carbon of the terminal ribose linked to the enzyme. Only the latter mechanism would appear to account for the different velocities observed for the NAD^+ glycohydrolase, ADP-ribosyl cyclase and cyclic ADP-ribose hydrolase activities: CD38 has been reported to possess these activities in a ratio of roughly 100:1:10 (Zocchi et al. 1993). If the NADase activity were a result of two consecutive reactions, the rate at which added cyclic ADP-ribose is hydrolyzed should at least equal that of NAD^+ hydrolysis. On the basis of kinetic studies using a calf spleen NADase, a reaction mechanism has been derived that includes an enzyme-stabilized ADP-ribosyl oxocarbenium ion intermediate that can be formed from either NAD^+ or cyclic ADP-ribose (Muller-Steffner et al. 1994). The proposed mechanism is depicted in Fig. 1. From this model, the formation of NAD^+ from cyclic ADP-ribose and nicotinamide would also be predicted. In fact, this reaction was shown to take place (H. Kim et al. 1993a), whereas free ADP-ribose is unlikely to be a substrate for the generation of an oxocarbenium ion intermediate, as it lacks the energy conserved in the glycosidic bond. Under in vivo conditions, the availability of a certain substrate, for example, water or pyridine base, to the catalytic center may be regulated by conformational changes. Consequently, either

cyclase or hydrolase may be favored depending on the conditions. A possible way for such a mechanism would be cGMP-dependent (Galione et al. 1993a) or calmodulin-dependent (Takasawa et al. 1995) phosphorylation. The successful crystallization of the enzyme from *Aplysia* (Prasad et al. 1996) will provide further insight into the catalytic mechanism of this class of enzymes.

3.6
Role of Cysteine Residues

Analysis of the primary structures of the *Aplysia* ADP-ribosyl cyclases and CD38 from several mammals revealed, besides a high degree of overall similarity, the conservation of most of the cysteines. Two cycteines corresponding to C119 and C201 of the human CD38 are present in all CD38 sequences, but not in those from *Aplysia californica* or *Aplysia kurodai* ADP-ribosyl cyclases. Introduction of cysteines at the respective positions of the enzyme from *Aplysia kurodai* by site directed mutagenesis conferred cyclic ADP-ribose hydrolase activity to this enzyme (Tohgo et al. 1994). It was also shown that the presence of both cysteines was required for this activity. CD38 mutants in which these cysteines (either individually or together) were substituted with the corresponding amino acids of the *Aplysia* enzyme retained cyclase activity, but lacked cyclic ADP-ribose hydrolase activity (Tohgo et al. 1994).

While the mammalian cyclases (CD38 and the enzyme isolated from bovine liver mitochondria) are highly sensitive towards reducing agents (Guida et al. 1995; Ziegler et al. 1996b), the *Aplysia* enzyme is not (Zocchi et al. 1995; Inageda et al. 1995). Treatment of CD38 with reducing agents (DTT or β-mercaptoethanol) led to aggregation of the protein into an oligomeric complex (Franco et al. 1994; Zocchi et al. 1995). Moreover, aggregation and concomitant enzyme inactivation were prevented by crosslinking the enzyme to the erythrocyte membrane with glutaraldehyde prior to the addition of reducing agents (Guida et al. 1995). These observations were interpreted to rule out any catalytic function of cysteine residues suggesting only a structural role, namely to maintain a functionally active monomeric structure. Perhaps, this apparent contradiction to the mutagenesis experiments (Tohgo et al. 1994) will be resolved, when the cysteine residues involved in potential disulfide bridge(s) will be identified. It would be interesting to test, whether the mutated CD38 (C119 K/C201E) having lost the hydrolase but retaining the cyclase activity is still sensitive

to reducing agents. Also, it has not been reported, whether the mutated *Aplysia* enzyme (K95C/E176C), gaining hydrolase activity, has also been rendered thiol-sensitive.

3.7
Synthesis of Other Pyridine Nucleotide Derivatives
with Calcium-Mobilizing Activity by ADP-Ribosyl Cyclases

According to a number of recent studies, the ability of most ADP-ribosyl cyclases to catalyze the hydrolysis of NAD^+ to ADP-ribose, as well as the synthesis and hydrolysis of cyclic ADP-ribose does still not exhaust the repertoire of these enzymes. At least some of these enzymes are capable of utilizing analogs of NAD^+ as substrates. For example, analogs containing a modified purine base, such as guanine or hypoxanthine, are used by the enzymes to form fluorescent cyclic nucleotides with the link between the ribose and the purine ring via the N^7 atom (see Sect. 5.3).

Importantly, the ADP-ribosyl cyclase from *Aplysia* as well as CD38 have been found to be able to form a cyclic product from $NADP^+$ (F.-J. Zhang et al. 1995; Vu et al. 1996). This product has been identified to be cyclic ADP-ribose-2'-phosphate (2'-P-cADPR). Moreover, this metabolite has also been shown to represent a novel calcium-mobilizing agent. Addition of 2'-P-cADPR to brain microsomes resulted in a calcium release from stores that were apparently distinct from those activated by IP_3, but sensitive to cyclic ADP-ribose (Vu et al. 1996).

In addition to 2'-P-cADPR, another metabolite of $NADP^+$, nicotinate adenine dinucleotide phosphate ($NAADP^+$), has been demonstrated to exert calcium-mobilizing activity when added to sea urchin egg homogenates (Chini et al. 1995; Lee and Aarhus 1995) or when microinjected into intact cells (Perez-Terzic et al. 1995). It was found that this compound can be synthesized by ADP-ribosyl cyclases in the presence of $NADP^+$ and nicotinic acid (Chini and Dousa 1995; Aarhus et al. 1995). The reaction obviously resembles the transglycosidation described for NADases. As opposed to 2'-P-cADPR, $NAADP^+$ mobilizes calcium from intracellular stores that are not directly activated by cyclic ADP-ribose or IP_3 (Chini et al. 1995; Lee and Aarhus 1995). $NAADP^+$ would therefore represent a novel calcium-mobilizing agent involved in a hitherto unknown signalling mechanism.

4
Calcium-Mobilizing Activity of Cyclic ADP-Ribose

As outlined before, cyclic ADP-ribose was actually discovered due to its ability to mobilize Ca^{2+} from internal stores in sea urchin eggs (Clapper et al. 1987). This experimental system has a number of advantages for studying the regulation of the intracellular Ca^{2+} level (Lee et al. 1994b). In recent years the potential of cADPR to raise the intracellular Ca^{2+} concentration has been established in a number of other systems, including pituitary cells (Koshiyama et al. 1991), rat dorsal root ganglion cells (Currie et al. 1992), brain (Mészáros et al. 1993; Takasawa et al. 1993b; White et al. 1993), bullfrog sympathetic neurons (Hua et al. 1994), T cells (Guse et al. 1995; Bourguignon et al. 1995), bovine oocytes (Yue et al. 1995), intestinal longitudinal muscle (Kuemmerle and Makhlouf 1995), cardiac and skeletal muscle (Mészáros et al. 1993; Morrissette et al. 1993; Sitsapesan et al. 1994; Sitsapesan and Williams 1995), opossum renal epithelial cells (Beers et al. 1995), liver nuclear envelope (Gerasimenko et al. 1995), pancreatic β-cells (Takasawa et al. 1993b), pancreatic acinar cells (Thorn et al. 1994; Gromada et al. 1995; Gerasimenko et al. 1996), and plant vacuoles (Allen et al. 1995). The physiological significance of cADPR as a calcium-mobilizing agent has been corroborated by investigations demonstrating the presence of this metabolite in a variety of cells and tissues (Walseth et al. 1991), along with the widespread occurrence of the enzymatic activity catalyzing its synthesis (Rusinko and Lee 1989). Therefore, cyclic ADP-ribose has emerged as a widely distributed potent intracellular agent controlling the level of Ca^{2+}. Its Ca^{2+}-mobilizing activity is comparable to that of IP$_3$ (Dargie et al. 1990).

The possibility exists that Ca^{2+} mobilization by cADPR could be mediated via a calmodulin-sensitive pathway. Calmodulin, which did not itself cause a Ca^{2+} efflux in sea urchin eggs (Tanaka and Tashjian 1995), was suggested to be a positive modulator for the cADPR-mediated calcium release, not only in sea urchin egg microsomes (Lee et al. 1994c; 1995) but also in mammalian microsomes (Takasawa et al. 1995). While in rat islet microsomes the calmodulin-activated calcium mobilization was abolished by inhibitors for calmodulin-dependent protein kinase II (Takasawa et al. 1995), in the sea urchin system calmodulin seemed to confer cADPR sensitivity through a more direct mechanism, possibly through interaction with a specific receptor (Lee et al. 1994c; Tanaka and Tashjian 1995).

As pointed out earlier, the intracellular level of cADPR may also be regulated by phosphorylation of the cyclase in a cGMP-dependent manner.

A number of current investigations have been focused on the identification of the receptor for cADPR and its mode of action. Although there is good evidence for cADPR to interact with ryanodine receptors (Sitsapesan et al. 1995), a specific, physiologically relevant binding site has yet to be established. The existence of a highly specific site exhibiting a binding affinity of about 17 nM has been demonstrated in sea urchin egg microsomes (Lee 1991).

4.1
Relationship with Inositol Trisphosphate – Dependent Calcium Release

The role of IP$_3$ as a major endogenous agent triggering the efflux of Ca^{2+} from the endoplasmic or sarcoplasmic reticulum has been well known and characterized (Berridge 1993). The extent of cADPR-induced calcium release from sea urchin egg homogenates has been found to be comparable to that induced by IP$_3$ (Dargie et al. 1990; Lee 1991). In addition, the concentration of cADPR required to achieve a half-maximal effect, about 18 nM, was about five to seven times lower than that of IP$_3$. Therefore, cADPR may be regarded as an even more potent Ca^{2+}-releasing agent.

It was an important observation that cyclic ADP-ribose triggered calcium release from internal stores independently of IP$_3$ (Dargie et al. 1990; Galione et al. 1991). That is, egg homogenates desensitized to IP$_3$ remained fully responsive to cADPR and vice versa (Dargie et al. 1990; Galione et al. 1991). These experiments, which have been confirmed in other systems (Lee et al. 1994b), suggest that elevation of internal calcium levels may be evoked by independent mechanisms depending on the external signal. The calcium release induced by IP$_3$ and cADPR did not appear to be additive (Dargie et al. 1990). It was suggested, therefore, that the stores from which Ca^{2+} was released by these two agents were overlapping. However, the possibility that different calcium stores may be activated by different agents cannot be excluded (Gromada et al. 1995).

Observations that normal development after fertilization of sea urchin eggs proceeded when calcium stores were activated by both IP$_3$ or cADPR (Galione et al. 1993b; Lee et al. 1993a) raise the possibility of localized increases of the Ca^{2+} concentration depending on the messenger. Although the occurrence of ordered calcium waves has been a well documented phenomenon, a spatially limited rise in the calcium concentration would represent yet another mode of regulation by this ion.

The possible existence of other calcium-activating agents derived from NADP$^+$ has been demonstrated recently (F.-J. Zhang et al. 1995; Vu et al. 1996). NAADP$^+$ was shown to stimulate Ca^{2+} release from sea urchin egg homogenates independently of both cADPR and IP$_3$ (Chini et al. 1995; Lee and Aarhus 1995). Also, 2'-P-cADPR exerted calcium release activity from brain microsomes. Further experimentation should explore the physiological significance of these metabolites .

4.2
Calcium Release via a Ryanodine-Sensitive Mechanism

Since the discovery of cADPR it has been of major interest to establish its mode of action that eventually leads to calcium release. First evidence that cyclic ADP-ribose may exert its function via a ryanodine receptor-dependent pathway was presented by Galione et al. (1991). In their experiments on sea urchin eggs cADPR-dependent Ca^{2+} release was modulated by effectors of calcium-induced calcium release (CICR) a pathway that proceeds via the ryanodine receptor (Endo 1977). Thus, the cADPR-induced (but not the IP$_3$-induced) Ca^{2+} release was selectively blocked by procaine and ruthenium red. Moreover, cADPR desensitized the microsomes to ryanodine receptor agonists such as ryanodine and caffeine, but not to IP$_3$ (Galione et al. 1991). These as well as studies using other systems suggested the ryanodine receptor as a possible target of cADPR.

Several isoforms of ryanodine receptors have been described (Ogawa 1994). The mammalian type 1 ryanodine receptor is expressed in skeletal muscle, while the type 2 receptor has been found in cardiac muscle and brain (Ogawa 1994). A third isoform is expressed in the brain as well as in other tissues (Meissner 1994; Giannini et al. 1995). There appear to be homologs of the type 1 and type 3 ryanodine receptors in other species including bird, fish, amphibia, and insects (Sitsapesan et al. 1995). Ryanodine receptor–Ca^{2+}-channel complexes exist as homotetramers with molecular masses of the subunits of approximately 550 000 (Ogawa 1994).

Paralleling the diversity of ryanodine receptors, cADPR appears to interact differently with different isoforms (Sitsapesan et al. 1995). Studies in several laboratories on mammalian ryanodine receptors have led to partially controversial results (reviewed in Sitsapesan et al. 1995) regarding the ability of cADPR to interact with certain isoforms and to activate Ca^{2+} fluxes. Apparently, the influence of various effectors on channel activity and ryanodine binding depends strongly on the conditions of the experi-

ment, for example, the luminal Ca^{2+} concentration. Despite these uncertainties, the available evidence suggests that cADPR may activate primarily the type 2 ryanodine receptors (Lee et al. 1994b; Sitsapesan et al. 1995). The regulation of ryanodine receptors appears to be quite complex involving binding of effectors at different sites. Binding of cADPR to the receptor greatly enhances Ca^{2+} release from the endoplasmic reticulum, but only at elevated intracellular Ca^{2+} levels. Therefore, cADPR may function as a modulator of CICR by influencing the sensitivity of the ryanodine receptor to Ca^{2+} (Galione and White 1994).

Considering the experimental systems studied so far the activating ability of cADPR on ryanodine receptors is most potent and specific in sea urchin eggs. Perhaps, some of the controversy can be resolved when this receptor will be isolated and characterized.

Using a specific photoaffinity probe, 8-azido-cADPR, two specific cADPR-binding proteins with molecular masses of 140 000 and 100 000 were identified in sea urchin egg homogenates (Walseth et al. 1993). Since the size of these proteins would not suggest them to be ryanodine receptor molecules, it was proposed that they may represent proteins that interact with the receptor (Walseth et al. 1993). It would indeed be an interesting experiment to test the ability of these proteins to mediate the influence of cADPR on type 1 and type 2 ryanodine receptors, as has been suggested (Sitsapesan et al. 1995).

An important contribution emphasizing the potential of cADPR to be a second messenger was provided by the observation that cGMP stimulated the release of Ca^{2+} from sea urchin egg homogenates via a cADPR-sensitive pathway (Galione et al. 1993a). It was found that stimulation by cGMP occurred only in the presence of NAD^+ and was abolished by prior desensitization to cADPR, but not IP_3. The cGMP-induced Ca^{2+} release was sensitive to effectors of the ryanodine receptor. Also, it has been reported that nitric oxide (NO) induces mobilization of intracellular calcium via a cGMP- and cADPR-, but not IP_3-dependent pathway (Willmott et al. 1996). Furthermore, there are indications that the activation of ADP-ribosyl cyclase may have been mediated by a cGMP-dependent kinase (Lee et al. 1994b). A mechanism has been proposed (Galione and White 1994; Lee et al. 1994b) suggesting the activation of guanylate cyclase by extracellular stimuli, such as NO (Publicover et al. 1993). As a consequence, enhanced production of cyclic ADP-ribose, perhaps, via phosphorylation, and opening of the ryanodine receptor-associated calcium channel would take place. Such a pathway would be consistent with the latency of about 30 s of

cGMP-induced Ca^{2+} release in sea urchin eggs (Whalley et al. 1992; Galione et al. 1993a) as well as with the sensitivity of this mechanism to ryanodine receptor effectors.

