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Sensitivity of DNA and Nucleotides to Oxidation by Permanganate and Hydrogen Peroxide

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Introduction

For a number of cytotoxic and tumorigenic agents, active oxygen species such as hydroxyl radical are postulated to be responsible for indirect DNA damage (Cerutti 1981). Besides the formation of strand breaks, hydroxylation of DNA bases is assumed to occur, as shown earlier with ionizing radiation. It was the aim of this work to determine in vitro the DNA-damaging potency of hydrogen peroxide (H_2O_2), and to compare that with the potency of the strong oxidant permanganate ion.

Materials and Methods

Chemicals

Chemicals were obtained from Merck (Darmstadt, FRG) or Fluka (Buchs, Switzerland) and were of analytical grade. Nucleotides and calf thymus DNA were from Sigma (St. Louis, Mo., USA); [^{14}C]thymidine from the Radiochemical Center (Amersham, Buckinghamshire, GB).

Oxidation of Nucleotides and DNA

Oxidation with $KMnO_4$ was performed at 0 °C as described by Frenkel et al. (1981). The H_2O_2 reaction was carried out in 0.2 *M* sodium phosphate, pH 7.8, at 37 °C with 25 μ*M* $FeCl_3$. Single-stranded DNA was obtained by heat denaturation of double-stranded calf thymus DNA followed by rapid cooling at −30 °C. The concentration of the oxidant is given in Table 1 and Figs. 1 and 2.

Isolation of Oxidized DNA

The reaction mixture was dialysed; DNA was precipitated with ethanol and stored at −20 °C overnight. The DNA was centrifuged for 20 min at 1000 *g*,

