

Induction of 1,*N*²-etheno-2'-deoxyguanosine in DNA exposed to β -carotene oxidation products

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Received 13 January 2004; accepted 14 January 2004

First published online 3 February 2004

Edited by Barry Halliwell

Abstract Epidemiological studies testing the effect of β -carotene in humans have found a relative risk for lung cancer in smokers supplemented with β -carotene. We investigated the reactions of retinal and β -apo-8'-carotenal, two β -carotene oxidation products, with 2'-deoxyguanosine to evaluate their DNA damaging potential. A known mutagenic adduct, 1,*N*²-etheno-2'-deoxyguanosine, was isolated and characterized on the basis of its spectroscopic features. After treatment of calf thymus DNA with β -carotene or β -carotene oxidation products, significantly increased levels of 1,*N*²-etheno-2'-deoxyguanosine and 8-oxo-7,8-dihydro-2'-deoxyguanosine were quantified in DNA. These lesions are believed to be important in the development of human cancers. The results reported here may contribute toward an understanding of the biological effects of β -carotene oxidation products.

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Key words: β -Carotene; β -Apo-8'-carotenal; 1,*N*²-Etheno-2'-deoxyguanosine; Etheno adduct; 8-Oxo-7,8-dihydro-2'-deoxyguanosine; DNA damage; Retinal

1. Introduction

Many studies suggest a protective role of β -carotene (β C) against cancer [1–3]. However, the Alpha-tocopherol, Beta-carotene Cancer Prevention Study and the Beta-carotene and Retinol Efficacy Trial [4–6] have shown that β C increases the incidence of lung cancer in heavy smokers and asbestos workers. To explain this paradox, it has been suggested that β C, under the free radical-rich atmosphere produced by the

chemicals in cigarette smoke and the resultant inflammatory response in the lung, can function as a pro-oxidant with the generation of β C oxidation products and other reactive species [6]. It is noteworthy that in vitro incubations of β C with postnuclear fractions of lung tissue from ferrets exposed to tobacco smoke showed an enhanced formation of β C oxidation products (apo-carotenals) concomitantly with a decrease in β C concentrations [7]. The same effect has also been observed in human bronchial epithelial cells supplemented with β C and exposed to cigarette smoke. The oxidation of β C by cigarette smoke generates various products, including 4-nitro- β C, β -apo-carotenals, and β C epoxides [8]. In fact, it has already been reported that β C acts as an antioxidant at low oxygen tensions, but at high oxygen tensions β C is a pro-oxidant, possibly by reacting with oxygen generating reactive carotenoid peroxy radical [9]. β -Ionone, β -apo-carotenals, and β C-5,8-endoperoxide were also detected as singlet oxygen oxidation products of β C [10]. Interestingly, β C oxidation products increase the amount of binding of metabolites of the important cigarette smoke carcinogen benzo[*a*]pyrene to calf thymus DNA [11]. Paolini and co-workers [12] have also found that β C produces a significant increase in carcinogen-metabolizing enzymes in rat lung, including activators of polycyclic aromatic hydrocarbons, and that this induction is associated with the generation of oxidative stress.

This laboratory and co-workers recently reported that *trans,trans*-2,4-decadienal oxidation products can bind to adenine and guanine in DNA yielding adducts with an extended etheno ring [13–15]. In addition to 1,*N*⁶-etheno-2'-deoxyadenosine (ϵ dAdo) and 1,*N*²-etheno-2'-deoxyguanosine (1,*N*²- ϵ dGuo), six novel etheno adducts were identified. The suggested reaction mechanism for the formation of etheno adducts involves initial aldehyde oxidation to reactive intermediates, such as epoxides, diepoxides, and/or hydroperoxides, with subsequent reaction with the DNA bases giving rise to the described adducts [14,15]. Recently, Lee and co-workers [16] showed unequivocally that 4,5-epoxy-2-(*E*)-decenal is a precursor to the formation of ϵ dAdo and 1,*N*²- ϵ dGuo adducts, providing an important contribution that validates the proposed mechanisms for *trans,trans*-2,4-decadienal reaction.