At present it cannot be clearly distinguished whether cADPR acts as a true second messenger or as a modulator of CICR via the ryanodine receptor (Galione and White 1994).

4.3
Possible Physiological Roles of Calcium Mobilization by Cyclic ADP-Ribose

Cyclic ADP-ribose has been implicated in the regulation of calcium levels in a variety of cells. In all cases studied cADPR-induced Ca^{2+} release was independent of the IP$_3$ pathway. Moreover, there is ample evidence that mobilization of Ca^{2+} from internal stores proceeds via a ryanodine-sensitive mechanism. Although a great number of processes in the cell are regulated by alterations of the calcium concentration (Carafoli 1987; Berridge and Irvine 1989; Berridge 1993; Clapham 1995), the individual signalling mechanisms (via IP$_3$ or cADPR or, perhaps, yet another messenger) have to be established.

For at least two physiological events there clearly seems to be an essential role for cADPR. First, the propagation of calcium waves in sea urchin eggs at fertilization was shown to be mimicked by microinjection of cADPR agonists. Second, a number of studies have demonstrated an involvement of cADPR in the mechanism of insulin secretion in pancreatic islet cells.

The fertilization response in sea urchin eggs is characterized by a transitory rise in intracellular Ca^{2+} at the sperm-egg fusion site that then spreads across the egg as a propagating wave (Jaffe 1991). This calcium wave appears to be both necessary and sufficient to promote the activation of the quiescent egg (Whitaker and Steinhardt 1985). The occurrence of the calcium transient is independent of the influx of extracellular Ca^{2+} (Whitaker and Swann 1993) and, therefore, mediated by the activation of intracellular stores. Two different mechanisms have been shown to contribute to the release of calcium: both IP$_3$- and cyclic ADP-ribose-dependent pathways may be activated at fertilization (Galione et al. 1993b; Lee et al. 1993b; Shen and Buck 1993). When the IP$_3$-dependent calcium release system was blocked by heparin, the fertilization response changed only insignificantly. On the other hand, inhibition of the ryanodine-sensitive pathway, which is likely to involve cADPR, by ruthenium red did also not substantially affect

the fertilization response (Galione et al. 1993b). That the cADPR-dependent pathway could indeed be blocked without abolishing the fertilization response was demonstrated using the specific antagonist 8-amino-cADPR (Lee et al. 1993b). Microinjection of either IP_3 or cADPR have been shown to induce calcium transients resembling those at fertilization as well as the cortical exocytosis reaction (Lee et al. 1993b). Most importantly, if both pathways were blocked simultaneously neither calcium transients nor a cortical reaction could be observed nor could these phenomena be induced by adding sperm (Galione et al. 1993b, Lee et al. 1993b). In this case the eggs would become polyspermic (Galione et al. 1993b). These experiments suggest a functional redundancy of calcium signalling pathways. Although the functional significance of such a phenomenon would have to be explored, the structural similarity of IP_3 and ryanodine receptors may support such a notion (Furuichi et al. 1994). The existence of independent alternatives for activating calcium stores may also indicate the possibility of spatially limited regulatory events.

The involvement of cADPR in the secretion mechanism of insulin from pancreatic islets was initially suggested on the basis of the following observations (Takasawa et al. 1993b): cADPR induced the release of Ca^{2+} from microsomes of pancreatic islets via a ryanodine-sensitive mechanism. The cADPR concentration in pancreatic islets was raised by glucose and, finally, insulin secretion from digitonin-permeabilized islets was stimulated by cADPR. These findings indicate that glucose-induced insulin secretion may be mediated by a CICR mechanism involving cADPR and a ryanodine-sensitive channel. The presence of a CICR mechanism in islet cells has been demonstrated. It was, however, rather insensitive to ryanodine (Islam et al. 1992; Hellmann et al. 1992; Roe et al. 1993). The potential role of cADPR metabolism in insulin secretion was also supported by experiments in which transgenic mice were investigated that overexpressed CD38 in pancreatic islet cells (Kato et al. 1995). Recently, it was also reported that, besides CD38, the GPI-anchored ADP-ribosyl cyclase BST-1 is expressed in pancreatic islet cells (Kajimoto et al. 1996) lending further support for the potential role of cADPR in these cells.

5
Methods of Detection of ADP-Ribosyl Cyclase Activity

The activity of ADP-ribosyl cyclases can be measured in two principal ways: by determining the formation of cADPR, or by testing the biological effect of the product formed from NAD$^+$ during the enzymatic reaction. The biological assay has been initially used to verify the identity of cADPR. The physical methods that have been developed over the past few years are certainly preferable, because of their ease and efficiency.

5.1
Biological Assay

The original assay that led to the discovery of cyclic ADP-ribose (Clapper et al. 1987) may be utilized to determine the calcium-mobilizing activity of metabolites. Calcium fluxes are monitored with purified microsomes from sea urchin eggs by following changes in the calcium concentration of the medium using specific fluorescent dyes such as Fura 2. Addition of cADP-ribose will lead to release of calcium from the microsomes which is detected as a decrease of fluorescence. Since IP$_3$ and pyridine nucleotide derivatives other than cADP-ribose may also exert calcium-mobilizing effects, this assay is not specific. However, specificity can be verified using the cADPR antagonist 8-amino-cADPR (Walseth and Lee 1993).

5.2
Detection of Cyclic ADP-Ribose by Chromatographic Procedures

Cyclic ADP-ribose can be separated by all methods conventionally used for nucleotides. For quantitative determinations, HPLC procedures have been described using ion-exchange or reverse-phase columns and UV-detection. These techniques provide a good separation of ADP-ribose, cyclic ADP-ribose, NAD$^+$ and nicotinamide as well as analogs of NAD$^+$ and their derivatives (e.g., Graeff et al. 1994b; H. Kim et al. 1993a).

Thin layer chromatography enables a sufficiently sharp separation of NAD$^+$, ADP-ribose and nicotinamide in various solvent systems (Galione et al. 1993a). Although adequate sensitivity can only be achieved using radioactive substrates, this method permits the simultaneous analysis of several samples.

The problem of unsatisfactory separation of ADP-ribose from cyclic ADP-ribose in some systems can easily be overcome by treating the samples to be analyzed with phosphodiesterase I from snake venom: This enzyme cleaves ADP-ribose to AMP and ribose phosphate, whereas cyclic ADP-ribose does not serve as substrate.

5.3
Fluorescence Assay Using Analogs of NAD$^+$

ADP-ribosyl cyclases can utilize substrates other than NAD$^+$ to synthesize cyclic purine nucleoside diphosphoriboses (Graeff et al. 1994b, 1996; Zhang and Sih 1995). When the adenine moiety of NAD$^+$ is replaced by guanine, hypoxanthine, xanthine, or 1,N^6-etheno-adenine, the ADP-ribosyl cyclase from *Aplysia*, as well as human CD38 will produce analogous cyclic products. However, cyclization occurs between the anomeric carbon of the terminal ribose and the N^7 of the purine ring (Zhang and Sih 1995; Graeff et al. 1996), rather than the N^1 position, as is the case with NAD$^+$ as substrate. As a consequence of the cyclization at the N^7 position, the products are fluorescent. Since the NAD$^+$ analogs themselves as well as their noncyclic purine nucleoside diphosphoribose derivatives are not fluorescent, the cyclyzation reaction can be easily followed fluorimetrically.

Figure 2, right panel, demonstrates that the *Aplysia* ADP-ribosyl cyclase can be renatured after SDS-PAGE in such a way that its activity can be visualized fluorimetrically directly within the gel using nicotinamide hypoxanthine dinucleotide as substrate. The NADase from *Neurospora crassa* does not catalyze a cyclization reaction, but its position in the gel can be detected using ε-NAD$^+$ as substrate (Fig. 2, left panel). The fluorescence enhancement of (cyclic)-ε-ADP-ribose, as compared to ε-NAD$^+$, is due to the separation of the fluorescent ε-adenine ring from the quenching nicotinamide (Barrio et al. 1972). Since this separation occurs also in the cyclization reaction, the ADP-ribosyl cyclase from *Aplysia* can be visualized using ε-NAD$^+$, too (Fig. 2, left panel).
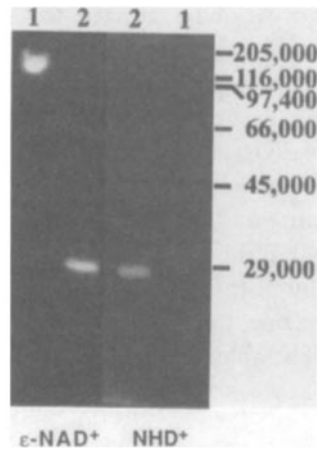
Fig. 2. "Activity staining" of SDS-PAGEs for NADase and ADP-ribosyl cyclase activities. NADase from *Neurospora crassa* (*lanes 1*) and ADP-ribosyl cyclase from *Aplysia californica* (*lanes 2*) were separated in duplicate on a 10% SDS-PAGE. After a renaturation procedure the gel was cut in half. One part was then incubated with $1,N^6$-etheno-NAD⁺ (ε-NAD⁺; *left panel*), the other with NHD⁺ (*right panel*). NADase activity (*left panel*) and ADP-ribosyl cyclase activity (*right panel*) were then visualized under UV-light

5.4
Radioimmunological Detection of Cyclic ADP-Ribose

Takahashi et al. (1995) have used an antiserum raised against cADP-ribose coupled to bovine serum albumin to detect cADP-ribose levels in HL-60 cells. This assay is highly sensitive. Its specificity is greatly enhanced, if the samples are treated with 5'-nucleotidase and snake venom phosphodiesterase to degrade other nucleotides that exhibit cross-reactivity, for example, NAD⁺ and NMN.

5.5
Detection of Cyclic ADP-Ribose Hydrolase Activity

The enzymatic hydrolysis of cyclic ADP-ribose or its analogs can be assessed in several ways, similar to those for its synthesis. For example, the resistance of cyclic ADP-ribose towards phosphodiesterase from snake

venom as discussed above can be utilized to follow the formation of ADP-ribose by converting it specifically to AMP and ribose phosphate.

Enzymatic activity of cyclic ADP-ribose hydrolases can also be measured using fluorescent analogs of cyclic ADP-ribose, such as cyclic GDP-ribose or cyclic IDP-ribose (Graeff et al. 1996). The absence of a substitution at the N^7 position of the purine ring (which is a result of the hydrolysis) renders the molecule again nonfluorescent. Therefore, the hydrolase activity would be proportional to the decrease of fluorescence. Similarly to the synthesis reaction, although this assay has been successfully applied to CD38 and the *Aplysia* enzyme, the possibility exists that not all hydrolases of cyclic ADP-ribose will be able to utilize these analogs as substrates.

6
Mitochondrial NAD⁺ Glycohydrolase Activity and Regulation of Calcium Fluxes

It has long been known that mitochondria possess a high capacity to sequester calcium from the surrounding medium (Carafoli 1987; Denton and McCormack 1990). Therefore, these organelles had been thought to represent a potential store that may be activated as a regulatory event. However, no specific mechanism has been described that would trigger instantaneous calcium release from mitochondria. The known Ca^{2+} signalling pathways, via IP_3 or ryanodine receptors, do not activate mitochondrial calcium stores. Nevertheless, recent investigations have provided clear evidence, that the metabolic state of mitochondria influences the occurrence of cytosolic calcium waves (Jouaville et al. 1995). Moreover, the activity of calcium-dependent mitochondrial dehydrogenases was strongly influenced by changes in the cyctosolic calcium concentration (Hajnóczky et al. 1995). Therefore, there is likely to be a calcium signalling pathway across the mitochondrial membranes.

Several years ago studies on isolated mitochondria have revealed a correlation between the pyridine nucleotide status and calcium movements across the inner membrane of these organelles (Lehninger et al. 1978; Lötscher et al. 1979, 1980). In a number of investigations Richter and coworkers (reviewed in Richter and Kass 1991) have obtained evidence that treatment of rat liver mitochondria with prooxidants resulted in the oxidation and subsequent degradation of mitochondrial pyridine nucleotides. Concomitantly, efflux of Ca^{2+} from the mitochondria was observed. Since

under these conditions an enhanced degree of ADP-ribosylation was de-
tected within the organelles, it was hypothized that this protein modifica-
tion may be related to a calcium release pathway. The proposed model
(Richter and Kass 1991) suggested prooxidant-induced hydrolysis of NAD$^+$
followed by nonenzymatic ADP-ribosylation of specific mitochondrial
proteins by the liberated ADP-ribose (Fig. 3, pathway 1). Free ADP-ribose
has indeed been shown to be able to covalently modify mitochondrial
proteins (Hilz et al. 1984; Frei and Richter 1988). However, there are reports
demonstrating the occurrence of mitochondrial ADP-ribosylation using
NAD$^+$ as substrate under conditions that lead to virtually complete inhibi-
tion of NADase activity, and, therefore, no formation of free ADP-ribose
(Masmoudi and Mandel 1987; Ziegler et al. 1996b). It was concluded from
these studies that, besides the possibility of nonenzymatic ADP-ribosyla-
tion, mitochondria contain monoADP-ribosyl transferase activity (see
Fig. 3). These findings raised the question as to the function of the NADase,
as this enzyme appeared not to be required for mitochondrial ADP-ribo-
sylation using NAD$^+$ as substrate. Considering the ability of some known
NADases to form cyclic ADP-ribose it was an obvious experiment to test
the mitochondrial enzyme in this regard too. Recently, it was established
that the NADase from bovine liver mitochondria catalyzes the formation
of cyclic GDP-ribose and cyclic IDP-ribose from the corresponding NAD$^+$
analoga (Ziegler et al. 1996b). These findings suggest that the previously
observed prooxidant-induced calcium release from mitochondria may
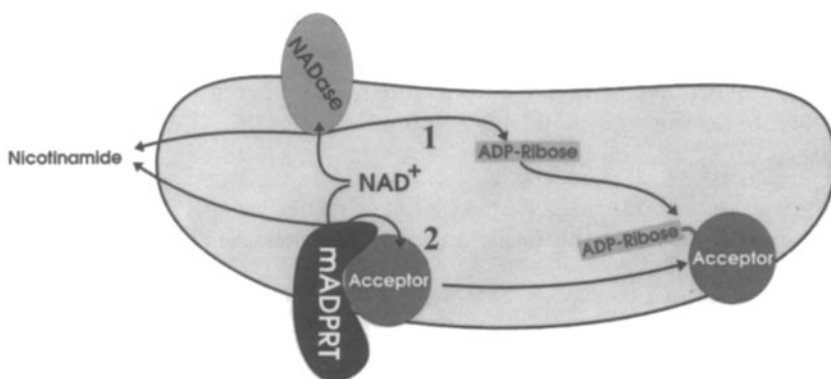

Fig. 3. Two possible pathways of mitochondrial ADP-ribosylation. *1*, Nonenzymatic,
via free ADP-ribose generated by the mitochondrial NAD$^+$ glycohydrolase; *2*, enzy-
matic, by mono(ADP-ribosyl) transferase

actually be mediated by a cADPR-dependent mechanism. It is interesting in this regard that ADP-ribosyl cyclase as well as NADase activities are lost after treatment of the mitochondrial enzyme with dithiothreitol (Ziegler et al. 1996b). On the other hand, it has been observed that prooxidant-induced calcium efflux is also sensitive to reducing agents (Schweizer and Richter 1996) and requires the oxidation of vicinal thiols in mitochondria (Schweizer and Richter 1994; Schweizer et al. 1994). Similarly, both NADase activity and Ca^{2+} efflux (Hofstetter et al. 1981) are inhibited by ATP.

