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In vitro Inhibition by *N*-Acetylcysteine of Oxidative DNA Modifications Detected by ^{32}P Postlabeling

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Reactive oxygen species are involved in the pathogenesis of cancer and other chronic degenerative diseases through a variety of mechanisms, including DNA damage. We investigated by ^{32}P and ^{33}P postlabeling analyses the nucleotidic modifications induced *in vitro* by treating calf thymus DNA with H_2O_2 and $CuSO_4$, interacting in a Fenton type reaction. Six different enrichment procedures and three chromatographic systems were comparatively assayed. The chromatographic system using phosphate/urea, which is more suitable for detecting bulky DNA adducts, was rather insensitive. In contrast, the system using acetic acid/ammonium formate revealed high levels of mononucleotidic modifications. In terms of ratio of adduct levels in treated and untreated DNA, the enrichment procedures ranked as follows: nuclease P_1 (19.6), no enrichment (18.3), digestion to trinucleotides (17.6), digestion to monophosphate mononucleotides (8.4), digestion to dinucleotides (3.4), and extraction with butanol (<1.0). The system using formic acid/ammonium formate was quite efficient in detecting 8-hydroxy-2'-deoxyguanosine. Labeling with ^{33}P further enhanced the sensitivity of the method. The oxidative damage was so intense to produce a strong DNA fragmentation detectable by agarose gel electrophoresis, and nucleotidic modifications were more intense when DNA fragmentation was greater. The DNA alterations produced by H_2O_2 alone were significantly lower than those produced following reaction of H_2O_2 with $CuSO_4$. The thiol *N*-acetylcysteine (NAC) was quite efficient in inhibiting both nucleotidic modifications and DNA fragmentation produced *in vitro* by either H_2O_2 or the ·OH generating system. These results support at a molecular level the findings of previous studies showing the ability of NAC to inhibit the genotoxicity of peroxides and of reactive oxygen species generated by electron transfer reactions.

Keywords: Reactive oxygen species, hydrogen peroxide, hydroxyl radical, *N*-acetylcysteine, DNA damage, ^{32}P post-labeling

INTRODUCTION

Reactive oxygen species (ROS) play a primary role in the pathogenesis of cancer and other chronic degenerative diseases.[1–3] *In vitro* studies using ROS-generating systems led to a better understanding of the mechanisms underlying the genotoxic activity of these molecules. The investigated endpoints covered genotoxic damage in

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mammalian cells[4] and bacteria,[5,6] and molecular alterations including DNA fragmentation,[7] single base modifications such as formation of 8-hydroxy-2′-deoxyguanosine (8-OH-dG),[8–11] and generation of mutagenic DNA derivatives such as glyoxal.[12] Other ROS-induced modifications consist in the formation of nucleotide dimers,[8,9] adenine *N*-1-oxide,[13] ribonucleotides,[14] and bulky DNA adducts.[10]

^{32}P postlabeling has a very high sensitivity and can reveal at one time a wide range of the aforementioned oxidative alterations by testing low amounts of biological material. A first objective of the present study was to further validate this technique in the evaluation of ROS-induced nucleotidic modifications. With this aim, a variety of procedures were compared for the enrichment and labeling of nucleotidic modifications as well as for their chromatographic mobility. Generation of highly reactive hydroxyl radical (·OH) was obtained by a Fenton type reaction[15] involving the interaction between copper sulfate ($CuSO_4$) and hydrogen peroxide (H_2O_2).[9,16] Since the molecular damage induced by ·OH is generally accompanied by DNA fragmentation,[7,9] this parameter was also assessed. A further objective was to evaluate the ability of the thiol *N*-acetylcysteine (NAC), which had previously been shown to inhibit the genotoxic damage produced by ROS in bacterial test systems,[5,6,17] to affect the formation of ^{32}P postlabeled DNA modifications produced by H_2O_2 and ·OH.

MATERIALS AND METHODS

Reagents

Calf thymus DNA, micrococcal nuclease, nuclease P_1, spleen phosphodiesterase, *Crotalus durissimus terrificus* snake venom phosphodiesterase, DNAse I from bovine pancreas, human prostatic acid phosphatase, ethidium bromide and DNA molecular weight marker (16-2 kb ladder) were purchased from Sigma Chemical Co. (St. Louis, MO), T4 polynucleotide kinase from Boehringer Mannheim GmbH (Germany), H_2O_2 from Carlo Erba OTC (Mozzate, Como, Italy) and BDH Italia (Milan, Italy), $CuSO_4$ and agarose from BDH Italia, TLC polyethylenimine/cellulose sheets from Macherey-Nagel (Düren, Germany), AT-$\gamma^{32}P$ ($\geq$3,000 Ci/mmol) and AT-$\gamma^{33}P$ ($\geq$1,000 Ci/mmol) from ICN Biomedicals (Irvine, CA). NAC was a gift from Zambon Group (Vicenza, Italy).

Treatment of DNA

Calf thymus DNA was dissolved in deionized water at a concentration of 1 mg/ml. Based on the indication of preliminary experiments, $CuSO_4$ was added at a final concentration of 1 mM, and NAC at 25, 50, 100 or 200 mM. The pH of each reaction mixture was adjusted to 8.0 by adding 0.4 N NaOH. Unless otherwise specified, H_2O_2 was added at a final concentration of 50 mM. The samples were then incubated at 37°C for 5 min, frozen at −80°C for 10 min, and lyophilized. Suitable controls were tested by omitting either hydrogen peroxide, copper sulfate and/or NAC.

Agarose Gel Electrophoresis

Twenty μg DNA were cast using 0.8% (w/v) agarose dissolved in 40 mM Tris, 20 mM acetic acid, 1 mM EDTA·Na_2, pH 8.0.[18] Gels were run for 15 h at 2 V/cm and 52 mA. Following staining with ethidium bromide, DNA was then viewed under UV light (365 nm) and photographed on Polaroid 667 film (Polaroid Ltd, St. Albas, Hertfordshire, UK). A densitometric analysis was obtained by image transfer on PC with a CLC-10 Canon scanner (Canon, Amstelveen, The Netherlands) and data process with the program NIH Image 1.57 running on a Macintosh Quadra 700. A molecular weight marker with ladder fragments ranging between 2 and 16 kb was used as a reference standard. Each experiment was repeated 5 times. The mean proportion of DNA detected in the lowest portion of gel containing DNA fragments shorter

than 6 kb was evaluated as related to treatment of calf thymus DNA.