The promutagenic properties of etheno bases have been well determined by in vitro primer extension assays and site-specific mutation in *Escherichia coli* and in mammalian cells. In vitro misincorporation studies showed that 1,*N*²- ϵ dGuo tends to strongly block replication at and beyond the site of substitution and to favor the misincorporation of dATP and

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Abbreviations: β C, β -carotene; 1,*N*²- ϵ dGuo, 1,*N*²-etheno-2'-deoxyguanosine; dGuo, 2'-deoxyguanosine; ϵ dAdo, 1,*N*⁶-etheno-2'-deoxyadenosine; *N*²,3- ϵ dGuo, *N*²,3-etheno-2'-deoxyguanosine; *N*²,3- ϵ Gua, *N*²,3-etheno-guanine; 1,*N*²- ϵ Gua, 1,*N*²-etheno-guanine; 8-oxodGuo, 8-oxo-7,8-dihydro-2'-deoxyguanosine; HPLC, high performance liquid chromatography; ESI/MS, electrospray ionization mass spectrometry; LC/ESI/MS-MS, liquid chromatography/electrospray ionization tandem mass spectrometry; MRM, multiple reaction monitoring

dGTP across it [17]. Another study, in which miscoding opposite this adduct in an *E. coli*/M13MB19-based system was examined, showed that it is mutagenic in *E. coli* *uvrA*[−], with more abundant G→A mutations, followed by G→T mutations [18]. 1,*N*²-edGuo also generates deletions, rearrangements, double mutants and base pair substitutions at sites near the 1,*N*²-edGuo site [19]. In studies using highly sensitive techniques it was shown that etheno-DNA adduct levels were increased in target tissues for carcinogenesis in experimental animals exposed to vinyl chloride or ethyl carbamate, in hepatic DNA from patients with metal storage diseases, and in colonic polyps of familial adenomatous polyposis patients [20].

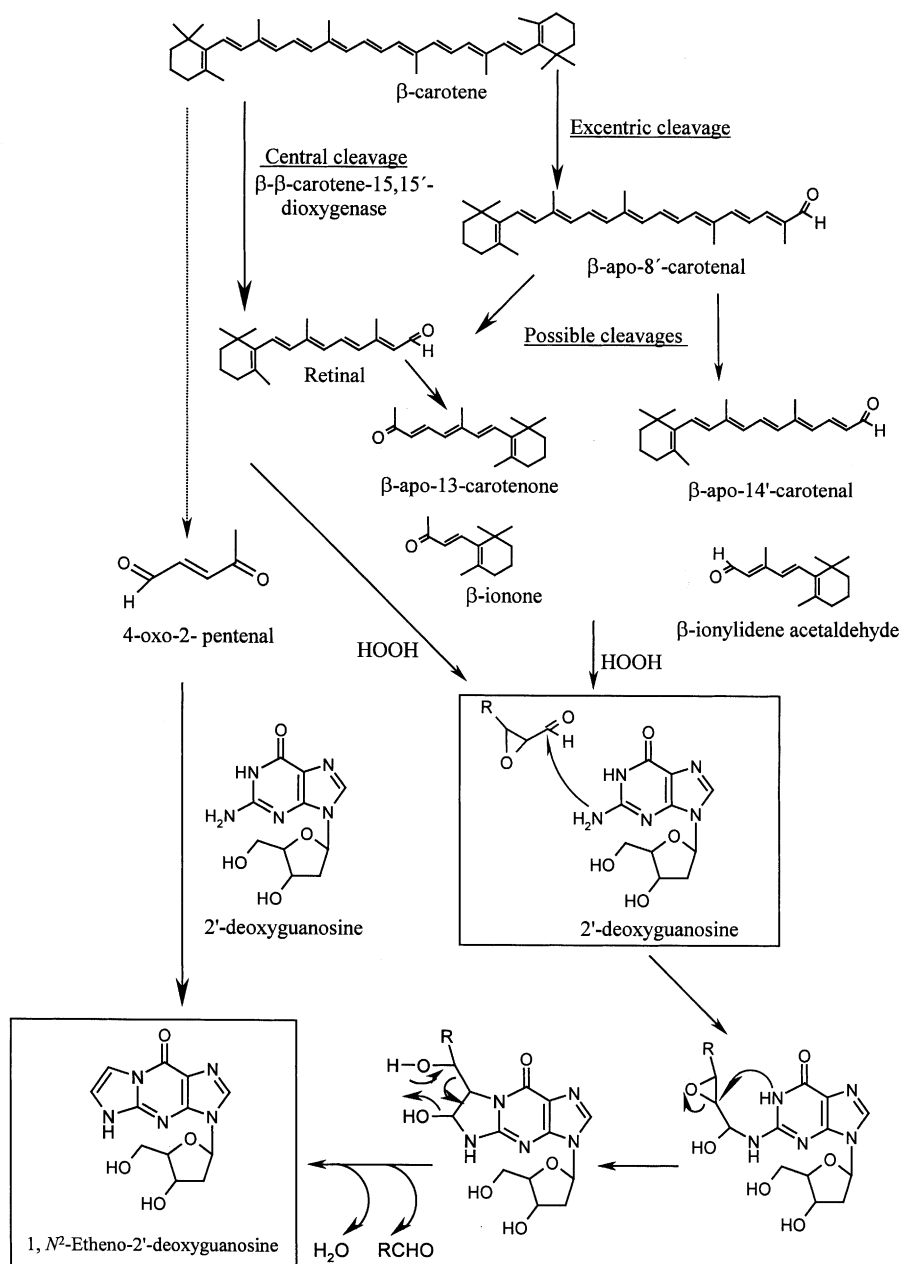
Because retinal and β -apo-8'-carotenal are aldehydes generated during β C oxidation [21], we decided to investigate