Taken together, a mechanism for prooxidant-induced calcium release from mitochondria appears to emerge that includes the oxidation of mito-chondrial pyridine nucleotides (and, perhaps, the formation of a disulfide bridge within the NADase), followed by the synthesis of cyclic ADP-ribose from NAD^+ by the NADase. Although a mitochondrial target for cyclic ADP-ribose has not been described, it is a possibility that it would trigger calcium release from the organelles. Further studies should, therefore, establish whether intramitochondrial cyclic ADP-ribose would cause cal-cium release. Alternatively, as pointed out above, other metabolites with calcium-mobilizing activity could possibly be formed by the NADase. A possible approach to these questions would be the use of submitochondrial particles (exposing the matrix side of the inner membrane to the outside) with fluorescent calcium indicators entrapped.

The novel role of mitochondrial NADase indicates that formation of free ADP-ribose for nonenzymatic ADP-ribosylation may not be its physiologi-cal function. Nevertheless, mitochondrial ADP-ribosylation, presumably catalyzed by specific enzyme(s), has repeatedly been found (Masmoudi and Mandel 1987; Boyer et al. 1993; Ziegler et al. 1996b). In view of the possible mechanism of calcium release discussed above, it is likely to be involved in regulatory processes unrelated to calcium fluxes. Therefore, it should be of great interest to establish the role of this protein modification in mitochon-dria.

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The Role of Poly(ADP-Ribosyl)ation

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Contents

1
Introduction

Two classes of post-translational modification by ADP-ribose, poly(ADP-ribosyl)ation and mono(ADP-ribosyl)ation are found in eukaryotes. Although the biological roles of these protein modifications are still unclear, their involvement in metabolic and regulatory pathways of cellular biology is undisputed. Poly(ADP-ribosyl)ation is principally a nuclear reaction and, hence, in contrast to mono(ADP-ribosyl)ation, limited to eukaryotes. So far poly(ADP-ribosyl)ation could not be detected in prokaryotic organisms. The modification of nuclear proteins by poly(ADP-ribose) is induced by DNA-damaging agents. Polymer synthesis and transfer onto protein acceptors is catalyzed by the enzyme poly(ADP-ribosyl) transferase, here abbreviated as ADPRT (EC 2.4.2.30). Due to the different enzymatic activities of this protein, also other names are used in the literature, for example, poly(ADP-ribosyl) synthetase or polymerase. ADP-ribose polymers are degraded by the catabolic enzymes poly(ADP-ribose) glycohydrolase, phospodiesterase, and (ADP-ribose) protein lyase.

In this review we focus on ADPRT as polymer synthesizing enzyme, and its interaction with and modification of target proteins and the consequences onto cellular processes (for reviews see Althaus and Richter 1987; de Murcia et al. 1991; Boulikas 1991; de Murcia and Menissier-de Murcia 1994; Lindahl et al. 1995; and the special issues of *Molecular and Cellular Biology* vol. 138 (1994) and *Biochimie* vol. 77 (1995).

2
Poly(ADP-Ribose)

2.1
Discovery of Poly(ADP-Ribose)

About 30 years ago the groups of Paul Mandel, Takashi Sugimura, and Osamu Hayashi, motivated by different intentions, independently made the observation that proteins were covalently modified by the addition of ADP-ribose residues. The first published observation (Chambon et al. 1963) was a 1000 times enhanced incorporation of (^{14}C-adenine)-labelled ATP into the acid-insoluble fraction of a nuclear preparation from chicken liver stimulated by nicotinamide mononucleotide (NMN). The polymer product

was first considered to be poly(A) but was identified as poly(ADP-ribose) 3 years later by the same group (Chambon et al. 1966). During this time, two different Japanese groups made the same observation of an increase of insoluble acid-precipitable material in nuclear preparations after addition of NMN. The analaysis of these products confirmed Mandel's conclusion about the structure of poly(ADP-ribose) (Fujimura et al. 1967a,b; Nishizuka et al. 1967).

Further investigations of hydrolysis products of poly(ADP-ribose) led to the conclusion that NAD^+ was formed from NMN and ATP by NAD^+ pyrophosphorylase in nuclei, and the ADP-ribose moiety of NAD^+ was then converted to poly(ADP-ribose) with concomitant release of nicotinamide (Fig. 1).

Since then a large number of biological systems have been described, in which poly(ADP-ribose) seems to be involved in nuclear processes.

2.2
Structure of Poly(ADP-Ribose)

The first information concerning the structure of poly(ADP-ribose) was obtained by analyzing the degradation products following enzymatic digestion. From these results it was possible to deduce structural features for the complex macromolecule.

Hydrolysis of poly(ADP-ribose) with snake venom phosphodiesterase results in a major hydrolysis product of 2'(or 3')-(5''-phosphoribosyl)-5'AMP, which was named φ-ADP-ribose (Chambon et al. 1966), or phosphoribosyl AMP, abbreviated as PR-AMP (Fujimura et al. 1967b), indicating that the polymer consists of ADP-ribose monomers covalently linked via (2'-1'') glycosidic bonds.

The identification of a further product of hydrolysis, 2'-[1''-ribosyl 2''-(or 3''-)(1'''-ribosyl)]adenosine-5',5''5'''-tris(phosphate) $(PR)_2$-AMP) revealed the existence of a branched portion of poly(ADP-ribose) (Miwa et al. 1981). The molar ratio of phosphodiesterase hydrolysis products 5'AMP, $(PR)_2$-AMP, and PR-AMP is about 1:0.5:24, which is consistent with one branch per polymer chain with an average length of 25 ADP-ribose units (Juarez-Salinas et al. 1983). Branching of polymers synthesized *in vitro* could be visualized by electronmicroscopy as impressive "Christmas-tree-like" structure (de Murcia et al. 1983; Hayashi et al. 1983), and distinct positions of branching were assigned for polymers attached to histones (Boulikas 1989). Synthesized polymers detached from protein could be
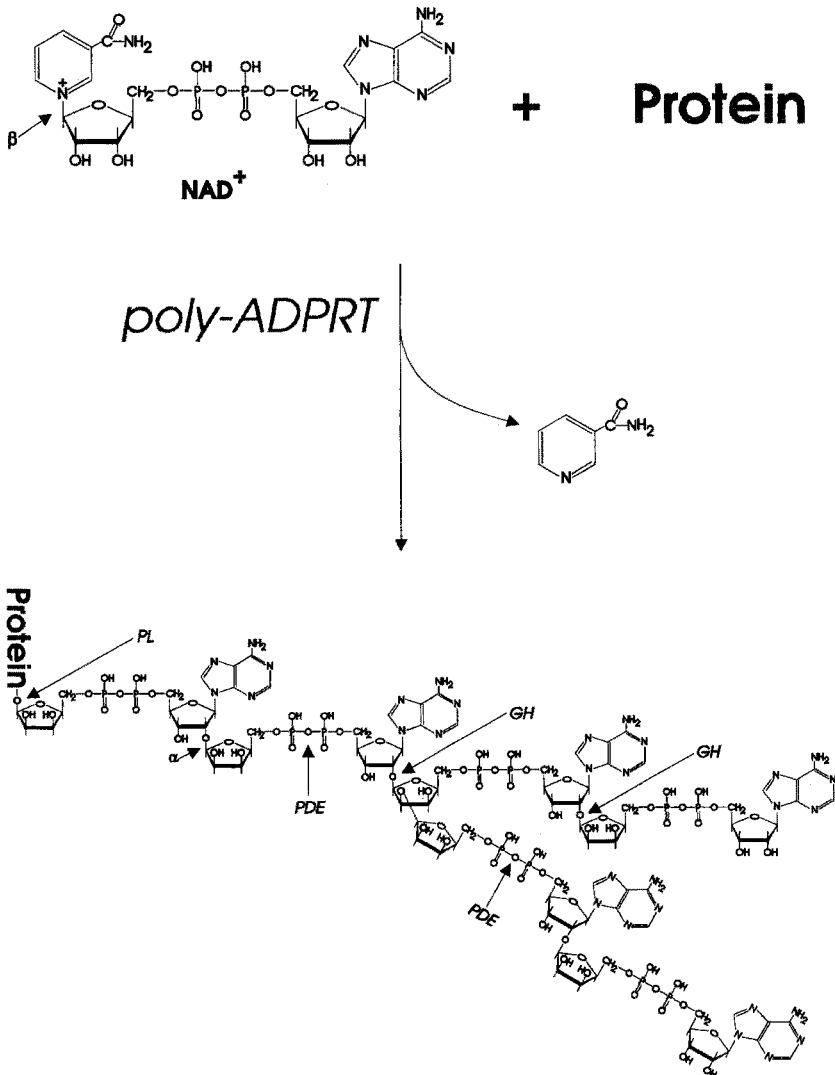
Fig. 1. Poly(ADP-ribosyl)ation reaction. ADPRT catalyzes the transfer and the poly-merisation of (ADP-ribose) moieties to protein acceptors using NAD^+ with concomi-tant release of nicotinamide. The conformation (α or β) of glycosidic bonds in NAD^+ and poly(ADP-ribose), respectivly, and potential cleavage sites for enzymes involved in polymer catabolism are indicated: *PL*, protein lyase; *PDE*, phosphodiesterase; *GH*, poly(ADP-ribose) glycohydrolase

separated electrophoretically (Tanaka et al. 1978), and the length of poly-
mers could be determined (Alvarez-Gonzalez and Jacobson 1987; Panzeter
and Althaus 1990; Fig. 2B). While a lot of investigations have been done on
the structure of poly(ADP-ribose), the nature of the 5'phosphoribosyl
glutamic ester, which is the attachment site of polymers to proteins, is not
well documented in the literature.

    Some physicochemical properties of poly(ADP-ribose)polymers, re-
viewed by Althaus and Richter (1987), resemble that of other polymers such
as poly(A), DNA, and RNA. Yet, the thermal hyperchromicity of poly(ADP-
ribose) is considerably lower than that of poly(A). No hyperchromic effect
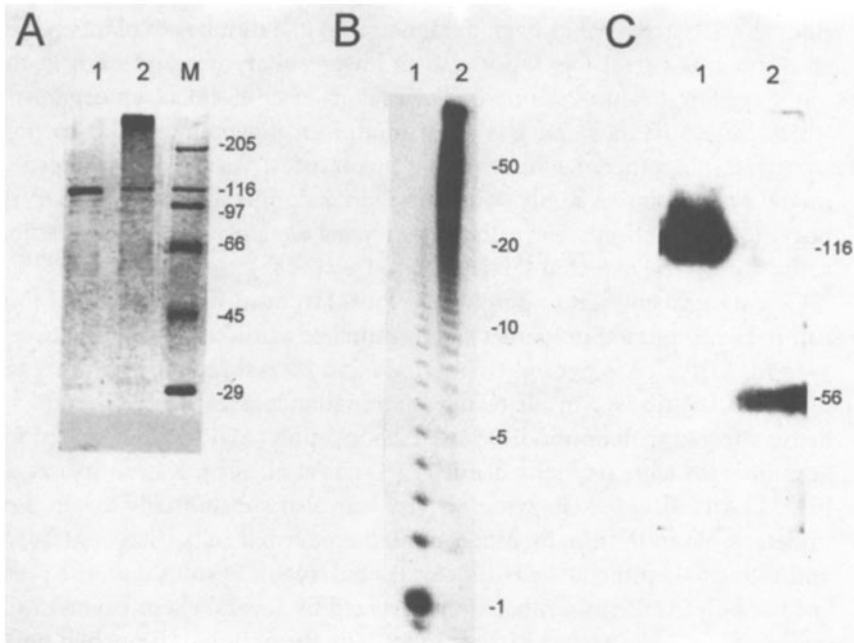
Fig. 2 A–C. Automodification of human ADPRT. Purified recombinant human full-
length ADPRT was incubated with 0.1 μM [^{32}P]NAD$^+$ (1) and 1 mM [^{32}P]NAD$^+$ (2) in
the presence of DNA. **A** Probes were subjected to 8% SDS-PAGE and Coomassie stained.
Relative sizes of marker proteins (M) are indicated. **B** Autoradiogram of polymer size
analysis according to Panzeter and Althaus (1992) of both probes. Relative polymer
lengths are indicated. **C** Autoradiogram of an activity blot (according to Simonin et al.
1991) of recombinat full-length ADPRT (1) and the 56-kDa C-terminal catalytic domain
of ADPRT (2). In brief, proteins were seperated by 10% SDS-PAGE and transferred onto
nitrocellulose. Blots were incubated with [^{32}P]NAD$^+$, washed, and autoradiographed

was observed when poly(ADP-ribose) was mixed with poly(U). A helical conformation of long-chain poly(ADP-ribose) has been proposed (Minaga and Kun 1983a,b), coupled with helical forces in long-chain polymers under formation of higher order helices.

2.3
Occurrence of Poly(ADP-Ribose)

Poly(ADP-ribosyl)ation reactions seem ubiquitous in higher eukaryotes, and known sequences of the polymer synthesizing enzyme ADPRT from different species show high homology (Uchida and Miwa 1994). Poly(ADP-ribosyl)ation activity has been demonstrated in a number of plants (Chen et al. 1994; O'Farrel 1995), but also in lower eukaryotes and even in the dinoflagellate *Crypthecodinum cohnii* (Werner et al. 1984), an organism, which lacks histones and has a chromatin arrangement similar to prokaryotes. Therefore, such primitive eukaryotes provide an interesting model system to reevaluate some functional significance of poly(ADP-ribosyl)ation reactions. Nevertheless in yeast no poly(ADP-ribosyl)ation activity could be detected (Hayashi and Ueda 1982).

The natural content of poly(ADP-ribose) in most tissues is rather low, compared to related polymers such as nucleic acids, and ranges between 3–30 ng ADP-ribose per mg DNA (Ueda and Hayashi 1985). The presence of poly(ADP-ribose) in all tested mammalian nucleated cells could be demonstrated with antibodies raised against poly(ADP-ribose) except for granulocytes with segmented nuclei (Kanai et al. 1974; Kawamitsu et al. 1984; Ikai et al. 1980). Enzyme activity was also substantially lowered or undetectable in terminally differentiated epidermal cells (Ikai et al. 1982) and intestinal epithelial cells (Porterus et al. 1979). Besides that, the presence of poly(ADP-ribose) has been revealed by several chemical methods (reviewed in Althaus and Richter 1987). The formation of branched polymers *in vivo* has been observed (Kanai et al. 1982), which was stimulated by carcinogen (Malanaga and Althaus 1994).

Other studies on the intranuclear distribution of the enzyme demonstrated that ADPRT is closely associated with the nuclear matrix ("scaffold") of interphase cells (Adolph and Song 1985a,b; Adolph 1987) and partially complexed to the attachment points of loop DNA on the chromatin matrix (see Table 2). An analysis of the *in vivo* distribution of poly(ADP-ribosyl)ation activity showed a preferential localization of the activity in polynucleosomal preparations (Giri et al. 1978), whereas immunoelectron

microscopy detected a preferential association of the enzyme ADPRT to the nucleosomal core (Leduc et al. 1986). Although ADPRT is primarily located in the nucleus, a cytoplasmic enzymatically active ADPRT has been found in the microsomal-ribosomal fraction of rat spermatogenic cells (Concha et al. 1989).