In electroelution experiments DNA was treated as indicated in the previous section but H_2O_2 was used at a final concentration of 3 mM rather than 50 mM in order to avoid an excessive DNA fragmentation. At the end of the electrophoretic run, each lane was divided into 3 parts containing DNA affected by an increasing rate of fragmentation (>14, 10–14, and <10 kb). Each area was cut under UV light (365 nm), and each DNA containing agarose fragment was then trapped in a dyalisis tube, and immersed for 3 h in the same buffer used for dissolving agarose under an 8 V/cm electric field.[18] Ethidium bromide was removed from agarose-electroeluted DNA by repeated washing in butanol, and DNA was precipitated with absolute ethanol. The quantitative and qualitative analyses of DNA used in each experiment were performed by using a Hitachi U3200 spectrophotometer (Hitachi, Tokyo, Japan). The 230/260 and 260/280 absorbance ratios were consistently lower than 0.5 and higher than 1.75, respectively.

Postlabeling of Modified Nucleotides

Untreated DNA and DNA treated with H_2O_2, $CuSO_4$ and/or NAC were subjected to different procedures, *i.e.*, without enrichment, nuclease P_1 digestion, butanol extraction, or depolymerization to either monophosphate mononucleotide, dinucleotide or trinucleotide adducts. In particular, for the first 3 procedures 6 μg DNA were digested by incubation with 0.5 U micrococcal nuclease and 6.8 μg spleen phosphodiesterase for 3.5 h at 37°C. Enrichment with nuclease P_1 was then achieved by adding 2.37 U enzyme. After incubation for 40 min at 37°C the reaction was stopped by adding 0.5 M Tris.[19] Enrichment with butanol was obtained as described by Gupta.[20] Omission of enrichment, nuclease P_1 digestion and butanol extraction were designed to produce mononucleotide biphosphate adducts (*pXp). In some experiments monophosphate mononucleotide (*pX) adducts, dinucleotide adducts (*pXpN), and trinucleotide adducts (*pXpNpN) were evaluated. The production of *pX and *pXpN was obtained as described by Randerath *et al.*[21] The production of *pXpNpN was obtained according to the DNA digestion protocol reported by Bykov *et al.*,[22] with minor modifications. Briefly, 10 μg DNA were digested at 37°C for 4 h with DNase I (645 mU/nmol nucleotide), and overnight with snake venom phosphodiesterase (1 mU/nmol nucleotide). The pH was adjusted to 5.0 by adding 0.1 N HCl, and the samples were incubated with prostatic acid phosphatase (5 mU/nmol nucleotide) for 4 h at 37°C. The reaction was stopped by heating the mixtures at 90°C for 15 min, and the pH was adjusted by adding 0.4 N NaOH. The samples were lyophilized and labeled by adding 8 U of polynucleotide kinase and 80 μCi AT-γ^{32}P, and incubated at room temperature (≥22°C) for 40 min. In addition, in some experiments nuclease P_1-enriched DNA samples were labeled as described above but using AT-γ^{33}P rather than AT-γ^{32}P.

Chromatographic Mobility of Radiolabeled Adducts

The samples subjected to various enrichment and labeling procedures were developed on polyethylenimine sheets on cellulose support according to three multidirectional thin layer chromatographic systems. The first system (A) used the following solvents: D1 and D4, 0.5 M ammonium formate, 0.5% acetic acid, pH 5.4; D2, 1.75 M ammonium formate, 0.5% acetic acid, pH 5.4; D3, 1.0 M ammonium formate, 1.0% acetic acid, pH 5.4. D1, D3 and D4 were developed in the same direction (from bottom to top in Fig. 2), whereas D2 was developed at 90° clockwise compared to the other directions. D1 and D4 were developed overnight by stapling blotting paper to the bottom of the sheet. The origin, placed 1.5 cm below the lower edge of the areas reported in Figures 2 and 3, was cut at the end of D2. The autoradiographs were exposed at room temperature for time periods ranging between 5 min

and 3 h. The second system (B) used the following solvents: D1, 1 M sodium phosphate, pH 6.0; D2, 3 M lithium formate, 7 M urea, pH 3.8; D3, 0.6 M lithium chloride, 7 M urea, 0.5 M Tris, pH 8.0; D4, 1.7 M sodium phosphate, pH 6.0. Direction of chromatographic development and stapling areas were as previously described.[19] The autoradiographs were then exposed for 24–72 h at −80°C. The third system (C), designed in order to detect 8-OH-dG in nuclease P_1 digested samples,[23] used 1.5 M formic acid, pH 1.5, and 0.6 M ammonium formate, pH 6.0, in D1 and D2, respectively.

Quantification of DNA Adducts

For each labeled sample an aliquot of normal nucleotides corresponding to a 1/140,000 fraction of normal nucleotides available in the sample were labeled with 1.87 U polynucleotide kinase and 16 μCi AT-γ^{32}P or AT-γ^{33}P. The labeled normal nucleotides were then separated by unidimensional TLC on polythylenimine/cellulose sheets, using 170 mM sodium phosphate, pH 6.8, as a solvent. The labeled nucleotides were identified by autoradiography, the corresponding areas were cut and read in liquid scintillation using a Tri-Carb 1600 TR counter (Packard, Meriden, CT). The same procedure was followed for quantifying DNA adducts. The number of modified nucleotides in each sample was expressed as the ratio of beta activity in modified nucleotides to that of normal nucleotides multiplied by the dilution factor. This parameter, referred to as RAL or Relative Adduct Labeling[19] was then transformed into the number of modified nucleotides for 10^8 normal nucleotides by taking into account that 1 μg DNA contains 1.95×10^{15} normal nucleotides. The quantitative data reported under "Results" for each experimental condition are the means of at least 3 independent experiments.