whether they react with 2'-deoxyguanosine (dGuo) producing potentially mutagenic lesions in DNA (Scheme 1).

2. Materials and methods

2.1. Chemicals

All the chemicals employed were of the highest purity grade commercially available. Potassium phosphate, dGuo, formic acid, and 2-chloroacetaldehyde were acquired from Merck (Darmstadt, Germany). The labeled [¹⁵N₅]dGuo was provided by Cambridge Isotope Laboratories (Andover, MA, USA). Chromatography grade acetonitrile and methanol were obtained from EM Science (Gibbstown, NJ, USA). Chloroform and ethanol were from Cinética Química (São Paulo, Brazil). Hydrogen peroxide was from Fluka Chemika (Buchs, Switzerland). All the other chemicals used were from Sigma (St. Louis, MO, USA). Water was purified on a Milli-Q system (Millipore, Bedford, MA, USA).



Scheme 1.

2.2. Spectroscopy

^1H nuclear magnetic resonance (NMR) spectra were acquired using a DRX-500 MHz NMR spectrometer (Bruker, Germany) at 27°C. The samples were dissolved in dimethylsulfoxide ($\text{DMSO}-d_6$) and the solvent peak was used as the reference. UV spectra were obtained with a Hitachi U3000 spectrophotometer (Tokyo, Japan).

2.3. DNA reactions

Calf thymus DNA (5 mg) dissolved in 1 ml of 50 mM potassium phosphate buffer pH 7.4 (neutral pH incubation) or 0.2 M carbonate–bicarbonate buffer pH 9.4 (basic pH incubation) was added to a solution of retinal (5 mg) or β -apo-8'-carotenal (5 mg) or βC (5 mg) prepared in acetonitrile (0.5 ml) and the resulting mixture was stirred at 37°C for 72 h. Additional reactions were also performed in the presence of 50 mM H_2O_2 . The unreacted carotenoid was removed by two extractions with ethyl acetate. DNA precipitation was carried out by the addition of an appropriate volume of 3 M sodium acetate buffer pH 5 and 1 ml of ethanol. The suspension was cooled to -20°C overnight and centrifuged at 1000 g for 5 min at 4°C. The resulting pellet was washed with 70% ethanol and dissolved in 1 ml of water. The DNA (200 μg) was added to 4 μl of 1 M sodium acetate buffer (pH 5) and 2 pmol of labeled [$^{15}\text{N}_5$]1, N^2 -edGuo, followed by digestion with 2 U of nuclease P1 at 37°C for 30 min. 12 μl of 1 M Tris–HCl buffer (pH 7.4), 12 μl of phosphatase buffer and 12 U of alkaline phosphatase were then added for an additional 1 h incubation at 37°C. The final volume of the solution was adjusted to 200 μl with water. The enzymes were precipitated by the addition of chloroform and the resulting aqueous samples were subjected to liquid chromatography/electrospray ionization tandem mass spectrometry (LC/ESI/MS-MS) analysis (100 μl DNA/injection). The amounts of the reagents and labeled internal standard were proportionally adjusted for hydrolysis and analysis of other DNA quantities. This assay permitted the complete hydrolysis of 60–70% of the initial DNA samples.