3
The Synthesis of Poly(ADP-Ribose)

The synthesis of poly(ADP-ribose) is catalyzed by the multifunctional nuclear enzyme ADPRT (EC 2.4.2.30). A total of 10^6–10^7 molecules of ADPRT are present in mammalian cell nuclei. ADPRT catalyzes the transfer of (ADP-ribose) moieties from its substrate NAD^+ to protein acceptors (heteromodification) as well as to the enzyme itself (automodification; Fig. 1). ADPRT has been isolated and characterized from different species. The K_m for NAD^+ of purified ADPRT is about 50 μM (reviewed in Althaus and Richter 1987). The enzymatic activity of ADPRT is strongly enhanced by the presence of DNA breaks (Benjamin and Gill 1980). Molecular biological recombinant DNA technologies enabled the overexpression of enzymatically active recombinant ADPRT constructs in *Escherichia coli* cells (Gradwohl et al. 1989; Ikejima et al. 1989) or the baculovirus system (Giner et al. 1992) facilitating structural and functional analysis. Nevertheless the physiological role of ADPRT in cellular processes is still unknown.

3.1
Structure of Poly(ADP-Ribosyl) Transferase

Following limited proteolysis and functional analysis (Nishikimi et al. 1982; Kameshita et al. 1984) three distinct domains of ADPRT could be identified (Fig. 3): A 46 000 aminoterminal fragment including the DNA binding domain (DBD), a central 22 000 polypeptid fragment containing the main acceptor sites for automodification with poly(ADP-ribose), and a 54 000 carboxyterminal fragment bearing the NAD^+-binding domain. This organization of serial functional elements is also reflected by an "identity profile" obtained from a sequence alignment of ADPRT from various species (de Murcia et al. 1994; Miwa et al. 1995). Regions with strongest conservation correspond to the functional domains and subdomains of
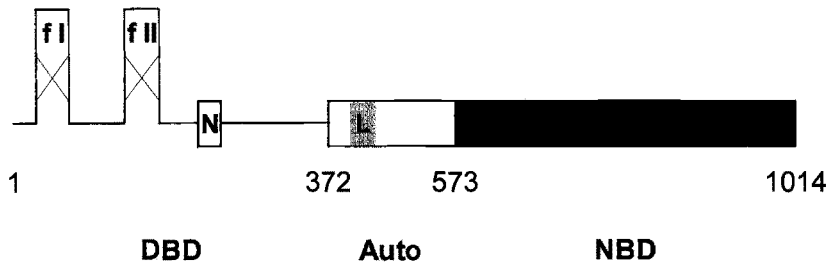
Fig. 3. Schematic representation of functional domains of human ADPRT. The DNA binding (*DBD*), automodification (*Auto*), and catalytic NAD$^+$-binding (*NBD*) domains and relative amino acid positions of the human ADPRT enzyme are indicated. *fI, fII*, Zn^{2+} coordinated finger motifs; *N*, nuclear localization signal; *L*, putative leucine zipper region

ADPRT; more than 90% identity was found within the catalytic, C-terminal NAD$^+$-binding domain.

ADPRT isolated from mammalian species (Mandel et al. 1977; Niedergang et al. 1979; Jump and Smulson 1980; Petzold et al. 1981; Agemori et al. 1982) yielded proteins with apparent molecular weights of about 112 000–135 000. The use of affinity chromatography (Burtscher et al. 1986) allowed to purify the enzyme to homogeneity. Both, protein and full-length cDNA sequences have been obtained for human (Alkhatib et al. 1987; Cherney et al. 1987; Kurosaki et al. 1987; Schneider et al. 1987; Uchida et al. 1987); mouse (Huppi et al. 1989); bovine (Saito et al. 1990); chicken (Ittel et al. 1991); *Xenopus* (Uchida et al. 1993b), *Drosophila* (Uchida et al. 1993a), and *Sarcophaga peregrina* ADPRT (Masutani et al. 1994). The human ADPRT gene was localized on chromosome 1 (q41-q42) (Cherney et al. 1987; Herzog et al. 1989; Baumgartner et al. 1992) and its genomic DNA was analyzed (Auer et al. 1989). The promoter region of the rat (Potvin et al. 1992, 1993) and human ADPRT genes have been sequenced and characterized (Ogura et al. 1990a,b; Yokoyama et al. 1990; Schweiger et al. 1992; Oei et al. 1994; Schweiger et al. 1995).

3.1.1
DNA Binding Domain

DNA binding is a prerequisite for enzymatic activity of ADPRT. As reported by Zahradka and Ebisuzaki (1984), ADPRT is a metalloprotein and the N-terminal domain of ADPRT was found to bind to DNA in a Zn^{2+}-de-

pendent manner (Menissier-de Murcia et al. 1989; Mazen et al. 1989). Later the characterization of the aminoterminal DBD domain of human ADPRT revealed the presence of a repeated sequence of the form $CX_2CX_{28/30}HX_2C$ (residues 2–97 and 106–207) consistent with two zinc finger like motifs (Mazen et al. 1989) (indicated as fI and fII in Fig. 3). Crucial residues involved in metal coordination and DNA binding, identified by mutagenesis, are strictly conserved during evolution (reviewed in de Murcia et al. 1994). The second zinc finger (fII) plays a fundamental role in specific single strand DNA binding (Gradwohl et al. 1990), whereas the first finger (fI) is involved in the DNA-dependent mediation of the catalytic activity without affecting the DNA binding ability of ADPRT (Ikejima et al. 1990). Full enzymatic activity of ADPRT is absolutely dependent on binding to DNA strand breaks and hence on the integrity of the zinc finger domain (Simonin et al. 1990; Ikejima et al. 1990). In addition, besides the N-terminal zinc finger domain another region binding to "DNA with special secondary structures" has been postulated, located in the 36 000 automodification domain (Sastry et al. 1989; Thibodeau et al. 1993; own unpublished observations).

The binding-ability of ADPRT to DNA of different origins has been analyzed (Hengartner et al. 1991). DNase I protection experiments indicated that ADPRT specifically binds to single strand DNA breaks in a sequence-independent manner, symmetrically, covering 7–8 nucleotides on each side of the break (Menissier de Murcia et al. 1989; Gradwohl et al. 1990). The binding of ADPRT to a DNA nick induces a V-shaped sharp bend, with the enzyme located at the kink as visualized by electronmicroscopy studies (Le Cam et al. 1994). It has been reported that ADPRT binds also preferentially to supercoiled DNA (Zahradka and Ebisuzaki 1984) DNA loops (Gradwohl et al. 1987), and cruciform structures (Sastry and Kun 1990).

A bipartite nuclear localization signal (Fig. 3) has been found in mammalian ADPRT (Schreiber et al. 1992). The coupling of DNA binding and nuclear localization signal, reviewed by La Casse and Lefebvre (1995), may allow to coordinate regulation of both motifs.

3.1.2
Automodification Domain

The main acceptor of poly(ADP-ribosyl)ation is ADPRT itself both *in vitro* (Yoshihara et al. 1977; Kawaichi et al. 1981) and *in vivo* (Kreimeyer et al. 1985; Adamietz 1987). The automodification domain contains 15 highly

conserved glutamate residues, which may include most of the acceptor sites for poly (ADP-ribose). ADP-ribosylation of bovine ADPRT *in vitro* led to a modification of 28 amino acid residues (Desmarais et al. 1991). Some of these modified residues were even located within the catalytic domain (55 200 C-terminal part) and the DBD (42 500 N-terminal part).

Within the automodification domain of *Drosophila melanogaster* ADPRT a putative leucine zipper motif has been found suggesting that this region might be involved in protein-protein interaction (Uchida et al. 1993a).

Automodification of ADPRT interferes with DNA binding, probably due to electrostatic repulsion between the negatively charged ADP-ribose polymers and the DNA (Ohgushi et al. 1980; Zahradka and Ebisuzaki 1982). It has been reported by Kirsten et al. (1991) that automodification not only alters the DNA binding capability of the protein but converts it to a NAD^+ase.

3.1.3
Catalytic NAD^+ Binding Domain

The 40 000 C-terminal NAD^+ binding domain of ADPRT has been derived by recombinant DNA technologies (see Fig. 2C). This domain alone exerts all catalytic activities (NAD^+-binding, NAD^+ase activity, poly(ADP-ribose) synthesis and branching ability) of the whole enzyme with the exception of DNA-dependent activation (Simonin et al. 1993b). The K_m of this 40 000 protein for NAD^+ is quite comparable to that of the full-length ADPRT (50 μM), but its specific activity is approximately 500-fold lower than that of the full-length enzyme activated by DNA.

The catalytic C-terminal domain bears two conserved putative dinucleotide binding motifs GX3GKG (amino acids 888–894 in human ADPRT) and GXGKT (amino acids 950–954), although the latter one is not conserved in *Drosophila* (reviewed by de Murcia et al. 1994). Based on sequence similarities of the ADPRT NAD^+-binding domain to Leu and Glu dehydrogenases (Simonin et al. 1993a), a Rossmanfold (Rossman et al. 1975), consisting of a β1-αA-β2 arrangement and potential active-site residues within the C-terminal domain has been postulated.

Mutational analyses revealed, that a non conservative mutation of Lys-893 (K893I) and of Asp-993 (D993A) respectively completely abolished the catalytic activity (Simonin et al. 1990, 1993b) probably by interfering with the initial modification reaction. Moreover, a gain-of-function mutant

(L713F) has been described, with a 9 times increased k_{cat} *in vitro* (Miranda et al. 1995).

X-ray studies of the catalytic domain to provide a three-dimensional structure are in progress (Jung et al. 1994).

3.2
The Mechanism of Poly(ADP-Ribosyl)ation

The exact mode of modification and transfer of poly(ADP-ribose) is not totally understood yet. This process, reviewed by Alvarez-Gonzalez et al. (1994), could be divided into three enzymatic steps: (a) initiation reaction (Kawaichi et al. 1981); (b) (ADP-ribose) chain elongation (Ueda et al. 1979; Taniguchi et al. 1987; Alvarez-Gonzalez 1988); and (c) branching reaction (Miwa et al. 1979). In *in vitro* studies with limited amounts of NAD$^+$, ADPRT activated by DNA has been shown to produce polymers with an average length of seven ADP-ribose units (Fig. 2B). These oligomers synthesized in the priming reaction were attached to a small subfraction of enzyme molecules in a processive reaction mode (Naegli et al. 1989).

Controversy exists about the elongation of ADP-ribose chains: Ikejima et al. (1987) postulate a protein proximal mechanism of elongation, while several results indicate a protein-distal mechanism (Taniguchi 1987; Alvarez-Gonzalez 1988; Mendoza-Alvarez and Alvarez-Gonzalez 1993). Automodification experiments of Ueda and Hayashi (1985) and Bauer and Kun (1985) argued for an intramolecular mechanism, while on the other hand data by Holtlund et al. (1983) and Kameshita et al. (1984) are consistent with an intermolecular mechanism of automodification. Kinetic studies suggest that automodification occurs via an intermolecular and not an intramolecular reaction with an enzymatically active dimer-intermediate (Mendoza-Alvarez and Alvarez-Gonzalez 1993; Panzeter and Althaus 1994). Homodimerization regions within the aminoterminal part and the automodification domain of ADPRT have been found (Bauer et al. 1990a; Buki et al. 1995; Griesenbeck et al. submitted).

In addition to the catalytically active residues Lys-893 and Asp-993 identified by mutagenesis (Simonin et al. 1990, 1993b), an intermediate His-(ADP-ribose) within a 56 000 C-terminal proteolytic fragment has been proposed, which appears to be an intermediate of the modification reaction *in vivo* (Bauer et al. 1986, 1990b).

Further analyses are required to clarify the precise mechanism of the modification reaction.

4
The Catabolism of Poly(ADP-Ribose)

At high levels of DNA damage, the half-life of poly(ADP-ribose) polymers
in vivo may be less than 30 seconds, whereas in a slower catabolism of a
constitutive polymer fraction in undamaged cells a half-life of about 7.7 h
was observed (Alvarez-Gonzalez and Althaus 1989). Affectors of catabo-
lism, listed in Althaus and Richter (1987), are DNA intercalators (Tavassoli
et al. 1985) which may directly interact with the polymers and may facilitate
the action of endo- and exonucleolytic enzymes on poly(ADP-ribose).

To date, three different enzymes, poly(ADP-ribose) glycohydrolase,
phosphodiesterase, and ADP-ribosyl protein lyase are known to be in-
volved in the catabolism of poly(ADP-ribose). The cleavage sites of these
enzymes are indicated in Fig. 1. Some characteristics of these enzymes have
been reviewed by Althaus and Richter (1987).

No cDNAs of the poly(ADP-ribose) catabolic enzymes have been cloned
and sequenced yet.

4.1
Poly(ADP-Ribose) Glycohydrolase

The physiological counterpart of ADPRT is the poly(ADP-ribose) glycohy-
drolase. This enzyme cleaves the ribose-ribose bonds of linear and
branched portions of polymers (Fig. 1) yielding free ADP-ribose (Miwa and
Sugimura 1971; Ueda et al. 1972; Miwa et al. 1974). Nuclear poly(ADP-ribose)
glycohydrolase with an apparent M_r of 59 000 as well as a cytosolic form of
the glycohydrolase (M_r about 75 000) have been purified and characterized
from different organisms: guinea pig liver (Tanuma et al. 1986a; Maruta et
al. 1991), pig liver (Tavassoli et al. 1983), calf thymus (Hatakeyama et al.
1986; Thomassin et al. 1992), mouse mammary tumor cells (Tsai et al. 1992),
human erythrocytes (Tanuma and Endo 1990), and human placenta
(Uchida et al. 1993c). The biological relationship and differences between
the nuclear and cytoplasmic glycohydrolases have been determined
(Maruta et al. 1991; Tanuma et al. 1986b; Uchida et al. 1993c). The biochemi-
cal properties and function of poly(ADP-ribose) glycohydrolase are re-
viewed in Desnoyers et al. (1995) and a lot of potent inhibitors of glycohy-
drolase activity have been described (Tsai et al. 1992; Aoki et al. 1993; Slama
et al. 1995).

From *in vitro* studies it has been proposed, that poly(ADP-ribose) glycohydrolase operates in a biphasic, bimodal reaction mode (Hatakeyama et al. 1986). While large polymers are degraded to smaller polymers in a fast and processive reaction, further degradation then proceeds in a slowly distributive reaction mode. Rapid initial degradation of large polymers, which may be facilitated by initial endoglycosidic incision was observed by Ikejima and Gill (1988). Accordingly, Braun et al. (1994) proposed that the glycohydrolase reaction can be divided into three steps: (a) endoglycosidic cleavage; (b) endoglycosidic cleavage plus exoglycosidic, processive degradation; (c) exoglycosidic, distributive degradation.

Poly(ADP-ribose) turnover was investigated in an *in vitro* system, consisting of ADPRT and glycohydrolase acting simultaneously, to elucidate the *in vivo* situation (Menard et al. 1990; Brochu et al. 1994; Shah et al. 1995).