Statistical Analyses

Comparisons between individual treatments for all investigated parameters were made by Student's *t* test.

RESULTS

Effect of NAC on Oxidative DNA Fragmentation

In the absence of NAC, H_2O_2 alone produced a significant DNA fragmentation, which was further enhanced in the presence of $CuSO_4$ (Table I).

TABLE I Effect of NAC on DNA fragmentation produced by H_2O_2 or H_2O_2 plus $CuSO_4$ in calf thymus DNA, as assessed by agarose gel electrophoresis

NAC (mM)	Percent of DNA fragments lower than 6 kb (mean ± SD)		
	Calf thymus DNA	Calf thymus DNA + H_2O_2 (50 mM)	Calf thymus DNA + H_2O_2 (50 mM) + $CuSO_4$ (1 mM)
0	2.7 ± 0.9	20.1 ± 5.4[†]	68.6 ± 8.2[‡,¶]
25	2.4 ± 1.1	19.0 ± 4.4[†]	26.0 ± 4.7[†,#]
50	3.1 ± 0.8	16.2 ± 3.1[†]	17.2 ± 2.6[‡,#]
100	2.3 ± 0.8	11.1 ± 2.3[*,§]	7.1 ± 2.6[*,#]
200	2.1 ± 1.2	3.5 ± 1.0[‖]	4.2 ± 0.5[#]

* Significantly higher as compared to controls (untreated calf thymus) at $P \leq 0.05$
† Significantly higher as compared to controls (untreated calf thymus) at $P \leq 0.01$
‡ Significantly higher as compared to controls (untreated calf thymus) at $P \leq 0.001$
¶ Significantly higher than the corresponding value obtained with H_2O_2 alone, at $P \leq 0.001$
§ Significantly decreased as compared to the corresponding control (without NAC) at $P \leq 005$
‖ Significantly decreased as compared to the corresponding control (without NAC) at ≤0.01
Significantly decreased as compared to the corresponding control (without NAC) at ≤0.001

The copper salt alone did not produce any significant change (data not shown). Addition of NAC did not affect the background levels of DNA fragmentation in untreated DNA, whereas it resulted in a concentration-dependent decrease of damage in calf thymus DNA treated with either H_2O_2 or $H_2O_2/CuSO_4$ (Figure 1A). Inhibition of H_2O_2-induced DNA damage was statistically significant at 100 and 200 mM NAC, i.e., in a slight molar excess compared to H_2O_2 (50 mM). Conversely, inhibition of $H_2O_2/CuSO_4$-induced DNA damage was highly significant even at the lowest tested NAC concentration, *i.e.*, 25 mM (Table I). In the presence of any NAC concentration, the damage produced by $H_2O_2/CuSO_4$ was not significantly different compared to the damage produced by H_2O_2 alone. At a 200 mM concentration of NAC, DNA fragmentation produced by either H_2O_2 or $H_2O_2/CuSO_4$ was no longer significantly different from control values (Table I).

^{32}P Postlabeling of Modified Nucleotides by Using Different Enrichment Procedures and Cromatographic Conditions

Figure 2 shows, as an example of a single experiment, DNA adduct patterns in untreated calf thymus DNA (upper row) or in DNA treated with 50 mM hydrogen peroxide plus 1 mM copper sulfate (lower row), by using in all cases except for 8-OH-dG detection the chromatographic system with acetic acid/ammonium formate (referred to as A in Materials and Methods). The best resolution of radioactive spots was obtained by either omitting enrichment or following digestion with nuclease P_1 or digestion to monophosphate mononucleotides, whereas the digestion to dinucleotides or trinucleotides yielded poorly resolved, diffuse zones. The formic acid/ammonium formate chromatographic system (C) resulted in the identification of a single radioactive spot (Figure 2) corresponding to the one identified by Devanoboyna and Gupta[23] as 8-OH-dG.

Irrespective of the enrichment procedure, ^{32}P postlabeled spots were consistently detected when testing untreated DNA. The total amounts of adducts/10^8 nucleotides (means $\pm$ SD of 3 experiments) in untreated DNA ranked as follows: digestion to dinucleotides (5,757 $\pm$ 1,143), digestion to trinucleotides (2,751 $\pm$ 849), no enrichment (2,509 $\pm$ 432), enrichment with butanol (2,182 $\pm$ 322), digestion to monophosphate mononucleotides (1,833 $\pm$ 411), enrichment with nuclease P_1 (1,368 $\pm$ 463), and nuclease P_1 digestion for 8-OH-dG detection (1,009 $\pm$ 198). When DNA was subjected to oxidative damage with $H_2O_2/CuSO_4$, the number and amounts of spots and radioactive areas were significantly higher as compared to untreated DNA by using all enrichment procedures with the exception of butanol extraction. In particular, the total amounts of adducts/10^8 nucleotides in treated DNA ranked as follows: digestion to trinucleotides (48,420 $\pm$ 7,333; $P < 0.001$), no enrichment (45,900 $\pm$ 9,984; $P < 0.01$), nuclease P_1 digestion (26,855 $\pm$ 3,753; $P < 0.001$), digestion to dinucleotides (19,797 $\pm$ 1,851; $P < 0.001$), digestion to monophosphate mononucleotides (15,827 $\pm$ 1,322; $P < 0.001$), nuclease P_1 digestion for 8-OH-dG detection (7,133 $\pm$ 1,103; $P < 0.001$), and enrichment with butanol (1,913 $\pm$ 238; not significant). Accordingly, the ratio of radiolabeled nucleotides in $H_2O_2/CuSO_4$-treated DNA to those in untreated DNA was <1 (butanol), 3.4 (digestion to dinucleotides), 7.1 (nuclease P_1 digestion for 8-OH-dG detection), 8.4 (digestion to monophosphate mononucleotides), 17.6 (digestion to trinucleotides), 18.3 (no enrichment), and 19.6 (nuclease P_1).