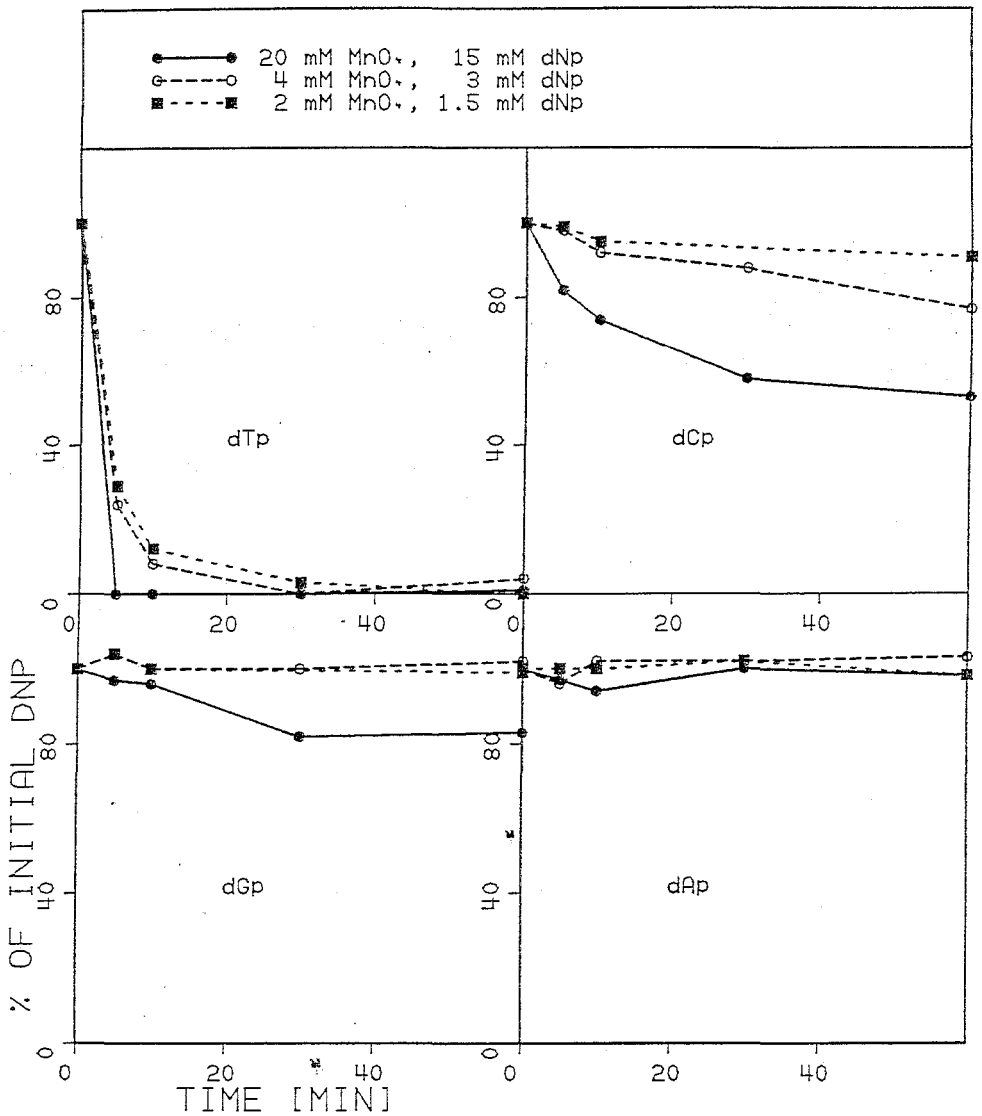

Fig. 1. Sensitivity of deoxyribonucleotides towards oxidation with $KMnO_4$ at 0 °C for various periods of time. *dTp*, thymidine monophosphate; *dCp*, deoxycytidine monophosphate; *dGp*, deoxyguanosine monophosphate; *dAp*, deoxyadenosine monophosphate

Table 1. Molar fraction and relative amount of nucleotides after oxidation for 48 h at 37° C of double-stranded DNA with H_2O_2. The least sensitive nucleotide dAp, was set to 100%. Oxidation of dAp cannot be excluded on the basis of these results.

dNp	Control		1 *M* H_2O_2	
	Molar fraction	Relative amount (%)	Molar fraction	Relative amount (%)
dTp	0.22	100	0.24	62
dCp	0.28	100	0.14	46
dGp	0.28	100	0.24	78
dAp	0.22	100	0.39	100

dTp, thymidine monophosphate; dCp, deoxycytidine monophosphate; dGp, deoxyguanosine monophosphate; dAp, deoxyadenosine monophosphate

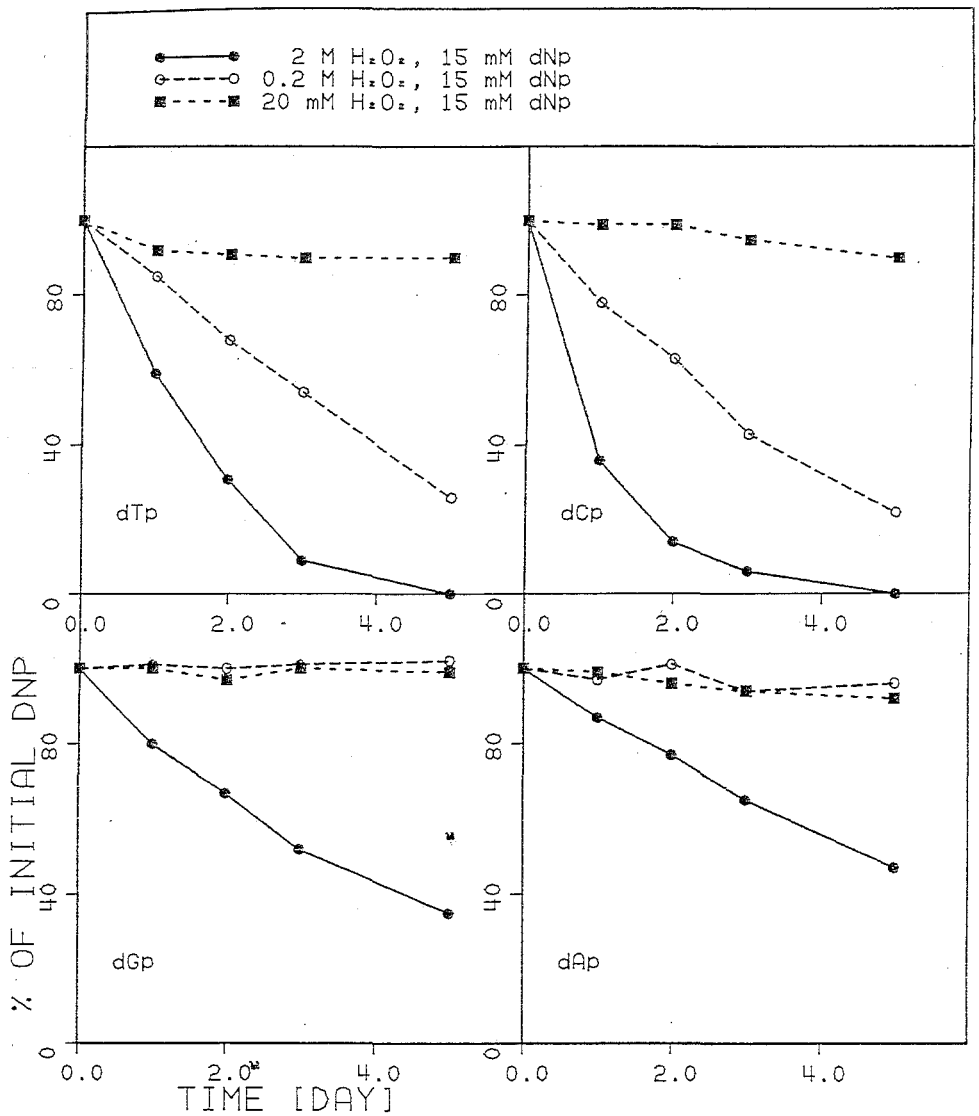

Fig. 2. Sensitivity of deoxyribonucleotides towards oxidation with H_2O_2 plus 25 μM Fe^{3+} at 37 °C for various periods of time. *dTp*, thymidine monophosphate; *dCp*, deoxycytidine monophosphate; *dGp*, deoxyguanosine monophosphate; *dAp*, deoxyadenosine monophosphate