2.4. Synthesis of 1, N^2 -edGuo standard

1, N^2 -edGuo standard was obtained by reaction of dGuo with chloroacetaldehyde, as described before [15,22].

2.5. Synthesis of [$^{15}\text{N}_5$]1, N^2 -edGuo internal standard

[$^{15}\text{N}_5$]1, N^2 -edGuo was obtained by reaction of [$^{15}\text{N}_5$]dGuo with chloroacetaldehyde with subsequent purification by high performance liquid chromatography (HPLC) as described by Loureiro and co-workers [15].

2.6. Isolation and characterization of 1, N^2 -edGuo from the reaction of retinal or β -apo-8'-carotenal with dGuo

Retinal (80 mg) or β -apo-8'-carotenal (200 mg) dissolved in acetonitrile (20 ml) was added to dGuo (40 mg) in 20 ml of 0.2 M carbonate–bicarbonate buffer (pH 9.4). H_2O_2 (50 mM) was then added to this solution. After 7 days of incubation at 50°C with stirring, the reaction mixture was extracted with ethyl acetate. The resulting products were isolated by HPLC (described below). One of the products in each incubation had spectroscopic properties identical to those of the authentic 1, N^2 -edGuo standard. The UV spectrum of the adduct is presented in Fig. 1A. The positive mode ESI/MS analysis (Fig. 1B) shows m/z 175.90 ($[\text{M}+\text{H}]^+-2\text{-D-erythro-pentose}$, 100% relative intensity) and 292.03 ($[\text{M}+\text{H}]^+$, 29.7% relative intensity). ^1H NMR (DMSO) δ 8.12 (s, 1H, H-2), 7.60 (d, 1H, $J=2.43$ Hz, H-6), 7.42 (d, 1H, $J=2.58$ Hz, H-7), 6.23–6.26 (t, 1H, H-1'), 4.92 (t, 1H, OH-5'), 5.27 (d, 1H, OH-3'), 4.33–4.38 (m, 1H, H-3'), 3.83–3.84 (m, 1H, H-4'), 3.56–3.60 (m, 1H, H-5'), 3.51–3.54 (m, 1H, H-5''), 2.59–2.62 (m), 2.23–2.27 (m, 1, 1H, H-2'H, H-2'').

2.7. HPLC separations

The products of the reaction of retinal and β -apo-8'-carotenal with dGuo in the presence of H_2O_2 were separated by HPLC (Shimadzu, Kyoto, Japan) system 1. Fractions with 20 min retention time for both reactions were collected and further purified by HPLC with a Luna C18 (2) (250 mm \times 4.6 mm i.d., 5 μm) analytical column (Phenomenex, Torrance, CA, USA) eluted with system 2 for the retinal reaction and with system 3 for the β -apo-8'-carotenal reaction. System 1: a Luna C18 (2) (250 mm \times 10 mm i.d., 10 μm) semi-preparative column (Phenomenex) eluted with a gradient of water and methanol (0–5 min, 2–5% methanol; 5–20 min, 5–30% methanol; 20–25 min, 30–100% methanol; 25–40 min, 100% methanol; 40–45 min, 100–2%

methanol; and 45–50 min, 2% methanol) at a flow rate of 4.6 ml/min. System 2: a gradient of water and acetonitrile (0–30 min, 8–16% acetonitrile) at a flow rate of 1 ml/min. System 3: a gradient of water and methanol (0–20 min, 18–30% methanol; 20–30 min, 30% methanol) at a flow rate of 1 ml/min.

2.8. LC/ESI/MS-MS analyses

LC/ESI/MS-MS analyses in the positive mode were carried out on a Quattro II mass spectrometer (Micromass, Manchester, UK). For the acquisition of mass spectra, a Shimadzu LC-10AD pump was used to pump the eluent, a mixture of water and acetonitrile (50:50), at a flow rate of 10 $\mu\text{l}/\text{min}$ directly to the spectrometer. The purified samples were dissolved in aqueous solutions containing 0.2% formic acid and injected into the mobile phase through a Rheodyne injector with a 20 μl Rheodyne loop (Rheodyne, Cotati, CA, USA). Full scan data in MS_1 were acquired over a mass range of 100–600 Da with different cone voltages.