Copper alone (1 mM) was devoided of detectable effects on calf thymus DNA (data not shown). Further assays were carried out in order to evaluate whether nucleotidic modifications may arise, under our experimental conditions, from treatment of calf thymus DNA with H_2O_2 alone (50 mM). As shown in Table II, H_2O_2 produced the same 4 spots detected after treatment with $H_2O_2/CuSO_4$ (see Figure 3 for their identification). In the

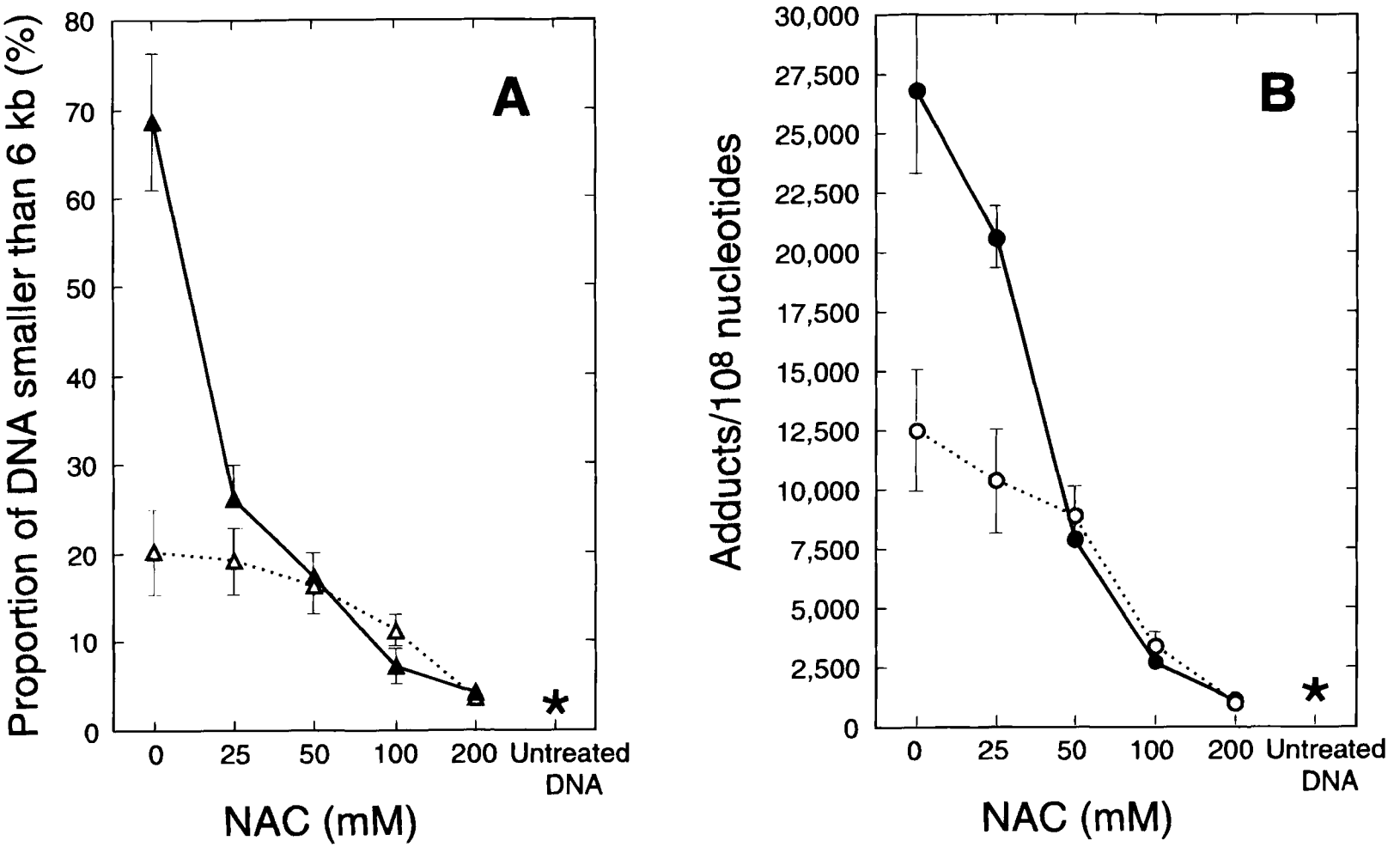

FIGURE 1 Inhibition by NAC of oxidative DNA fragmentation and nucleotidic modifications. The two panels show the dose-dependent inhibition by NAC of DNA fragmentation (A, mean ± SD of 5 experiments) and of total DNA adducts detected by ^{32}P postlabeling (B, mean ± SD of 3 experiments) following treatment of calf thymus DNA with either 50 mM H_2O_2 (open triangles and dotted lines) or 50 mM H_2O_2 plus 1 mM $CuSO_4$ (full triangles and continuous lines). The background levels of DNA adducts and DNA fragmentation in untreated DNA are also shown. DNA adducts were enriched by nuclease P_1 digestion and resolved by chromatography in acetic acid/ammonium formate.