4.2
Phosphodiesterase

Phosphodiesterases have been isolated from different species. The snake venom phosphodiesterase (EC 3.1.4.1) splits the pyrophosphate bond of poly(ADP-ribose) endonucleolytically (Sugimura 1973) yielding PR-AMP, as described earlier (see Sect. 2.2), and 5'AMP released from the polymer terminus. In contrast, phosphodiesterase isolated from rat liver (EC 3.1.3.1) cleaves poly(ADP-ribose) exonucleolytically, and also hydrolizes NAD^+, NADH, and ADP-ribose (Futai et al. 1968). Hydrolysis proceeds from the AMP-terminus of each polymer to the bound protein and does not produce oligomers of PR-AMP. Phospodiesterase isolated from tobacco cells also cleaves the phyrophosphate bonds in poly(ADP-ribose), ATP, NAD^+, inorganic pyrophosphate, cyclic nucleotides, dinucleotides, and in the cap-structure of the 5'terminus of mRNA (Shinshi et al. 1976). Besides that human urinic (Ito et al. 1987) and bull seminal (Codini et al. 1992) phosphodiesterase activities have been described.

4.3
ADP-Ribosyl Protein Lyase

Very little is known about the third enzyme of poly(ADP-ribose) catabolism, the ADP-ribosyl protein lyase. This enzyme cleaves the protein proximal ADP-ribose-glutamic ester bond (Fig. 1) (Okayama et al. 1978). The elimination reaction catalyzed by the lyase yields an unsaturated sugar

identified as 5'-ADP-3''-deoxypent-2''-enofuranose (Oka et al. 1984). The removal of the proximal ADP-ribose residue bound to the acceptor protein has been proposed to be the rate-limiting step in the catabolism of carcinogen-induced polymers (Wielkens et al. 1982).

Williams et al. (1984) have ascribed a heriditary neurological disorder in humans to a defect in activity of the lyase.

5
The Function of Poly(ADP-Ribosyl)ation

The physiological role of poly(ADP-ribose) metabolism in cells is still unclear. Several hypotheses suggest an involvement in central nuclear processes, such as DNA repair, differentiation, transcriptional regulation, cancerogenesis, chromosomal stability, or apoptosis, which are not necessarily mutually exclusive. Undoubtedly, the role of ADPRT contributes to a complex metabolic pathway. Loetscher et al. (1987) proposed, that poly(ADP-ribose) serves a hitherto unrecognized function by signalling altered metabolic conditions to the chromatin and thus modulating its functions dependent on the metabolic state of the cell.

Initiation of DNA synthesis is blocked by ADPRT and automodification reverses this inhibition by diminishing the DNA binding ability of the protein, indicating that ADPRT is a primarily structural DNA binding protein, whose catalytic activity serves to modulate its interaction with DNA (Nobori et al. 1989). Thus, ADPRT is suggested to be a critical regulatory component of a DNA binding multiprotein system, playing a central role in defining DNA structures in the intact cell (Rice et al. 1992). Following the observation that automodification of ADPRT interferes with DNA binding, a shuttle mechanism as a dynamic DNA-protein interaction model has been proposed (Zahradka and Ebisuzaki 1982). In this model, the dissociation of modified ADPRT from DNA and, consequently, the inactivation of the enzyme is reversed by subsequent action of poly(ADP-ribose) glycohydrolase, which reestablishes the DNA binding activity of ADPRT and, thus, reinitiates the conditions for enzyme activation. Ten years later Realini and Althaus (1992) proposed a "histone-shuttle model." This mechanism involves four distinct reaction intermediates, as shown *in vitro*. In the first step, ADPRT bound to DNA automodifies itself in the presence of histone-DNA complexes. In the second step the negatively charged ADP-ribose polymers bind noncovalently to histones, and the

histone-polymer-ADPRT complex dissociates from the DNA. The depro-
teinized DNA then becomes susceptible to nuclease digestion. In the third
step, poly(ADP-ribose) glycohydrolase degrades ADP-ribose polymers
and thereby eliminates the binding sites for histones. In the last step,
histones reassociate with DNA, and the resulting histone-DNA complex is
equivalent to the starting condition. This shuttle model would enable
nucleosomal unfolding of chromatin in processes such as regulation of
DNA excision repair. However, an *in vivo* occurence of the intermediates
suggested by the histone-shuttle model has not been proven yet. Further-
more, such a putative conformational change in chromatin seems not to
be required for DNA repair (Scicchitano and Hanawalt 1989).

5.1
Analytical Methods

Different experimental approaches have been designed to study poly(ADP-
ribosyl)ation reactions. Detection of poly(ADP-ribosyl)ation *in vitro* and
in vivo (reviewed in Althaus and Richter 1987; Shah et al. 1995), structure
analyses, and kinetic studies led to a considerable progress in this field.

One way to get information about the role of ADP-ribosylation in
cellular processes and to study the reaction mode is the application of
specific (ADP-ribosyl)ation inhibitors (reviewed in Althaus and Richter
1987; Rankin et al. 1989; Banasik et al. 1992; Banasik and Ueda 1994). Besides,
inhibitors of poly(ADP-ribosyl)ation are expected to provide novel
chemotherapeutic drugs against malignancies. Therefore, a great number
of investigations have been carried out, *in vitro* and *in vivo*, to determine
the structural requirements for potent ADPRT inhibitors (reviewed in
detail in Griffin et al. 1995). A very potent ADPRT inhibitor is 3-aminoben-
zamide with a K_i of about 2 μM (Purnell and Whish 1980), and the high
affinity of ADPRT to this nicotinamide analog was utilized for an efficient
enzyme purification with an affinity column (Burtscher et al. 1986). Several
experiments were performed using this inhibitory effect to study the influ-
ence of ADPRT on processes such as DNA repair.

"Knocking-out" ADPRT activity in *in vivo* systems is another approach
to elucidate the physiological role of this enzyme, and modern technologies
offered the possibilities to produce recombinant enzymes (Ikejima et al.
1989; Gradwohl et al. 1989; Giner et al. 1992), and to create directed mutants
of ADPRT (Gradwohl et al. 1990; Simonin et al. 1993a,b; Cherney et al. 1991).
Table 1 summarizes various approaches undertaken in this field. The physi-

ological significance of results obtained by these experiments and presented in Table 1 are discussed in Sect. 5.3. The following four main strategies have been developed.

(a) ADPRT antisense RNA (antisense to full-length or the 5'-part of the messenger RNA) expressed in human or rodent cells inhibited endogenous translation of ADPRT mRNA. Using this method Stevnser et al. (1994) found that ADPRT appears to stimulate gene-specific repair of DNA after damaging by alkylating agents, but not the repair of UV-induced pyrimidine dimers. In addition, cells depleted of ADPRT activity with antisense RNA showed changes in chromatin structure and cell morphology, and strand rejoining of DNA single strand breaks seems to be delayed (Ding et al. 1992). ADPRT is probably also required in early stages of differentiation of some cell types (Smulson et al. 1995), and its involvement in the regulation of the IFN-γ inducible MHC class II expression has been discussed (Qu et al. 1994).

(b) In another series of experiments ADPRT activity is decreased by transdominant inhibition by overexpression (tde) of the N-terminal DNA binding domain of the enzyme. This domain exhibits only the DNA binding ability but is catalytically inactive. Overexpression or microinjection of this ADPRT domain has been shown to repress the enzymatic activity of the resident ADPRT. Whereas cell-proliferation of hamster CV60 cells was not affected by trans-dominant inhibition (Küpper et al. 1995) such experiments combined with DNA damaging by alkylating agents or γ-irradiation led to the conclusion that ADPRT is involved in the DNA excision repair pathway (Molinette et al. 1993). The constitutively transdominant expression of the ADPRT DNA binding domain in a stable HeLa cell line had drastic consequences for these cells when treated with genotoxic agents (Schreiber et al. 1995).

(c) By Chatterjee et al. (1987) a strategy for selection of cell variants deficient in ADPRT activity has been developed. Spontaneous mutants from rodent cells with a drastic decrease of ADPRT enzymatic activity showed prolonged doubling time, increased frequencies of sister chromatid exchange, and an increased temperature-sensitivity, which could be restored by reintroducing the wild-type ADPRT gene (Chatterjee et al. 1989; Yoshihara et al. 1992).

(d) The ultimate success was the cloning of homozygote ADPRT negative mice, although it was quite surprising that ADPRT negative mice are alive, healthy and even fertile (Wang et al. 1995).

Table 1. "Knocking-out" of ADPRT activity *in vivo*

Method	Results/conclusions	Reference
ADPRT anti-sense RNA expression		
in HeLa cells	Delay in DNA strand break rejoining altered cell morphology	Ding et al. 1992
in HeLa cells	Stimulation of the gene-specific repair of DNA damage caused by alkylating drugs; no influence on the repair of UV-induced pyrimidine dimers	Stevnser et al. 1994
in human leukemic cells	Involvement in the interferon-γ induced major histocompatible complex class II gene expression	Qu et al. 1994
in HeLa cells	Influence on genomic stability, chromatin organization and carcinogen cytotoxity; decrease in cellsurvival after treatment with cytotoxic drugs	Ding and Smulson 1994
in mouse preadipocytes	Importance for early stages of differentiation 3T3-L1 preadipocytes	Smulson et al. 1995
in mouse kerationocytes	Suitable system for studying the role of ADPRT in epidermis cells	Rosenthal et al. 1995
Trans-dominant inhibition by transient overexpression	(tde) of the ADPRT DNA binding domain	
in monkey cells	Inhibition of transient ADPRT activity	Küpper et al. 1990
in hamster CV 60 cells	Decrease in ADPRT activity after γ-irradiation; influence on cell survival after damaging; no influence on cell-proliferation	Küpper et al. 1995
in CV 1 monkey cells and micro-injection of ADPRT-DNA binding domain into human fibroblasts	Positive correlation between ADPRT activity nd repair of DNA adamage induced by alkylating agents; no effect on repair of DNA damage induced by UV-irradiation	Molinete et al. 1993
constitutively in stable HeLa cell lines	Prevention of cell recovery, apoptosis and sister chromatid exchange following DNA damage induced with alkylating agents; importance	Schreiber et al. 1995
for the G$_2$ checkpoint		

Table 1. Continue

Method	Results/conclusions	Reference
Cell lines deficient in ADPRT activity		
mutant hamster cells, defective in poly(ADP-ribose) synthesis (5%–11% ADPRT activity)	Prolonged doubling times; increased frequencies of sister chromatid exchanges	Chatterjee et al. 1989
mutated ADPRT in mouse cell line (8% ADPRT activity)	Prolonged doubling times; increased temperature sensitivity	Yoshihara et al. 1992
in vivo knock-out systems		
ADPRT knocked out mice	Proliferation following γ irradiation was impaired; susceptibility to spontaneousdevelopment of skin disease in older mice	Wang et al. 1995
pancreatic islet cells of ADPRT- knocked out mice	Positive correlation between ADPRT activity, NAD$^+$ depletition and cell death in pancreatic islet cells after exposure to DNA-	Heller et al. 1995
damaging radicals, nitric oxide or reactive oxygen intermediate		

5.2
Protein-Protein Interactions of ADPRT

A participation of ADPRT in regulatory processes is inevitably coupled to an interaction with partner proteins. Modification of acceptor proteins has been observed *in vitro* using reconstituted systems with purified ADPRT. Alternatively, proteins modified *in vivo* were isolated from "broken cell" systems or polynucleosomal preparations. Althaus and Richter (1987) reviewed in detail target proteins of poly(ADP-ribobosyl)ation known until 1987 and discussed the resulting effects of this modification. It is a general feature of identified acceptor proteins that they physiologically act on deoxynucleotide or nucleotide polymers. Generally, modification of such potential target enzymes suppressed their catalytic activity *in vitro*, probably due to the fact that modification decreases their DNA binding ability as it has been reported for the automodification reaction of ADPRT itself (Yoshihara et al. 1981; Ferro and Olivera 1982; Zahradka and Ebisuzaki 1982). Target proteins of heteromodification are histones *in vitro* (Caplan et al. 1979; Tanaka et al. 1979), and *in vivo* (Burzio et al. 1979; Lichtenwalner and Suhadolnik 1979), topoisomerases I and II (Jongstra-Bilen et al. 1983; Ferro and Olivera 1984; Adamietz 1985; Darby et al. 1985), DNA polymerase α and β (Yoshihara et al. 1985), DNA ligase I and II (Yoshihara et al. 1985), high mobility group proteins (Levy-Wilson 1981; Poirier et al. 1982; Faraone-Mennella et al. 1982; Tanuma et al. 1983), and low mobility group proteins (Faraone-Mennella et al. 1984).

Since 1987 a lot of work has been done on identification and further characterization of potential acceptors of poly(ADP-ribose) and proteins interacting with ADPRT (Table 2). In recent years the protein-protein interaction of ADPRT with histones, low- and high-mobility group proteins, DNA polymerases, topoisomerases and ligases, already known to be target proteins of ADP-ribosylation, has been investigated and described in more detail. Protein-Protein interaction of ADPRT with DNA polymerase α led to a stimulation of polymerase activity *in vitro* (Simbulan et al. 1993), while modification of polymerases α and ε with poly(ADP-ribose) decreased their activity (Eki 1994; Eki and Hurwitz 1991). The affinity of ADPRT to the nuclear matrix (D'Erme et al. 1990; Kaufmann et al. 1991; Quesada et al. 1995) and cell cycle dependent patterns of modification (Adolph 1987) suggest an involvement of ADPRT in chromatin arrangement in DNA replication or recombination during the cell cycle. Modification of histones and HMG proteins with poly(ADP-ribose) *in vitro* resulted

Table 2. Influence of interaction with ADPRT and poly(ADP-ribosyl)ation on proteins

Target protein	Description/functional relevance	Reference
Histones		
Histones	Interaction between DNA, ADPRT, and histones *in vitro*; influence of binding from ADPRT to histones on DNA topology; auto(ADP-ribosyl)ation topolgical decreases constraints on DNA	Sastry and Kun 1988
Histones	Presence of at least 60 ADP-ribosylated variant histones in isolated nuclei from mouse myeloma cells in culture	Boulikas 1988
Histones	Presence of oligo(ADP-ribosylated) histones in relation to cell proliferation; ocurrence of poly(ADP-ribosylated) histone intermediates in nuclear processes involving DNA strand breaks; assembly of poly(ADP-ribosyl)ated histone complexes and des-position on DNA during replication	Boulikas 1989, 1990
H1. H2B	Modification of chromatin (H2B is main target); reconstitution with unmodified H1	Huletsky et al. 1989
H1, H2B, H2A, H3, H4	Modification in response to treatment with alkylating agents	Krupitza and Cerutti 1989a
H1, H2B	Pattern of modification in dependence of ageing	Quesada et al. 1990a
H1, H2A, H2B, H3, H4	Rearrangements of nucleosome structure	Thibeault et al. 1992
HMG-proteins and histones	Resistance to proteolytic cleavage of modified proteins *in vitro*	Boulikas and Poirier 1992
HMG	Pattern of modification during differentiation of mouse preadipocytes	Janssen and Hilz 1989
Histones and low mobility proteins a, β, and γ	Pattern of modification during spermatogenesis	Quesada et al. 1989
H1, H2A, H2B, H3, H4 and protamines	Noncovalent interactions with poly(ADP-ribose)	Panzeter et al. 1992
H1, H2B	Protein-protein interaction between the automodification domain of ADPRT and the C-terminus of H1, and the N-terminus of H2B, respectively	Buki et al. 1995
Topoisomerases		
Topo I	Modification in response to treatment with alkylating agents inhibits enzymatic activity	Krupitza and Cerutti 1989b
Topo II	Modification inhibits enzymatic activity	Schröder et al. 1989
Topo II	Modification in untreated HeLa nuclei *in vivo*; no increase in	Scovassi et al.1993