dried in vacuo, and dissolved in 8 mM $CaCl_2$ and 20 mM sodium succinate, pH 6.0. Digestion to nucleotides was performed with 2.5 U micrococcal endonuclease (EC 3.1.31.1, Sigma) and 0.05 U spleen exonuclease (EC 3.1.16.1, Boehringer Mannheim, FRG; Rotkreuz, Switzerland) for 16 h at 37 °C.

Separation of Oxidation Products

The nucleotides were separated by HPLC on a Lichrosorb RP18 (7 µm) column (4.2 mm × 250 mm), eluting at a flow of 1.5 ml/min with 50 mM sodium phosphate buffer, pH 5.8, containing 3% methanol for 5 min, followed by a linear gradient to 20% methanol in 10 min.

Isolation of Thymidine Glycol

The reaction products of the oxidation of [^{14}C]thymidine were separated on a Lichrosorb RP18 (7 μm) column (4.2 mm × 250 mm), eluting at a flow of 0.75 ml/min with 1% aqueous methanol for 15 min, followed by a linear gradient to 100% methanol in 5 min.

GC/MS Analysis

The reaction products were silylated in 0.2 ml pyridine with 0.5 ml *N,O-bis*-(trimethylsilyl)trifluoroacetamide for 30 min at 150 °C. Pyridine was blown off with nitrogen and the residue dissolved in hexane; 1 μl was injected on a capillary column (19 m × 0.32 mm), coated with SE-54 (film thickness, 0.10 μm). Elution was at 60 kPa He, at a temperature of 60 °C for 2 min, followed by a gradient of 10 °C/min.

Results

Sensitivity of Nucleotides

Figs. 1 and 2 show that the pyrimidines (top) were more sensitive than the purines (bottom), both with $KMnO_4$ (Fig. 1) and H_2O_2 (Fig. 2). After 20 min incubation with 20 m*M* $KMnO_4$, a reduction of the optical density to 10% and 80% was observed for thymidine monophosphate and cytidine monophosphate, respectively, whereas no reduction was detectable for the purines. With 20 m*M* H_2O_2, no effect was detectable at all. Only after 2 days of incubation with 0.2 *M* H_2O_2 was a reduction to 70% and 60% observed for thymidine monophosphate and cytidine monophosphate, respectively. No oxidation of the purines was detectable at this H_2O_2 concentration.

Oxidation Products of Thymidine

Oxidation of [^{14}C]thymidine with 20 m*M* $KMnO_4$ yielded only one product, which was identified by mass spectrometry as thymidine glycol. With 2 *M* H_2O_2, at least three different products could be detected. Thymidine-5,6-glycol represented about 30% of the products. Fragmentation by mass spectrometry of another product was compatible with 5-hydroxy-5-methyl-hydantoin deoxyriboside. This compound has been postulated by Teoule and Cadet (1971) as a possible product of thymidine irradiation.

Sensitivity of DNA

Single-stranded (ss) and double-stranded (ds) DNA was oxidized with H_2O_2 and the loss of optical density at 254 nm was taken as a measure of the damage. It was shown that single-stranded DNA was more sensitive to oxidation than double-stranded DNA. Incubation with 2 *M* H_2O_2 resulted in a time-dependent decrease of optical density. After 2 days with 2 *M* H_2O_2, the optical density was

reduced to 30% and 70% for ssDNA and dsDNA, respectively. The sensitivity of the four nucleotides in intact DNA was dCp > dTp > dGp > dAp, i.e., the same ranking as found with isolated nucleotides (Table 1). The yield of high-molecular, oxidized DNA decreased with increasing concentration of H_2O_2. Under the present incubation conditions, only 30% of the DNA could be retained during dialysis. The loss is thought to be due to strand breaks.

Discussion

Thymine was not the most sensitive target base for an oxidation by hydroxyl radical in this in vitro system. A quantification of thymidine glycol must therefore lead to an underestimation of the DNA damage mediated by the hydroxyl radical. H_2O_2 was found to be an astonishingly mild DNA oxidizing agent in vitro and it is questionable whether, in vivo, base hydroxylations occur to a relevant extent. The hypothesis of an oxidative stress as one possible genotoxic process in carcinogenesis must be carefully examined and the relative importance of strand breaks and DNA hydroxylations has to be assessed in vivo.

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