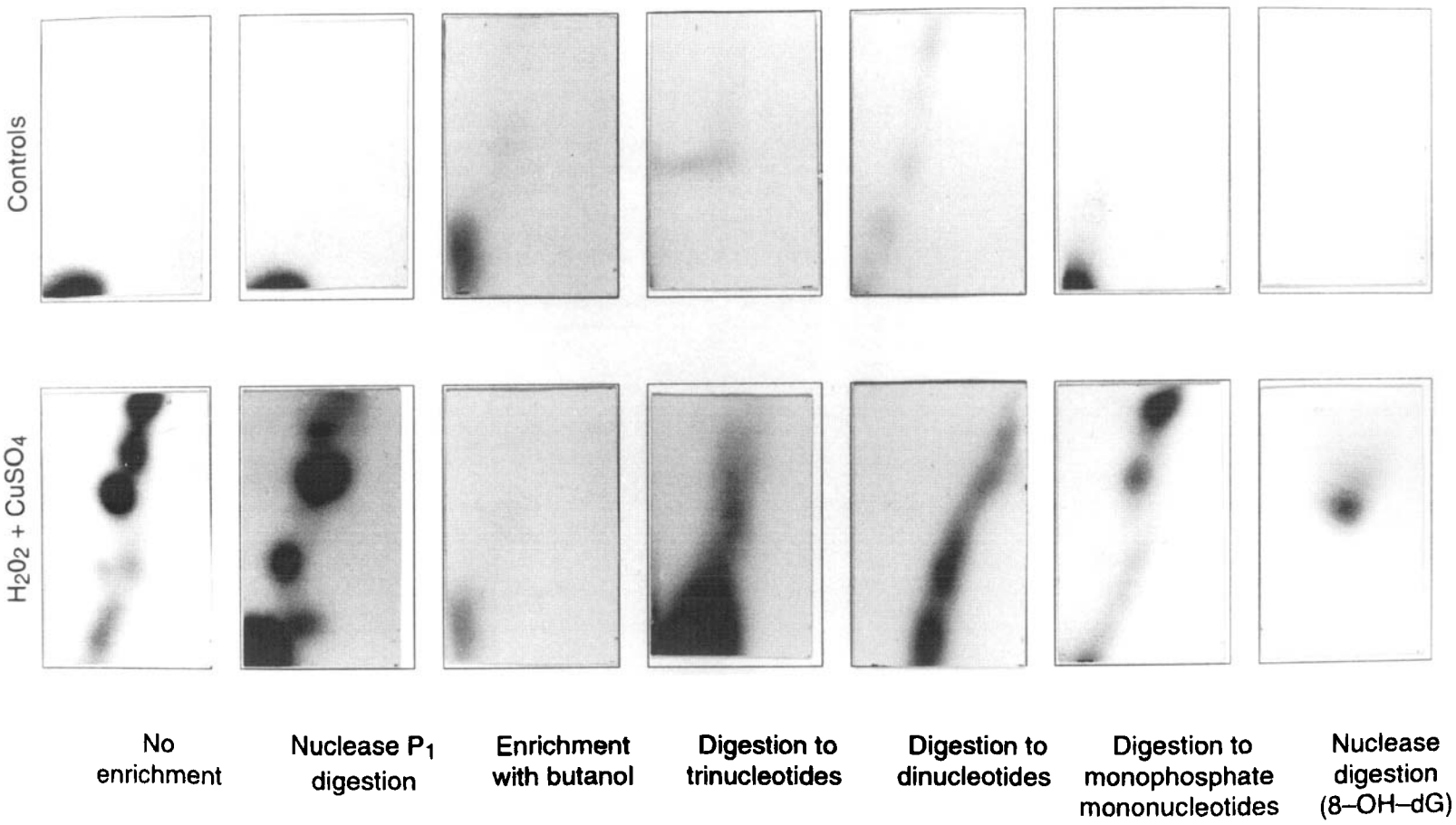

FIGURE 2 Comparison of different enrichment procedures for the detection of oxidative DNA damage by ^{32}P postlabeling.
Autoradiographs show the patterns of nucleotidic modifications, detected by ^{32}P postlabeling followed by chromatography in acetic acid/ammonium formate, in calf thymus DNA, either untreated (upper row) or treated with 50 mM H_2O_2 plus 1 mM $CuSO_4$ (lower row). The different procedures used for the enrichment of adducts (see "Materials and Methods") are indicated below each pair of autoradiographs.

TABLE II Effect of NAC on ^{32}P postlabeled spots formed after oxidative DNA damage

Oxidant	NAC (mM)	Autoradiographic spot (adducts/10^8 nucleotides)				
		1	2	3	4	Total
H_2O_2 (50mM)	0	915±244	3,650±1,631	5,625±879	2,284±1,154	12,475±2,776
	25	613±177	3,035±856	5,010±1,122	1,669±792	10,327±2,484
	50	516±229	2,960±1,063	3,633±1,063¶	1,745±989	8,845±1,492
	100	398±139¶	977±267¶	1,134±362‖	829±134	3,338±628‖
	200	147±54§	300±103¶	313±70‖	238±104¶	998±221‖
H_2O_2 (50mM) + $CuSO_4$ (1mM)	0	4,104±693†	3,010±465	13,554±1,836†	6,194±1,226*	26,855±3,753†
	25	3,685±359‡	2,621±365	8,232±815*,¶	6,099±1,095†	20,637±1,548†
	50	874±380*,§	1,092±692¶	4,110±353§	1,769±397§	7,878±600§
	100	321±81‖	540±198§	672±172‖	1,135±282§	2,666±241‖
	200	95±15‖	146±82‖	170±113‖	731±245§	1,142±313‖

Modified nucleotides were enriched by nuclease P_1 digestion and separed by using the chromatographic system with acetic acid/ammonium formate. The results are means±SD of 3 experiments

* Significantly higher as compared to the corresponding spot induced by H_2O_2 alone, at $P \leq 0.05$

† Significantly higher as compared to the corresponding spot induced by H_2O_2 alone, at $P \leq 0.01$

‡ Significantly higher as compared to the corresponding spot induced by H_2O_2 alone, at $P \leq 0.001$

¶ Significantly decreased as compared to the corresponding control (without NAC) at $P \leq 0.05$

§ Significantly decreased as compared to the corresponding control (without NAC) at $P \leq 0.01$

‖ Significantly decreased as compared to the corresponding control (without NAC) at $P \leq 0.001$