The 1, N^2 -edGuo adduct in DNA samples was detected by multiple reaction monitoring (MRM) as described before [23]. Briefly, the DNA hydrolysates containing 1 pmol of the [$^{15}\text{N}_5$]1, N^2 -edGuo internal standard were injected into the HPLC/ESI/MS-MS system [23]. The m/z 292 \rightarrow 176 (1, N^2 -edGuo) and 297 \rightarrow 181 ([$^{15}\text{N}_5$]1, N^2 -edGuo) transitions were monitored with a dwell time of 1 s. All the other parameters of the mass spectrometer were adjusted for acquisition of the best $[\text{M}+\text{H}]^+ / [\text{M}+\text{H}]^+-2\text{-D-erythro-pentose}$ transition. The specificity of the above described method for the detection of 1, N^2 -edGuo, instead of its isomer $N^2,3$ -edGuo, was observed as described before by Ham and co-workers [24] for detection of these adducts in the reaction of ethyl linoleate with dGuo. The MRM specific transitions for $N^2,3$ -edGuo (m/z 176 \rightarrow 81) and 1, N^2 -edGuo (m/z 176 \rightarrow 148) were monitored simultaneously. For these experiments the cone voltage was kept at 35 V, the collision energy was set at 30 eV, and the pressure of argon in the collision cell was adjusted to 1.0×10^{-3} mbar. The data were processed using MassLynx software (Micromass).

2.9. dGuo quantification in DNA samples

The following HPLC system was used to quantitatively determine dGuo in the DNA hydrolysates: a Luna 5 μ C18 (2) (250 mm \times 4.6 mm i.d., 5 μm) analytical column (Phenomenex) was eluted with an isocratic flow of 25 mM potassium phosphate buffer pH 5.5 and 6% methanol at a flow rate of 1 ml/min for 45 min, and the absorbance was monitored at 254 nm. A standard calibration curve prepared within the range of dGuo expected to be present in the DNA hydrolysates was used for this quantification.

2.10. Analysis of 8-oxodGuo with HPLC-electrochemical detection (HPLC/EC)

Samples (35 μg) of digested DNA previously incubated with carotenoids were injected into the HPLC/EC system, consisting of a Shimadzu model LC-10AD pump connected to a Luna C18 reversed-phase column (250 mm \times 4.6 mm i.d., 5 μm) (Phenomenex). The isocratic eluent was 8% methanol in 25 mM potassium phosphate buffer pH 5.5 at a 1 ml/min flow rate. Coulometric detection was provided by a Coulochem II detector (ESA, Chelmsford, MA, USA). The potentials of the two electrodes were set at 130 and 280 mV. The molar ratio of 8-oxodGuo to dGuo in each DNA sample was determined based on coulometric detection at 280 mV for 8-oxodGuo and absorbance at 254 nm for dGuo in each injection.

3. Results and discussion

The reaction of retinal and β -apo-8'-carotenal with dGuo in the presence of H_2O_2 led to the formation of several products which were separated by HPLC. Products with a 20 min retention time for the retinal/dGuo/ H_2O_2 and for β -apo-8'-carotenal/dGuo/ H_2O_2 reactions showed UV absorption spectra similar to that of 1, N^2 -edGuo. These products were isolated and characterized by UV, ^1H NMR and ESI/MS spectra analyses. One of them in each incubation displayed identical spectroscopic features to 1, N^2 -edGuo, as inferred from the comparison with the authentic standard (Fig. 1A). The ESI/MS spectrum of the isolated adduct in the positive mode (Fig. 1B)