DNA polymerases

pol α, HSSB (single-strand DNA binding protein)	Modification inhibits replication *in vitro*	Eki and Hurwitz 1991
pol α, pol β, pol γ, primase	Physically association of pol α with ADPRT *in vivo*; dose-dependent stimulation of pol α activity by addition of ADPRT *in vitro*; no effect on pol β, pol γ, primase activity	Simbulan et al. 1993
pol α, pol β, pol ε no effect on pol δ	Modification inhibits polymerase activity of pol α and pol ε *in vitro*;	Eki 1994
Nuclear matrix proteins		
Scaffold proteins	Modification pattern of nonhistone proteins in dependence of cell cycle; increased modification in S phase	Adolph 1987
Nuclear matrix	Major poly(ADP-ribosyl)ation site during replication *in vivo*	Alvarez-Gonzalez and Ringer 1988
Nuclear matrix	Binding of ADPRT to attachment points of loop DNA on the chromatin matrix	D'Erme et al. 1990; Quesada et al. 1995
Nuclear matrix	Cross-linking of ADPRT to the nuclear matrix	Kaufmann et al. 1991
Nucleolar phosphoproteins	Modification of numatrin/B23 and nucleolin/C23 in isolated nuclei	Leitinger and Wesierska-Gadek 1993
Nucleolar protein B23	Increased expression after exposure to radiation; modification by ADP-ribosylation and phosphorylation	Ramsamooj et al. 1995
Others		
Protein kinase C	Phosphorylation of ADPRT in vitro inhibits ADPRT activity	Tanaka et al. 1987
Protein kinase C	Phosphorylation of ADPRT within the automodification- and NAD^+-binding domain inhibits ADPRT activity and DNA binding.	Bauer et al. 1992
p66 (mouse)	Poly(ADP-ribosyl)ation of a 66 000 protein *in vivo*; copurification with ADPRT	Zhang and Ebisuzaki 1989
O^6-Methylguanine-DNA-methyltransferase	Induction by poly(ADP-ribosyl)ation	Lefebvre and Laval 1989
HeLa nuclear ribonucleases	Modification inhibits ribonuclease activity *in vitro*	Quesada et al. 1990b
Heterogeneous ribonucleo proteins (hnRNPs) of HeLa cells	ADP-ribosylation of hnRNP A1 and A2/B1 *in vivo*	Prasad et al. 1994

in a resistance to proteolytic cleavage of the modified proteins (Boulikas and Poirier 1992) and repressed nucleases in their activity (Quesada et al. 1990a,b).

Phosphorylation of ADPRT by proteinkinase C inhibited ADPRT activity *in vitro* (Tanaka et al. 1987; Bauer et al. 1992) and phosphorylation of ADPRT *in vivo* in permeabilized human leukocytes was observed (Bauer et al. 1994), suggesting a biological significance of ADPRT in cellular signal transduction.

Several experiments were performed to study the noncovalent interaction of poly(ADP-ribose) with proteins. Affinity chromatography with poly(ADP-ribose)-agarose beads (Panzeter et al. 1992) made it possible to screen for proteins which may interact with polymers, as has been shown for histones. Another approach to detect noncovalent interactions between polymers and cellular proteins transblotted onto nitrocellulose has been described by Nozaki et al. (1994). The interaction of poly(ADP-ribose) polymers of automodified ADPRT *in vivo* with adjacent proteins, predominantly histones, may provoke alteration the chromatin conformation through noncovalent interactions with histones (Panzeter et al. 1993).

Nevertheless, up to now no functional relevant ADPRT partner protein has been clearly established *in vivo*, which would allow to assign ADPRT to any distinct regulatory process.

5.3
Influence on Cellular Processes

5.3.1
DNA Repair

Numerous reports have demonstrated that alkylating agents and ionizing radiation lower intracellular NAD^+ levels (reviewed in Althaus and Richter 1987), and it has been shown that the major cellular pathway of NAD^+ catabolism refers to ADPRT activity (Whish et al. 1975; Smulson et al. 1975). Accordingly, depletion of the intracellular NAD^+ pool after treatment with alkylating agents or radiation has been found to be due to ADPRT activity stimulated by the presence of DNA single-strand breaks (Miller 1975). Since Durkacz et al. (1980) proposed a strong link between poly(ADP-ribosyl)ation reactions and DNA excision repair, several analyses in different systems have been performed to determine the role of ADPRT in DNA repair (reviewed in detail by Althaus and Richter 1987). Recent efforts to

study the participation of ADPRT in DNA repair revealed that ADPRT is not a necessary repair enzyme (reviewed in Lindahl et al. 1995), but it is able to stimulate the excision repair pathway. In cell-free HeLa extracts, depleted of endogenous DNA and ADPRT enzyme, DNA repair of γ-irradiated plasmids occured in a NAD^+ independent manner, whereas after addition of purified ADPRT DNA repair was accelerated in the presence of NAD^+ (Sathoh and Lindahl 1992; Satoh et al. 1993). In contrast to conclusions deduced from the histone-shuttle model (Realini and Althaus 1992), Satoh and Lindahl (1992) proposed a model of a histone independent participation of ADPRT in the excision repair pathway. Polymer synthesis during DNA repair was investigated in an *in vitro* system (Satoh et al. 1994) in agreement with a proposed model for ADPRT cycling and DNA strand break rejoining (Smulson et al. 1994). Screening for ADPRT activity in dependence on DNA damage showed, that the polymer metabolism *in vivo*, especially the formation of branched polymers, is enhanced (Malanga and Althaus 1994). Analyses with ADPRT anti-sense RNA expression or transdominant inhibition of ADPRT support a correlation between ADPRT activity and DNA repair after γ-irradiation or exposure to alkylating agents *in vivo* (see Table 1).

ADPRT negative mice showed no drastic decrease in their DNA repair capability (Wang et al. 1995), implying that ADPRT is not directly participating in DNA excision repair.

5.3.2
Antirecombination and Genomic Stability

Eukaryotic cells contain substantial amounts of repeated DNA sequences, and frequent recombination between such repetitive elements would lead to genomic instability. During recombination events DNA breaks appear. ADPRT is able to bind rapidly and tightly to such breaks, suggesting a possible participation of this enzyme in recombination processes. Farzaneh et al. (1988) discussed the involvement of this enzyme in eukaryotic DNA recombination. They observed that ADPRT activity stimulates the integration of donor DNA into the host genome during DNA transfection of eukaryotic cells.

A mutant hamster cell line, defective in ADPRT activity, showed prolonged doubling times and increased frequencies of sister chromatid exchanges (Chatterjee et al. 1989). Furthermore, inhibition of ADPRT activity by 3-aminobenzamide led to an increase of recombination events in several

animal and plant cell systems, as shown by an enhanced sister chromatid exchange frequency (Oikawa et al. 1980; Puchta et al. 1995). Studies with the ADPRT inhibitor 3-methoxybenzamide in mouse cells showed influences of this drug on different recombination pathways in mammalian cells. 3-Methoxybenzamide inhibited illegitimate recombination by repressing intrachromosomal homologous recombination (Waldman and Waldman 1991), whereas no effect on extrachromosomal recombination events could be observed (Waldman and Waldman 1990).

ADPRT inhibitors potentiate the recombinogenic but not mutagenic action of alkylating agents in somatic *Drosophila* cells *in vivo* (Magnusson and Ramel 1990). An increase in antibody class switching in mouse lymphoma cells following the application of ADPRT inhibitors was observed, due to an enhanced rearrangement in genomic DNA segments (Shockett and Stavnezer 1993). Local poly(ADP-ribose) synthesis in the vicinity of DNA strand interruptions causes a negative charge repulsion between the polymer and the DNA, probably to prevent accidental homologous recombination within tandem repeat DNA sequences (Satoh et al. 1994; Chatterjee and Berger 1994).

From these observations a regulatory role of ADPRT in recombination events has been suggested.

5.3.3
Gene Expression and Differentiation

Slattery et al. (1983) found that ADPRT copurifies with a fraction of transcription factors of the polymerase II system. Therefore, ADPRT was suggested to be a secondary factor in gene transcription by suppressing nick-induced pol II dependent transcription. However, no direct correlation between gene transcription and poly(ADP-ribosyl)ation could be observed *in vitro* (reviewed in Zahradka and Yau 1994), but a role in the signal transduction pathway leading to an activation of rRNA gene expression has been proposed (Mishima et al. 1993).

Differential expression of human ADPRT mRNA has been observed (Mc Nerney et al. 1989; Cesarone et al. 1990; Menegazzi et al. 1991; Chabert et al. 1992; Alcivar et al. 1992). While this observation could be partially ascribed to a difference in stability of the transcripts (Negroni and Bertazoni 1993), expression of ADPRT is suggested to be mainly regulated by transcription (Ogura et al. 1990a,b). A mechanism of autoregulation of the human ADPRT gene has been proposed (Oei et al. 1994; Schweiger et al. 1995).

Studies with a chimeric protein, in which the catalytic domain of ADPRT was fused to the DNA binding domain of the glycocorticoreceptor revealed, that ADPRT activity can be targeted to specific DNA sequences and represses gene expression (Rosenthal et al. 1994). Transcription of the ADPRT gene turned out to be regulated during the cell cycle of concanavalin A stimulated rat thymocytes (Wein et al. 1993) and after stimulation of human lymphocytes with a nonmitogenic dose of TPA (Menegazzi et al. 1992). Inhibition of glycohydrolase activity indicated that poly(ADP-ribosyl)ation of histone H1 and HMG 14 and 17 is associated with the suppression of the glucocorticoid-sensitive mouse mammary tumor virus mRNA synthesis (Tsai et al. 1992). Applying hormones to rats and measuring ADPRT activity led to the suggestion that the enzymatic activity is controlled by thyroid hormones (Cesarone et al. 1994).

Cytotoxic effects of carcinogens seem to be consistently enhanced by ADPRT inhibitors. Drugs interacting with ADPRT inhibited carcinogen-induced cellular transformation (Kun et al. 1983; Milo et al. 1985), prevented tumorgenesis (Tseng et al. 1987), and led to an altered chromatin structure, resulting in gene amplification (Bürkle et al. 1990). Concerning cancer research, numerous investigations have been done to develop potent specific ADPRT inhibitors (reviewed in Griffin et al. 1995). The relevance of ADPRT in transcription regulation is supported by the observation that in cells of ADPRT negative mice proliferation following γ-irradiation was impaired (Wang et al. 1995).

ADPRT seems also to be regulated on a posttranscriptional level (Herzog et al. 1989; Bhatia et al. 1990). A potential role of ADPRT in differentiation processes has been discussed. Treatment of *Trypanosoma* with the specific ADPRT inhibitor 3-aminobenzamide resulted in a delay of the morphological switch of the parasites surface proteins (Cornelissen et al. 1985), which could enable the infected host to a fitting antigen response. A treatment of *Leishmania mexicana amazonensis* with ADPRT inhibitors diverted this parasite from differentiation to proliferation, indicating that poly(ADP-ribosyl)ation is required for the initiation of differentiation (Taylor and Williams 1988). The down-regulation of ADPRT is suggested to be required for the process of IFN-γ induced MHC class II expression in murine leukemia cells (Taniguchi et al. 1993; Nomura et al. 1991; Tomoda et al. 1992), as well as in human leukemia cells (Otsuka et al. 1991; Hiromatsu et al. 1992; Qu et al. 1994). The activity of ADPRT is controlled by regulation of protein levels during differentiation of human leukemia and neutrophilic cells (Bhatia et al. 1995).

These results suggest that modulation of ADPRT gene transcription is required for cellular differentiation and maturation of certain cell lineages.

5.3.4
Cell Cycle

Poly(ADP-ribosyl)ation of nuclear proteins was investigated in dependence on the cell cycle (Adolph 1987). A specific pattern of modified nuclear proteins was found in synchronized HeLa cells. While more than 100 acceptor proteins could be detected in interphase nuclei, in the metaphase only ADPRT itself served as acceptor of poly(ADP-ribose) polymers. As shown by Tanuma and Otsuka (1991) the activity of poly(ADP-ribose) glycohydrolase is doubled during the G_1 phase of HeLa cells. In response to genotoxic treatment HeLa cells deficient in ADPRT activity accumulated in the $G_{2+}M$ phase, suggesting an ADPRT function critical for the G_2 checkpoint (Schreiber et al. 1995). Accordingly, ADPRT content as measured immunologically in rat fibroblasts was found to increase from G_1 to S and $G_{2+}M$ phases. Quiescent cells showed a lower ADPRT content than cells in the G_1 phase indicating a cell cycle specific event activating ADPRT expression before the $G_{2+}M$ phase (Leduc et al. 1988). The ADPRT mRNA level during the cell cycle was analyzed and was found to culminate in the G_1 phase (Thibodeau et al. 1989). A possible cell cycle dependent biosynthesis of ADPRT has also been proposed for mouse SV40-3T3 cells (Sooki-Toth et al. 1987). ADPRT seems to be critical for the induction of G_1 arrest and is involved in the regulation of G_2 arrest (Masutani et al. 1995).

The expression of ADPRT cDNA in yeast cells, lacking ADPRT and poly(ADP-ribose) glycohydrolase activities, resulted in the synthesis of poly(ADP-ribose) polymers in yeast. These polymers then provoked a cell cycle retardation, as a result of a specific delay of the G_1 phase, a decreased cell viability in stationary cultures, and an increased sensitivity to radiation (Kaiser et al. 1992; Avila et al. 1994; Collinge and Althaus 1994). Therefore, it is likely that ADPRT is an element of the G_2 checkpoint.

5.3.5
Ageing/Apoptosis

A positive correlation between life-span and ADPRT activity of 13 different mammalian species has been observed (Grube et al. 1992). The higher

poly(ADP-ribosyl)ation capacity in long-living species might be more efficient in preventing genetic alterations.

Increased ADPRT activity after application of DNA damaging cytotoxic drugs led to apoptosis (Marks and Fox 1991). Inhibitors of ADPRT partially prevent cell lysis of cytolytic T lymphocytes (Redegeld et al. 1992). ADPRT inhibitors interacting with the zinc finger domain completely suppressed the proliferation of leukemic and other malignant human cells and cause endonuclease-mediated cell death (Rice et al. 1992). H_2O_2, in concentrations achieved in the proximity of stimulated leukocytes, induced injury and lysis of target cells. Cell lysis was found to be associated with the activation of ADPRT (Schrauffstatter et al. 1986). Toxicity of chemically generated nitric oxide towards pancreatic islet cells and inhibition of cellular respiration in cells exposed to peroxynitrite can be prevented by ADPRT inhibitors (Kallmann et al. 1992; Radons et al. 1994; Szabo et al. 1996). It is proposed that nitrooxygen-induced neurotoxicity is caused by NAD^+ depletion due to stimulated ADPRT activity, resulting in cell lysis (Zhang et al. 1994). After exposure to DNA-damaging radicals ADPRT negative mutant islet cells were more resistant to the toxicity of nitric oxide and reactive oxygen intermediates (Heller et al. 1995). During the onset of apoptosis (recently reviewed by Vaux and Strasser 1996) specific proteases (Tewari et al. 1995; Nicholson et al. 1995) were activated, resulting in the appearance of specific proteolytic ADPRT fragments (Kaufmann et al. (1993). The proteolytic products were considered as markers of apoptosis (Lazebnik et al. 1994). However, it seems unlikely that ADPRT cleavage during apoptosis is a relevant signal of cell death *in vivo*.