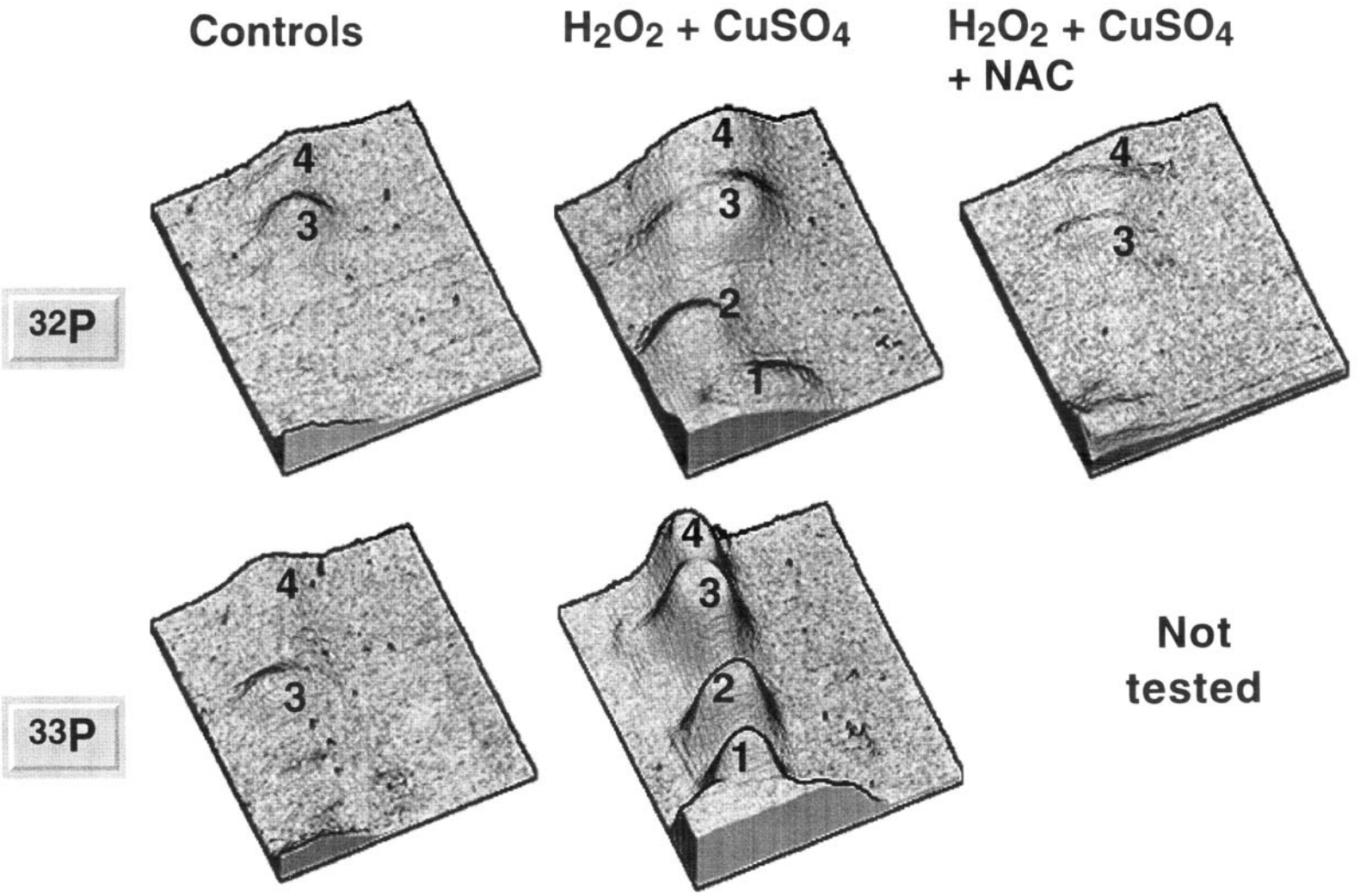

FIGURE 3 ^{32}P and ^{33}P postlabeling of DNA exposed to oxidants, and protective effect of NAC.
Nucleotidic modifications in calf thymus DNA, either untreated or treated with 50 mM H_2O_2 plus 1 mM $CuSO_4$, were evaluated by postlabeling with either ^{32}P or ^{33}P. The effect of 200 mM NAC on ^{32}P postlabeling was also assessed. DNA adducts were enriched by nuclease P_1 digestion and resolved by chromatography in acetic acid/ammonium formate. Tridimensional image analyses of autoradiographs were obtained by using the program NIH Image 1.57.

absence of NAC (0 mM in Table II), total spot levels and 3 individual spots (Nos. 1, 3 and 4) were significantly higher following reaction with $CuSO_4$. Conversely, the levels of spot No. 2 were slightly and nonsignificantly lower following reaction with $CuSO_4$.

The chromatographic system B, using urea in D2 and D3 (see "Materials and Methods"), failed to detect the presence of labeled spots in untreated DNA, irrespective of the enrichment procedure used (data not shown). Nuclease P_1 was the only enrichment procedure revealing with system B the presence of autoradiographic images, with 4 well resolved spots, accounting for a total level of 31.8 adducts/10^8 nucleotides. In the absence of enrichment procedures, a weak spot was observed in the upper part of the autoradiograph, corresponding to 1.2 adducts/10^8 nucleotides.

Postlabeling either of a standard control composed of a mixture of ATP, GTP, CTP and TTP, or of a DNA-free sample did not reveal any radioactive spot with any of the above reported enrichment and chromatographic procedures (data not shown).

Formation of Modified Nucleotides as Related to the Severity Oxidative DNA Fragmentation

The gel used for the electrophoresis of DNA treated with 3 mM H_2O_2 plus 1 mM $CuSO_4$ was divided into three areas, containing variously fragmented DNA. The DNA contained in each area was eluted as described in "Materials and Methods", and subjected to ^{32}P postlabeling analysis following nuclease P_1 enrichment and system A chromatography. The patterns of radioactive zones and the numbers of spots were similar when analyzing the DNA fragments of different size, and comparable to those obtained by testing 50 mM H_2O_2 plus 1 mM $CuSO_4$ (see Figures 2 and 3). However, there were quantitative differences in total adduct levels, which were $1,157 \pm 318$ adducts/10^8 nucleotides in DNA fragments greater than 14 kb, $2,759 \pm 444$ adducts/10^8 nucleotides in DNA fragments of 10–14 kb, and $3,939 \pm 617$ adducts/10^8 nucleotides in DNA fragments smaller than 10 kb (means $\pm$ SD of 3 experiments). Thus, the adduct levels were related to the extent of DNA fragmentation. When testing DNA fragments smaller than 10 kb, the adduct levels were significantly higher than those detected by testing either >14 kb fragments ($P = 0.001$) or 10–14 kb fragments ($P < 0.05$). In turn, adducts levels were significantly higher ($P = 0.05$) in 10–14 kb fragments as compared to >14 kb fragments.