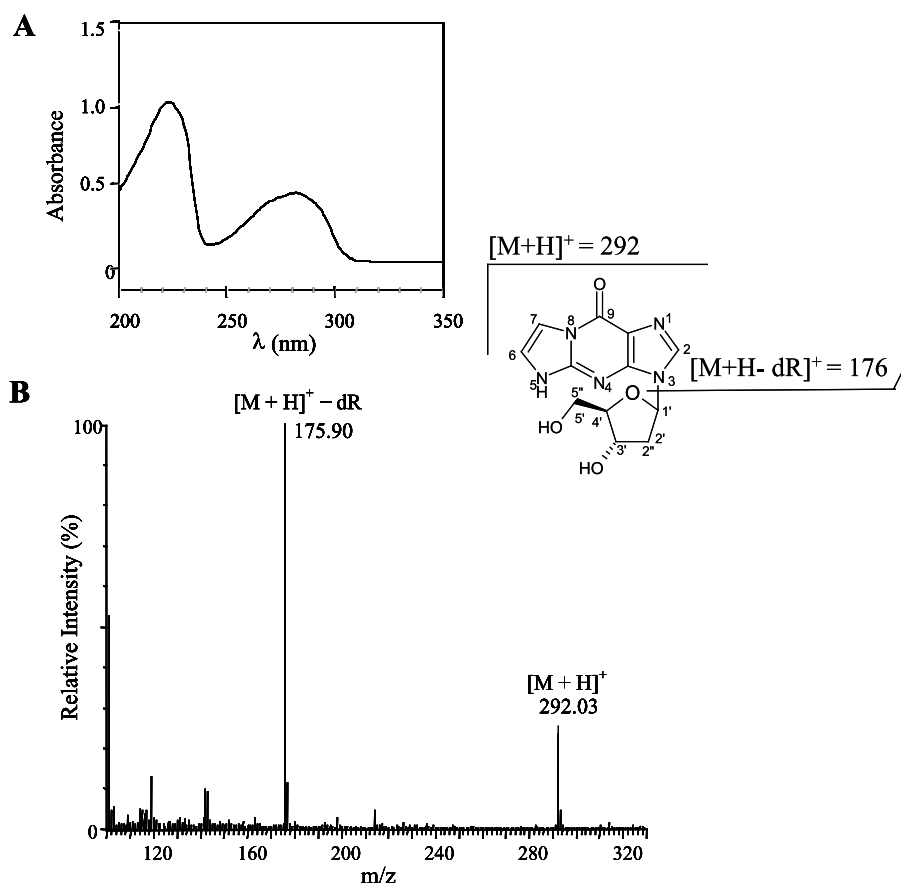


Fig. 1. A: UV absorption spectrum of the 1,*N*²-εdGuo adduct isolated from the reaction of retinal or β-apo-8'-carotenal with dGuo. B: ESI/MS spectrum of the isolated adduct. The cone voltage was 25 V. Conditions were as described in Section 2. dR, 2'-deoxyribose.

exhibits a protonated molecular ion $[M+H]^+$ at m/z 292.03 and a predominant fragment at m/z 175.90 ($[M+H]^+ - 2\text{-D-erythro-pentose}$). These assignments are consistent with the introduction of an etheno bridge in the dGuo molecule. This structure was confirmed by the ^1H NMR spectrum (Section 2). One plausible pathway for adduct formation is the one already described for the reaction of epoxy carbonyl compounds with nucleic acid bases [15]. It involves initial epoxidation of the aldehyde C=C double bond by peroxides. Reaction of the oxidation product with dGuo will produce the etheno adduct. It is noteworthy that etheno adduct formation is also observed in the absence of added peroxides. In this case, contaminant peroxides or the formation of reactive aldehydes could lead to adduct formation.

In order to verify if the reaction also occurs with calf thymus DNA, we used a sensitive and selective LC/ESI/MS-MS with MRM [23]. Calf thymus DNA was allowed to react with βC, retinal, or β-apo-8'-carotenal with and without H_2O_2 in neutral (pH 7.4) and basic (pH 9.4) conditions. We monitored the specific MRM transitions for *N*²,3-εGua (m/z 176 → 81) and 1,*N*²-εGua (m/z 176 → 148), as described in Section 2, to ensure that the isomer we detect at the m/z 292 → 176 transition is the 1,*N*²-εdGuo adduct (data not shown). LC/ESI/MS-MS was used to quantify the adduct against the respective $^{15}\text{N}_5$ -labeled internal standard. Fig. 2 shows the chromatographic behavior of the resulting product in control DNA, incubated without H_2O_2 at pH 9.4, as well as the calibration curve used to quantify the adduct. With this approach, 1,*N*²-εdGuo concentrations in calf thymus DNA in control incu-