6
Conclusions

The immediacy of the transient response of ADPRT activity, which apparently precedes DNA repair processes, as well as the high energy cost of polymer synthesis suggest that the poly(ADP-ribosyl)ation reaction catalyzed by ADPRT is an event of major importance in perturbed cell nuclei. However, it is still impossible to define the exact physiological role of ADPRT.

In 1980 Durkacz et al. suggested for the first time an involvement of poly(ADP-ribosyl)ation in DNA repair. Since then a lot of effort has been done to clarify the role of ADPRT in DNA excision repair. The influence of

specific ADPRT inhibitors on DNA repair indicated a participation of poly(ADP-ribosyl)ation reactions in the recovery from DNA damage. From these studies only the cytotoxic effects of carcinogens seem to be consistently enhanced by ADPRT inhibitors. Recent investigations using enzyme-depleted extracts or cells and even ADPRT "knocked-out" mice show that ADPRT does not directly participate in DNA repair. The absence of a striking difference in phenotype of ADPRT-negative mice (Wang et al. 1995) and their normal fetal and postnatal development revealed that poly(ADP-ribosyl)ation is not essential for mouse development and their cellular processes. This entangling result leads to the speculation of alternative, presently unknown mechanisms, which may substitute for the ADPRT function in these mice. Impaired proliferation and the onset of skin lesions in older mice suggest a function for ADPRT in response to environmental stress (Wang et al. 1995). Thus, these mice can provide a valuable system for testing the mutagenic and carcinogenic potential of agents in our environment and are also expected to aid studies on the role of ADPRT in other regulatory functions.

HeLa cells with depressed enzymatic ADPRT activity showed chromosomal instability, an increase in their doubling time, $G_{2+}M$ accumulation, and a marked reduction of cell survival in response to genotoxic treatment (Schreiber et al. 1995). It seems worthwhile to examine the role of poly(ADP-ribosyl)ation reactions under these aspects.

The future will provide discovery of physiological partner-proteins of ADPRT and hopefully allow to define the precise biological roles of this enzyme.

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Griesenbeck et al (submitted)

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A Discussion of Mechanisms of NO Genotoxicty: Implication of Inhibition of DNA Repair Proteins

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Contents

1
Introduction

It has been proposed that damage to DNA by various chemical agents leads to a number of diseases such as cancer. The repair of lesions induced on DNA by various xenobiotic agents is mediated by a series of DNA repair proteins. Through evolution, these proteins have evolved to insure the integrity of the genome Therefore, a balance exists between damage and repair, which is critical to the maintenance of the genome.

Under aerobic conditions, living organisms generate superoxide and peroxide which are products of oxygen metabolism. These agents, in the presence of metals, can form powerful oxidants which oxidize nucleic acids as well as induce strand breaks (Halliwell and Gutteridge 1989). Recently, another small molecule, nitric oxide, has been shown to be produced in vivo throughout the animal kingdom and to participate in the regulation of a number of key physiological roles (Ignarro 1990; Moncada et al. 1991; Feldman et al. 1992). This discovery was surprising since NO, in an aerobic environment, generates a number reactive nitrogen oxide species (RNOS) which are known to be toxic to living cells. This report will discuss some of the mechanisms by which NO is involved in various genotoxic mechanisms and the importance that the NO interaction with DNA repair proteins plays in these events.

To understand some of the mechanisms by which NO might mediate genotoxic events, a discussion of some of the chemistry and biochemistry of NO is useful. There are numerous reviews on biology of nitric oxide which cover the production of NO in various cells and biological conditions Nitric oxide is formed from the oxidation of arginine to citrulline, which is mediated by the enzyme nitric oxide synthase (NOS; Nathan and Xie 1994; Marletta 1993; Griffith and Stuehr 1995). This protein has similarities to the monoxygenase, cytochrome P450. NOS contains several cofactors such as calmodulin, tetrahydrobiopterin, FAD and FMN, as well as heme prothetic group. The oxidation of arginine to yield NO is mediated at the heme site. There are several isoforms of NOS which can be categorized into constitutive and inducible. The constitutive form (cNOS) is stimulated by an influx of extracellular calcium which binds to calmodulin thereby activating the enzyme. This isoform is present in specific cell types and modulates various physiological functions such as vascular tone (Ignarro 1990; Moncada et al. 1991). The inducible form (iNOS) is induced by combinations of several cytokines and/or endotoxins (Nathan and Xie 1994; Griffith and Stuehr

1995). iNOS is calcium independent and generates nitric oxide for pro-
longed periods of time yielding higher NO fluxes than cNOS. The duration
and amounts of NO production has a tremendous influence on the chem-
istry of NO in biological systems, and this is probably the major reason for
the two distinct isoforms of NOS. Thus, the chemistry of NO at different
NO fluxes can give insight into the different roles NO can play in biology.

2
Chemical Biology of NO

An important aspect of NO in various genotoxic mechanisms is the chem-
istry. A discussion of what we termed the "chemical biology of NO" is
useful. The chemical biology of NO can be defined as the pertinent chemical
reactions which occur in biological systems (Wink et al. 1996). These
reactions can be categorized into direct and indirect effects. Direct effects
are those reactions where NO directly interacts with specific biological
molecules. Indirect effects are the chemical reactions between RNOS de-
rived from NO and various biological molecules. Therefore, the direct
effects are the chemistry of NO, whereas the indirect effect is the chemistry
of RNOS. The advantage of separating the NO chemistry into these two
effects is that the concentrations of NO distinctly determine which effects
can occur. For instance, direct reactions occur at lower concentrations of
NO such as those that might be derived from cNOS. Yet, at higher concen-
trations, as expected from iNOS, indirect reactions may occur. Therefore,
the relevance of the chemical reactions might depend on the isoform of
NOS under a specific condition. The chemical biology provides insight into
the role NO plays in genotoxic mechanism.

Nitric oxide is a relatively unreactive molecule (see review in Wink et
al. 1996; Wink and Ford 1995). NO interacts directly in biological conditions
with metalloproteins such heme containing enzymes but doesn't react
directly with bio-organic molecules which contain thiol or amines. One
example of a direct effect is the binding of NO to the heme moiety in
guanylate cyclase which stimulates the conversion of GTP to cGMP (Ig-
narro 1990). However, under aerobic conditions, NO can react with oxygen
or superoxide to form reactive nitrogen oxide species such as N_2O_3 and
peroxynitrite (see review in Wink et al. 1996; Wink and Ford 1995). These
two species are proposed to be the primary RNOS formed in vivo. N_2O_3 can
be derived from several sources: the reaction of NO with O_2, acidic nitrite,

or as will be discuss below, from the NO/O_2- reaction. The primary mode of chemistry is to nitrosate amines and thiols to form nitrosamines and S-nitrosothiols (Williams 1988). The formation of these adducts occurs through the donation of NO+ to the amine or thiol complex (Eqs. 1, 2):

$$N_2O_3+RSH \rightarrow RSNO+NO_2- \qquad (1)$$
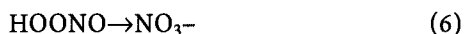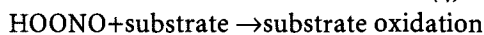$$N_2O_3+R'R''NH \rightarrow R'R''NNO+ NO_2- \quad (2)$$
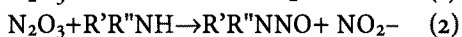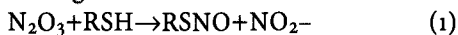
The conditions where these nitrosative adducts are formed in biological systems are referred to as nitrosative stress.

The reaction between NO and superoxide (Eq. 3) to form peroxynitrite has received a lot of attention in the literature, as being either a potential deleterious species or representing a detoxication mechanism for ROS formed from peroxide and metals (Pryor and Squadrito 1996):

$$NO+O_2^- \rightarrow OONO^- \qquad (3)$$
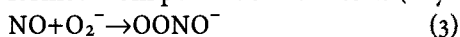
Peroxynitrite is relatively unreactive in basic solutions, slowly dismutating to nitrite and oxygen. In addition, peroxynitrite reacts with sulphydryl complexes resulting in the formation of disulfides, at a rate constant of 6×10^3 M/s (Radi et al. 1991). However, protanation at neutral pH of peroxynitrite forms a reactive intermediate (Eq. 4) which can oxidize various biological substrates (Eq. 5) or simply isomerize to nitrate (Eq. 6; Koppenol et al. 1992).

$$OONO^- \rightarrow HOONO \qquad (4)$$
$$HOONO+substrate \rightarrow substrate\ oxidation$$
$$HOONO \rightarrow NO_3- \qquad (6)$$

The reactive intermediates formed from protonation were thought to be nitrogen dioxide and hydroxyl radical in aqueous solution. However, it appears that this intermediate is less potent oxidant (Koppenol et al. 1992). A recent study has shown that peroxynitrite can oxidize its substrate by either one or two electrons (Pryor et al. 1994), showing a distinctly different reactivity pattern from either OH or NO_2. A very interesting reaction which could have biological implications is the nitration of tyrosine by peroxynitrite in the presence of metal ions (Ischiropoulous et al. 1992), which may contribute to the inhibition and alteration of key cellular proteins. However, the yield of this reaction is less than 5%. Overall, the predominant chemistry mediated by $OONO^-$ is oxidation, and therefore peroxynitrite represents a form of oxidative stress.

Though peroxynitrite may represent a mechanism by which NO can be activated to a potent oxidizing species, several recent reports suggest that oxidation mediated by this species is influenced by the relative amounts of NO and superoxide formed (Miles et al. 1995, 1996). It has been suggested

that N2O3 can be formed from the interaction of NO and superoxide. In the presence of excess of NO or superoxide, peroxynitrite is converted to nitrogen dioxide (Miles et al. 1996; Koppenol 1996; Eqs. 7, 8):

$$H+$$
$$OONO^- + NO \rightarrow H + NO_2 + NO_2^- \qquad (7)$$
$$H+$$
$$OONO^- + O_2 \rightarrow H + NO_3 \qquad (8)$$
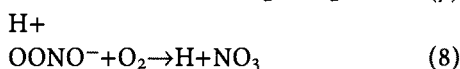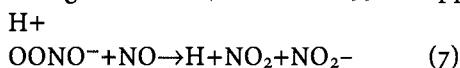
Nitrogen dioxide can rapidly react with NO to form the nitrosating species, N_2O_3 (Eq. 9):

$$NO_2 + NO \rightarrow N_2O_3 \qquad (9)$$

Thus relative amounts of NO and superoxide will impact the chemistry of peroxynitrite in vivo.

NO has been shown to generate chemical species which could alter biological macromolecules. However, several studies have shown that NO can abate oxidation reaction mediated by Fenton type reactions (Kanner et al. 1991; Rubbo et al. 1994; Hogg et al. 1993; Wink et al. b 1994). In the presence of reactive chemical species such as those derived from nitric oxide, superoxide or peroxide (via Haber-Weiss chemistry), there are two basic types of stress, oxidative and nitrosative. Oxidation occurs from the reaction of peroxide and metals to powerful oxidants like hydroxyl radical and hypervalent metals. The derivation of these types of oxidants are metal dependent oxidative stress. The reaction between O_2^- and NO to form peroxynitrite, where peroxynitrite mediates oxidative damage, can be termed metal independent oxidative stress. Since ROS derived from peroxide and $OONO^-$ do not mediate nitrosation (Miles et al. 1995; Wink et al. 1993), then nitrosative stress primarily arises from N_2O_3. Categorizing these effects can help to sort out when and where these types of reaction might occur and allow for discussion of different genotoxic mechanisms (Fig. 1).
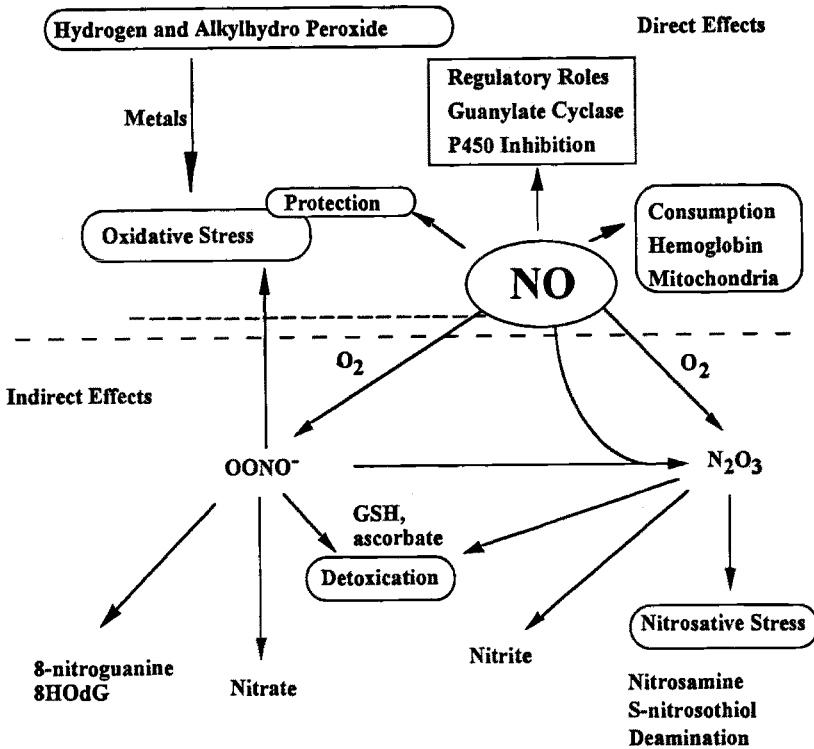
Fig. 1. The chemical biology of NO

3
Chemistry of NO and DNA

The presence of NO in an aerobic environment results in the formation of
RNOS which can nitrosate amines, forming potentially carcinogenic ni-
trosamines (Eq. 1; Williams 1988). It was first shown that induced macro-
phages could generate nitric oxide from iNOS. In the presence of secondary
amines, these induced macrophage formed nitrosamines (Marletta 1988).
It was proposed that nitrosamines formed from the activated immune
system might play a role in carcinogenesis associated with inflammation.
Liu et al. (1991, 1992) demonstrated that nitrosamines could be generated
from woodchuck liver chronically infected with hepatitis, suggesting that
there exists under certain conditions in vivo sufficient nitrosative stress to
form carcinogenic nitrosamines. These studies suggest that one mecha-

nism by which NO could be carcinogenic is via the formation of nitrosamines.

Though NO itself does not interact with bio-organic molecules such as DNA or proteins, RNOS such as N2O3 and peroxynitrite can alter DNA, resulting in a variety of lesions (Wink et al. 1996). It was shown that exposure of bacteria and mammalian cells to NO results in the formation of DNA single strand breaks (Arroyo et al. 1992; Nguyen et al. 1992). Further studies showed that deamination also occurred (Wink et al. 1991; Nguyen et al. 1992). It was proposed that the formation of RNOS via the autoxidation of NO was responsible for these lesions. In addition to cells being exposed to NO, several studies showed that when DNA was exposed to NO under aerobic conditions, cytosine, adenine and guanine underwent deamination (Wink et al. 1991; Nguyen et al. 1992). It was proposed that nitrosation of the exocyclic amine group resulted in a primary nitrosamine (Eq. 10). This was then followed by rapid deamination resulting in hydroxy group (Eq. 11):

$NH_2-R+N_2O_3 \rightarrow R-NHNO+NO_2-$ (10)
$R-NHNO \rightarrow R-NNOH \rightarrow R-OH+N_2$ (11)

This chemistry would result in the conversion of cytosine to uracil, guanine to xanthine, methylcytosine to thymine, and adenine to hypoxanthine. It has been proposed that this mechanisms involving NO could contribute to the spontaneous deamination which occurs in vivo.