Comparison of Postlabeling with ^{32}P and ^{33}P for Detecting Oxidative DNA Modifications

Untreated DNA and DNA treated with 50 mM H_2O_2 plus 1 mM $CuSO_4$ were analyzed by postlabeling following enrichment with nuclease P_1 and chromatography using ammonium formate (system A). The analysis of untreated DNA yielded $1,368 \pm 464$ adducts/10^8 nucleotides by ^{32}P postlabeling and 412 ± 85 adducts/10^8 nucleotides by ^{33}P postlabeling. The analysis of DNA exposed to the ·OH generating system yielded $26,855 \pm 3,753$ and $23,558 \pm 3,450$ adducts/10^8 nucleotides with ^{32}P and ^{33}P, respectively. Accordingly, the treated/untreated DNA ratio was 19.6 and 57.2, respectively. From a qualitative point of view the autoradiographic patterns observed with ^{32}P and ^{33}P postlabeling analysis were similar, ^{33}P yielding an even better resolution of spots as compared to ^{32}P (Figure 3).

Effect of NAC on Oxidative DNA Modifications Detected by ^{32}P Postlabeling

Addition of NAC consistently resulted in a dose-dependent inhibition of the formation of oxidative DNA modifications produced by 50 mM H_2O_2. Even more pronounced was the decrease of DNA modifications produced by 50 mM H_2O_2 plus 1 mM $CuSO_4$, the two curves being virtually overlapping at NAC concentrations equal or higher than 50 mM (Figure 1B). Irrespective of

H_2O_2 or $H_2O_2/CuSO_4$ treatment, in the presence of 200 mM NAC the levels of nucleotidic modifications were no longer significantly different from controls (untreated DNA). Table II shows that addition of NAC inhibited in a dose-related manner not only total DNA modifications but also the 4 individual spots produced by either H_2O_2 or $H_2O_2/CuSO_4$. In particular, inhibition by NAC of spot No. 1 was statistically significant at ≥ 100 mM (H_2O_2) or ≥ 50 mM ($H_2O_2/CuSO_4$), inhibition of spot No. 2 at ≥ 100 mM (H_2O_2) or ≥ 50 mM ($H_2O_2/CuSO_4$), inhibition of spot No. 3 at ≥ 50 mM (H_2O_2) or ≥ 25 mM ($H_2O_2/CuSO_4$), and inhibition of spot No. 4 at 200 mM (H_2O_2) or ≥ 50 mM ($H_2O_2/CuSO_4$). Therefore, also from these data it appears that NAC was even more effective in decreasing the nucleotidic modifications induced by $H_2O_2/CuSO_4$ than those induced by H_2O_2 alone. As to spot No. 2, it is noteworthy that, at all NAC concentrations, the levels of nucleotidic modifications were consistently higher in DNA treated with H_2O_2 alone than in DNA treated with $H_2O_2/CuSO_4$, although in no case the individual differences were statistically significant.

DISCUSSION

The present study investigated ROS-induced DNA modifications by evaluating DNA fragmentation and by performing postlabeling analyses with radioactive P under a variety of experimental conditions, including 6 different enrichment procedures, 2 radioactive markers of ATP (^{32}P and ^{33}P), and 3 chromatographic systems.

The results obtained showed the ability of ^{32}P postlabeling to detect nucleotidic modifications induced by ROS, thus confirming the findings of previous studies using different experimental conditions and/or detection procedures. The $H_2O_2/CuSO_4$ mixture was potently reactive by ^{32}P postlabeling and caused an intense DNA fragmentation, which could be even detected by agarose gel electrophoresis, which is not a highly sensitive method. The relationships between the two monitored endpoints was further supported by experiments analyzing by ^{32}P postlabeling variously fragmented DNA, electroeluted from agarose gel after treatment with the ·OH generating system. In fact, the levels of DNA adducts were higher where breaks were in larger amounts, as a demonstration of the parallelism and possible interconnection between nucleotidic modifications and DNA fragmentation.

The results of our study also confirmed the heterogeneous nature of nucleotidic modifications resulting from oxidative damage. In fact, ^{32}P postlabeling analyses showed the presence of multiple spots, whose number and resolution depended on the enrichment procedure used. Only a part of the modifications observed in DNA treated *in vitro* under Fenton reaction conditions has been tentatively characterized so far. Three spots were shown to represent intrastrand crosslink being derived from G-N and A-A sequences,[9,25] and were identical to modifications induced in kidney DNA after treatment of rats with the pro-oxidant carcinogen ferric nitrilotriacetate.[26] Other spots appear to be bulky adducts rather than small polar, hydroxylated or ring-opened nucleotide derivatives.[10] The bulky DNA lesions observed *in vivo* as the result of oxidative stress have been termed type II I-compounds.[3,26]

The chromatographic system using acetic acid/ammonium formate was by far more efficient and reliable in detecting ·OH-induced modifications than the system using phosphate/urea. There was an almost 1,000-fold difference in RAL following nuclease P_1 digestion, which was the only enrichment procedure showing a significant formation of radioactive spots by using the latter chromatographic system. Buffers containing ammonium formate are typically used for separating mononucleotides[20] and for detecting dinucleotide alterations induced, e.g., by UV light.[22] Randerath *et al.*[14] reported results quantitatively comparable to those obtained in the present study when using the chromatographic

system with ammonium formate for detecting H_2O_2/Fe^{2+} oxidative damage by ^{32}P postlabeling. Presumably, single nucleotides or dinucleotides tend to migrate too rapidly in phosphate/urea buffer, whereas bulky adducts have lower migration coefficients thereby remaining in the detection area of autoradiographs. The sharp differences between the two chromatographic systems suggest that, under our experimental conditions, mononucleotide and dinucleotide modifications were quantitatively prevailing over bulky adducts. This conclusion is also supported by the finding that, in agreement with Carmichael *et al.*,[9] extraction with butanol, which typically selects more hydrophobic bulky adducts, was the only enrichment procedure out of those tested which failed to detect the formation of oxidative DNA alterations even following chromatography in acetic acid/ammonium formate. Nucleotide modifications induced by ·OH were well detected even in the absence of any enrichment procedure, with a RAL ratio of treated to untreated DNA which was of the same order of magnitude as following enrichment with nuclease P_1 or digestion to trinucleotides.