bations were 0.57 and 5.44 adducts per 10^7 parent bases at pH 7.4 and 9.4, respectively. The basal level of 1,*N*²-εdGuo in calf thymus DNA was recently determined with LC/MS-MS to be 1.70 ± 0.09 adducts/ 10^7 dGuo [23]. Therefore, an increase in the 1,*N*²-εdGuo levels was observed after 72 h incubations at pH 9.4 (Table 1). This effect has already been described by Morinello et al. [25] who reported an induction in 1,*N*²-εdGuo formation in calf thymus DNA after 1 h incubation at pH 10.5. In fact, oxidation of deoxyribose in DNA can result in the formation of electrophilic products that have the potential

Table 1
Formation of 1,*N*²-εdGuo in DNA reacted with carotenoids

Treatment	1, <i>N</i> ² -εdGuo/ 10^7 dGuo	
	pH 7.4	pH 9.4
DNA (incubation only)	0.57 ± 0.18	5.4 ± 1.0
DNA/ H_2O_2	3.64 ± 0.69	186 ± 40
DNA/retinal	8.74 ± 1.56^a	320 ± 27^a
DNA/ H_2O_2 /retinal	24.55 ± 2.10^b	467 ± 70^b
DNA/β-apo-8'-carotenal	2.28 ± 0.42^a	1060 ± 134^a
DNA/ H_2O_2 /β-apo-8'-carotenal	29.24 ± 3.22^b	1284 ± 135^b
DNA/βC	4.44 ± 0.52^a	285 ± 37^a
DNA/ H_2O_2 /βC	24.75 ± 1.08^b	1033 ± 68^b

LC/ESI/MS-MS analysis of 100 μg DNA/injection. Adduct level (mean ± S.D., $n = 3-6$). The statistical comparisons were carried out using independent two population Student's *t*-test.

^aSignificantly different from (DNA/incubation only) control samples ($P < 0.05$) at both pHs.

^bSignificantly different from DNA/ H_2O_2 control samples ($P < 0.05$) at both pHs.