Further studies have examined the resultant mutations in a shuttle vector treated with nitrosative agents then transfected and retrieved from bacterial and mammalian cells to determine the types of mutations after repair in prokaryotic and eukaryotic systems. Plasmids were treated with nitrosating agents from NO gas (aerobic) (Routledge et al. 1993), NO donor compounds (Routledge et al. 1994a) and acidic nitrite (Routledge et al. 1994b) and were transfected into different cells. The plasmids were recovered and the mutations determined. As shown in Table 1, the types of mutations varied. Though many of the mutations were attributed to deamination, a recent study has suggested that these lesions were not due to deamination in human tumor lines (Schmutte Crideout et al. 1994).

Another potential genotoxic agent is peroxynitrite formed from the reaction between NO and superoxide. Several studies have shown that synthetically generated peroxynitrite can cause various lesions to DNA. Peroxynitrite concentrations ranging from 0.05 to 8 mM were used to induce DNA strand breaks in vitro (King et al. 1992; Salgo et al. 1995). Oxidation of guanine to form C8-oxoguanine (HOdG) in the presence of

Table 1. Summary of distribution of mutations in the psP189 vector transfected into Ad293 cells

Mutations	Plasmid treatment			
	NO donors	NO (gas)	Nitrite (pH 5.4)	OONO⁻
Transversions				
GC→TA	14	4	29	65
GC→CG	8	1	9	28
Transitions				
GC→AT	55	29	46	11
AT→GC	7	60	12	–

SIN-1, an NO donor thought to generate NO and superoxide, was observed (Inoue and Kawanishi 1995), though another study suggested that peroxynitrite did not increase HOdG levels in DNA (Yermilov et al. 1995a,b). In addition to oxidation products, 8-nitroguanine has also been detected as a product of peroxynitrite reacting with guanine, suggesting that nitration could be an important process (Yermilov et al. 1995a,b). In cell culture, deRojas-Walker et al. (1995) have suggested that oxidative damage to DNA in activated macrophage is due to the formation of peroxynitrite. Juedes and Wogan transfected into *E. coli* and AD293 cells a peroxinitrite-treated plasmid (Juedes and Wogan 1996): a treatment with 2.5 mM peroxynitrite resulted in primarily GC11→TA (65%) transversions; GC1→1CG (28%) transversions; GC1→1AT (11%) transitions, suggesting a different mutation spectra from agents which induce nitrosative stress (Table 1).

Most of the studies involving peroxynitrite where conducted in the presence of large boluses of the synthetically generated compound. These preparations are contaminated with excessive nitrite and hydrogen peroxide which could contribute to the observed results. Contrary to bolus delivery of peroxynitrite, the chemistry of peroxynitrite formed in vivo depends on the relative fluxes of NO and superoxide in that microenvironment, as discussed above (Eqs. 4, 7–9; Miles et al. 1996). The amount of peroxynitrite which directly reacts with the biological target is minimal, if the NO flux is in excess. In fact, XO and peroxide mediated damage to DNA is abated by the presence of NO (Pacelli et al. 1994). It is presumed that Fenton chemistry mediates the DNA strand breaks which is abated by NO. Furthermore, hydroxylation reactions are also quenched by the presence of NO (Miles et al. 1996). These results suggest that the presence of NO could abate the oxidation chemistry mediated by oxidants generated in the

classical Haber-Weiss chemistry. Taken together, these protective effects indicate that involvement of RNOS to modify DNA directly might be limited in vivo while NO could protect the chemistry of oxidative stress.

The conditions to induce strands breaks, oxidation and deamination of DNA require high concentrations of RNOS or NO which may not be physiologically relevant. In vivo, antioxidants and RNOS scavengers, such as ascorbate and GSH, are abundant decreasing the chance that these chemical species directly modify DNA. This prompts the question: "How can NO be involved in genotoxic mechanisms?" One mechanisms which we have begun to explore is that NO and RNOS may affect DNA repair mechanisms.

4
NO and DNA Repair Proteins

RNOS have a particular high affinity for amino acids containing thiol residues (Wink et al. a 1994) suggesting that enzymes which have thiol residues critical to their function may be inhibited. We have surveyed several purified DNA repair proteins. The first was the O^6-alkylguanine-DNA alkyltransferase (alkyl transferase) which, exposed to the NO donor DEA/NO, was inhibited (Laval and Wink 1994). The alkyl transferase proteins, involved in the repair of O^6-methylguanine and O^4-methylthymine residues, contain a thiol group in their active site (Ling-Ling et al. 1992; Zak et al. 1994). It was shown that NO inhibited the alkyl transferase activity, not only in the mammalian purified protein, but also in whole cells (Laval and Wink 1994). As shown in Fig. 2, the methyl group of O^6-methylguanine is repaired by the alkyl transferase protein by simply transferring the methyl group from the O^6 position of the methylated guanine to a cysteine residue within the protein. Exposure to NO in an aerobic solution results in nitrosation of the thiol, thereby preventing the methyl transfer (Fig. 2). Since this NONOate compound releases NO over a period of time, a time dependent experiment was done. The inhibition was time dependent suggesting that NO or RNOS was mediating these effects. Cysteine has been shown to scavenge the RNOS formed from the autoxidation of NO (Wink et al. a 1994). When the protein was treated with NO in the presence of 10 mM cysteine, its activity was not inhibited This result suggests that NO itself was not responsible for the inhibition, but the resulting RNOS presumably N_2O_3.
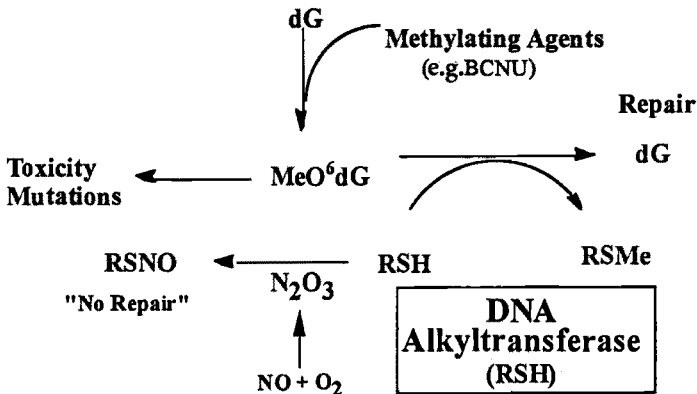
Fig. 2. Mechanism for O^6-methyl-guanine DNA methyltransferase inhibition by NO. (dG is one random deoxyguanine in DNA)

Another important class of DNA interacting proteins includes those containing zinc finger motifs (Schmeidescamp and Klevit 1994). Zinc finger motifs contain either two or four cysteine residues. One example is the *E. coli* formamidopyrimidine-DNA glycosylase (Fpg protein), which repairs alkylation damage to guanine, such as 2,6 diamino-4-hydroxy-5-*N*-methylformamidopyrimidine residues (Fapy) and oxidative damage such as 8-oxoguanine residues (rewieved by Laval 1996). This protein has a glycosylase activity, incises DNA at abasic sites by a β-δ elimination reaction, has a dRPase activity and contains a Zn finger motif which is mandatory for its various activities (O'Connor et al. 1993). It was shown that the Fpg protein activity was inhibited in the presence of aerobic NO (Wink and Laval 1994). It was suggested that N_2O_3 nitrosates the thiol residue, resulting in the ejection of the zinc. This degradation of the structural integrity of the protein does not allow its interaction with DNA, and hence inhibits repair in vitro and in vivo (Fig. 3). Another study showed that the zinc finger protein, LAC9, was degraded by the presence of NO (Kroncke et al. 1994). Using Raman spectroscopy, it was shown that *S*-nitrosothiol adducts were formed (Kroncke et al. 1994).

DNA ligases are enzymes which restore single strand breaks and are critical for the DNA integrity during processes such as replication and repair. The eukaryotic and T4 DNA ligases are active in the presence of ATP and act in two steps: the formation of protein-AMP intermediates then the ligation of DNA breaks (Lindahl and Barnes 1992). When T4 DNA ligase was exposed to the NO generator, DEA/NO {Et₂N[NO(NO)]Na}, a dose-
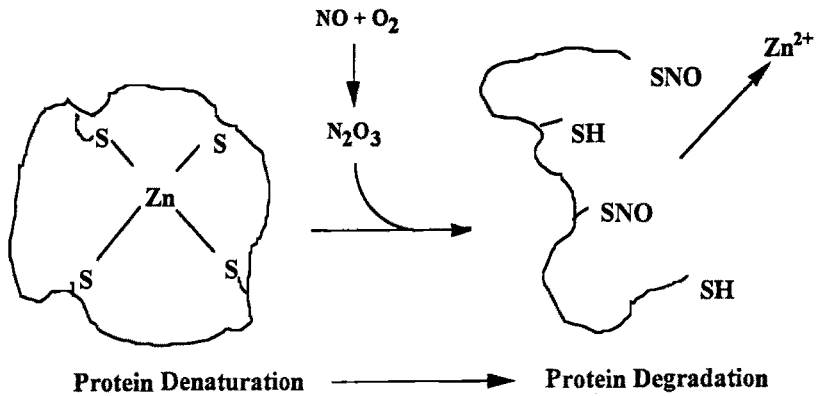
Fig. 3. Mechanism for the degradation of zinc finger motifs mediated by NO in the fpg protein

and time-dependent inhibition of these two steps, adenylylation of the protein and ligation of the substrate, was observed (Graziewicz et al. 1996). This inhibition was abated by the presence of cysteine, suggesting that RNOS rather than NO mediate the inhibition of the ligase activity. Recovery of the activity was observed neither with time nor in the presence of dithriothreitol, suggesting that the reaction is different from that observed in the case of the alkyltransferase protein. A critical lysine residue is present in the active site of the T4 DNA-ligase protein (Lindahl and Barnes 1992). This residue initially forms an intermediate with an adenyl group from ATP, then the adenyl group is transferred to the 5'P end of DNA (Fig. 4). In the final step, unadenylylated ligase is required for the generation of a phosphodiester bond. The initial adenylylation of the lysine is therefore a crucial step. In the presence of NO, nitrosation of the lysine would form a primary nitrosamine, which would rapidly rearrange to the diazonium salt followed by hydrolysis to yield the corresponding hydroxy adduct (Eq. 12):

$$\text{Protein-NH}_2 + \text{N}_2\text{O}_3 \rightarrow \text{-NH-NO} \rightarrow \text{Protein-N}_2\text{OH} + \text{OH}^- \tag{12}$$

It has been reported that the lysine residue is partially in the deprotonated form in the ligase protein (Engler and Richardson 1982). The lone pair electrons of the nitrogen atom that bind to adenylate group via nucleophilic attack provides a site for electrophilic attack. In the presence of NO, nitrosation of the partially deprotonated amine could occur via N_2O_3 which then results in a primary nitrosamine. Since primary nitrosamines undergo deamination (Williams 1988), this nitrosamine would result in a hydroxy group (Fig. 4). This chemistry represents a new mechanism by
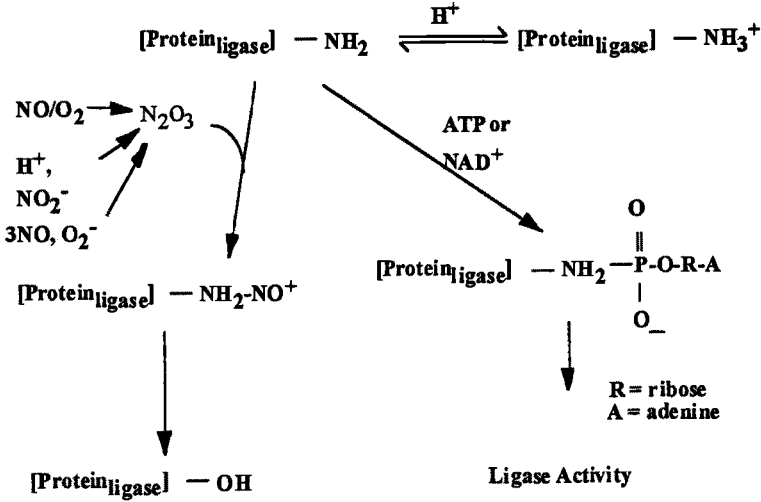
$$[Protein_{ligase}] \ — NH_2 \ \underset{\longleftarrow}{\overset{H^+}{\longrightarrow}} \ [Protein_{ligase}] \ — NH_3^+$$

NO/O$_2$ → N$_2$O$_3$

H$^+$,

NO$_2^-$

3NO, O$_2^-$

ATP or

NAD$^+$

$$O$$
$$\|$$
$[Protein_{ligase}] \ — NH_2 — P\text{-}O\text{-}R\text{-}A$

$$|$$

$$O\_$$

[Protein$_{ligase}$] — NH$_2$-NO$^+$

R = ribose
A = adenine

[Protein$_{ligase}$] — OH   Ligase Activity

Fig. 4. Mechanims for the inhibition of ligase mediated by NO

which NO (via RNOS) can interact with proteins suggesting that the pH of the protein site of the critical lysine residue is an important determinant for interaction with RNOS.

Different DNA ligases have been found in mammalian cells (Tomkinson et al. 1991). DNA ligase I is involved in the joining of Okazaki fragments during DNA replication (Li et al. 1994). A role in meiotic recombination has been suggested for DNA ligase II (Higashitani et al. 1990) and DNA ligase III activity has been related to DNA repair (Caldecott et al. 1994; Ljungquist et al. 1994). Recently, a fourth DNA ligase has been identified (Wei et al. 1995). It has also been shown that an inherited molecular defect in DNA ligase I resulted in immunosuppression, lymphoma and hypersensitivity to DNA damaging agents (Tomkinson et al. 1991). Therefore, inhibition of the DNA ligase activity could play a crucial role in the cells and could explain some previous observations. Exposure of cells to NO results in an increased number of DNA single strand breaks (Nguyen et al. 1992). However, when purified DNA is exposed to NO, even at doses resulting in RNOS concentration of 1 M, there is no formation of single strand breaks (Routledge et al. 1993). This implies that direct chemical modification of DNA by NO or RNOS does not occur to form DNA breaks, which, in vivo, are probably due to another mechanism. Our results suggest that one possibility is the inhibition of the DNA ligase activity by NO, resulting in

the accumulation of DNA breaks formed either during transcription or repair. The increase in DNA breaks due to NO-mediated inhibition of ligase could in turn activate the tumor suppressor gene, p53 (Messmer et al. 1994), or activate poly (ADP-ribose) synthesis (Zhang et al. 1994).

5
Conclusions

The involvement of NO in genotoxicity and carcinogenic mechanisms may be varied. On one hand NO can form RNOS which can modify DNA. Most of these experiments were done under very high concentrations of NO and RNOS and may have little or nothing to do with in vivo mechanisms. However, NO can affect DNA specific repair systems even in whole cells at lower NO and RNOS concentrations which might enhance the damage of another agent. Though NO might alter DNA directly, the most likely involvement of its genotoxic action is through the increase in sensitivity to other mutagenic agents. From this discussion, it appears that the primary source of RNOS is from iNOS. Thus genotoxicity either by direct chemical alteration of DNA or interference with the repair system would be from an iNOS source.

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