A well defined autoradiographic spot was also detected by using the formic acid/ammonium formate chromatographic system, in which the acidic environment slows down 8-OH-dG migration, whereas normal nucleotides migrate outside the detection area.[23]

Postlabeling of DNA exposed to $H_2O_2/CuSO_4$ by using ^{33}P was even more sensitive than ^{32}P postlabeling, especially due to the lower levels of modified nucleotides detected in untreated DNA. Compared to ^{32}P, ^{33}P has a lower specific activity and ionizing energy (0.25 *versus* 1.71 MeV), and therefore poses less stringent radioprotection measures. However, use of ^{33}P is limited by its cost, which is 15 times higher than cost of ^{32}P. On the whole, also taking into account the remarkable intensity of the molecular alterations produced *in vitro* by $H_2O_2/CuSO_4$, use of ^{33}P does not appear to be convenient for detecting this kind of DNA damage.

In agreement with a previous report,[24] a basal oxidative damage was present in commercially available calf thymus DNA. Of the two reactants, $CuSO_4$ was devoid of any detectable activity, whereas H_2O_2 produced a significant DNA fragmentation as well as nucleotidic modifications revealed by ^{32}P postlabeling following chromatography in acetic acid/ammonium formate. These modifications were qualitatively similar to those produced by the $H_2O_2/CuSO_4$ mixture, but their total levels were significantly and considerably lower in the absence of $CuSO_4$. H_2O_2 has been reported to be negative at ^{32}P postlabeling following chromatography in phosphate/urea.[9] Positivity of H_2O_2 following chromatography in phosphate/urea and contact transfer chromatography was ascribed to a possible contamination of the reagent used with trace metals.[10] The H_2O_2 products used in our study, which were from two commercial sources, yielded similar results. They had a declared contamination accounting for a maximum of 1 ppm iron, which means that 50 mM H_2O_2 contained at most 30 nM iron. We do not know whether this extremely low concentration of iron is sufficient to trigger a reaction with H_2O_2 detectable at ^{32}P postlabeling. It is noteworthy, however, that one of the 4 detected spots was likely to be specifically induced by H_2O_2, since it was not enhanced but rather tended to be decreased following reaction with $CuSO_4$. Such a trend was not statistically significant but was consistent both in the absence and in the presence of varying concentrations of NAC, which suggests that H_2O_2 was partially consumed in the reaction with $CuSO_4$. It is known that H_2O_2 is mutagenic in *Salmonella typhimurium* strains sensitive to ROS,[27,28] and is genotoxic in *Escherichia coli* strains lacking DNA repair mechanics.[29]

The thiol NAC is an analog and precursor of cysteine, which is the rate limiting amino acid in the intracellular synthesis of reduced glutathione (GSH).[30] NAC has been shown to possess multiple protective mechanisms, and to inhibit the chemical induction of mutations and cancers in a variety of experimental test systems, both *in vitro*

and *in vivo*.[17,31] The results of the present study showed that NAC was quite efficient in inhibiting both ROS-induced DNA fragmentation and nucleotidic modifications. Although our *in vitro* data cannot be extrapolated to the *in vivo* situation, this effect was quite remarkable, keeping in mind the almost complete destruction of DNA and the high levels of ^{32}P postlabeled spots occurring under our experimental conditions. Protection by NAC was statistically significant at approximately equimolar concentrations of H_2O_2 and NAC in the reaction mixture, and both DNA fragmentation and nucleotidic modifications were not significantly different from controls (untreated DNA) in a 4 molar excess of NAC. Inhibition of oxidative DNA damage by NAC was even more pronounced when calf thymus DNA was treated with the $H_2O_2/CuSO_4$ mixture than after treatment with H_2O_2 alone, which may be tentatively ascribed either to chelation of copper by NAC and/or to higher reactivity of NAC towards ·OH than towards H_2O_2. This conclusion is consistent with the finding that NAC reacts rapidly with ·OH and hypochlorous ion (−OCl), and more slowly with superoxide anion radical (O_2^-) and H_2O_2.[32,33] Since we worked in an acellular system, it is evident that NAC was effective *per se* and not as a precursor of cysteine and GSH. It is known that the interaction between NAC and H_2O_2 involves a nonenzymatic reduction of the peroxide with the concomitant formation of NAC disulfide.[34]

The results obtained *in vitro* by using molecular dosimetry end-points are in agreement with the findings of biological assays. In fact, NAC attenuated ROS genotoxicity in bacterial test systems by inhibiting the differential lethality of H_2O_2 in strains WP2 (wildtype) and CM871 (*uvrA*$^-$ *recA*$^-$ *lexA*$^-$) of *E coli*[29] as well as the mutagenicities of cumene hydroperoxide in *S. typhimurium* TA104 and of H_2O_2 in *S. typhimurium* TA102,[34] at about equimolar concentrations. TA102 and TA104 have been constructed to be reverted by oxidative mutagens.[27] Interestingly, NAC and other thiols decreased the "spontaneous" mutagenicity in TA104 (*uvrB*$^-$) but not in TA102 (*uvrB*$^+$), which suggests a prevailing role for DNA repair mechanisms rather than for oxidative mechanism in the "spontaneous" mutagenicity of these bacteria.[35] Additionally, NAC showed inhibitory effects in systems generating ROS by electron transfer reactions. In particular, NAC and other thiols completely inhibited, with a dose-related effect, the mutagenicity induced in *S. typhimurium* TA104 by the hypoxanthine/xanthine oxidase system (generating O_2^-) and of the same reactants plus superoxide dismutase (generating H_2O_2).[5] Moreover, NAC completely inhibited, again with a dose-related effect, the selective killing of *E. coli* WP2 and WP100 (*uvrA*$^-$ *recA*$^-$) due to ROS generation by electron transfer reaction following illumination of the chromophore rose bengal.[6]

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