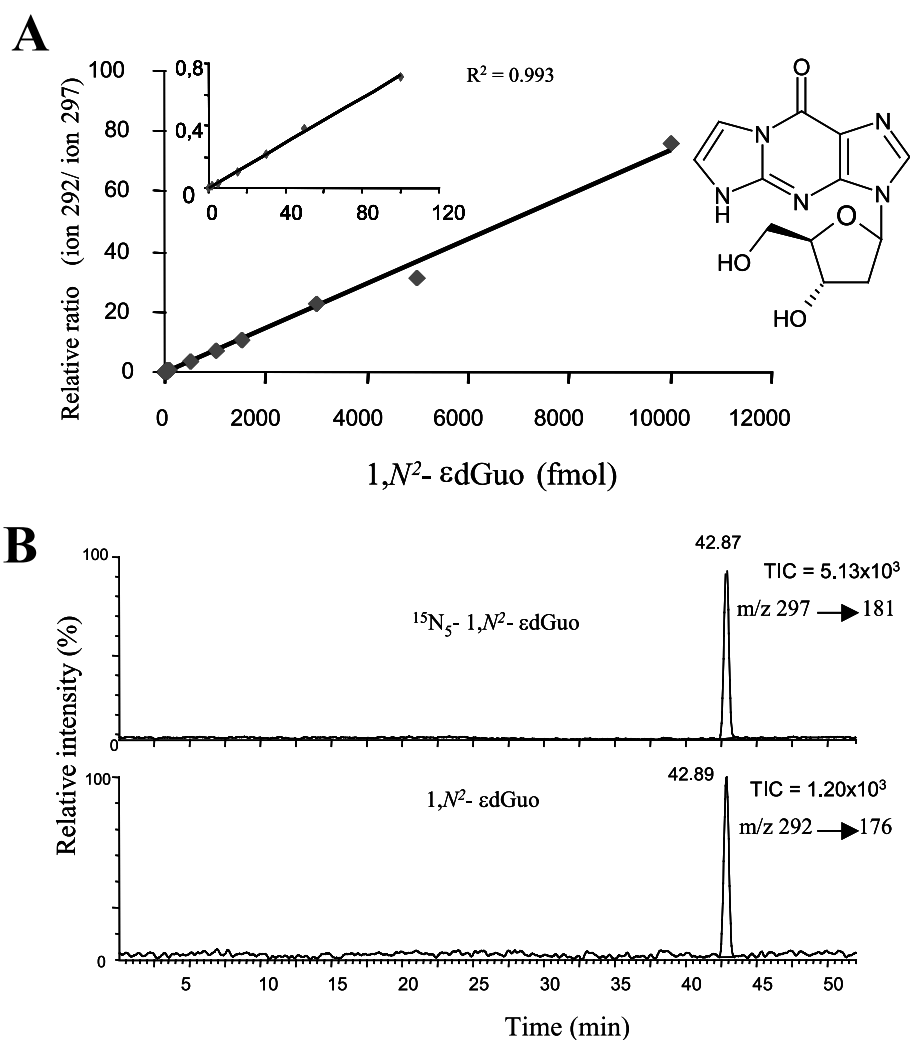


Fig. 2. Detection of 1, N^2 - ϵ dGuo in calf thymus DNA. A: LC/ESI/MS-MS calibration curve for 1, N^2 - ϵ dGuo obtained by plotting the relative ratio of the 1, N^2 - ϵ dGuo (m/z 292 \rightarrow 176) to the isotopically $^{15}\text{N}_5$ -labeled internal standard (m/z 297 \rightarrow 181) ions vs. increasing amounts of 1, N^2 - ϵ dGuo. B: Detection of the adduct formed in control DNA (incubated without H_2O_2 at pH 9.4, 37°C for 72 h).

to form nucleobase adducts as proposed by Awada and De-don [26].

A significant increase in the 1, N^2 - ϵ dGuo concentration was observed after DNA reactions with retinal, β -apo-8'-carotenol and βC at pH 7.4 and 9.4 (Table 1). The degradation of carotenoids in model systems has been extensively studied [11,21,27,28]. In a study at 30°C, apocarotenoids, epoxycarotenoids, and hydroxycarotenoids were identified, confirming that the first steps in carotenoid degradation are epoxidation, apocarotenol formation and hydroxylation [28]. It is noteworthy that 4-oxo-2-pentenal was detected as a βC degradation product. The chemical reactivity of this aldehyde is similar to 4-oxo-2-nonenal, the bifunctional electrophile described by Rindgen et al. [29] as the principal breakdown product of linoleic acid hydroperoxide. It has been shown to react with dAdo, dGuo and dCyd to form etheno adducts [30,31]. This reactive aldehyde can contribute to adduct formation in the reactions performed with and without the addition of H_2O_2 . Interestingly, 4-oxo-2-pentenal was shown to be formed after metabolic activation of N -nitrosopiperidine, a carcinogenic cyclic nitrosamine, leading to substituted etheno adduct formation [32,33].

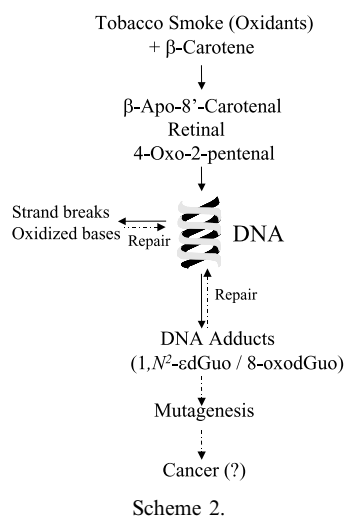
Murata and Kawanish [34] reported that retinal and retinol induced 8-oxodGuo formation in HL-60 cells but not in H_2O_2 -resistant HP-100 cells. Superoxide generation by auto-oxidation of retinoids is suggested to play a role in 8-oxodGuo formation in the presence of metals. Recently, formation of aldehydic compounds and increased levels of 8-oxodGuo were also measured in calf thymus DNA incubated with oxidized βC [35]. For comparison purposes, the levels of 8-oxodGuo in

Table 2
Formation of 8-oxodGuo in DNA reacted with carotenoids

Treatment	8-oxodGuo/ 10^7 dGuo	
	pH 7.4	pH 9.4
DNA (incubation only)	555 \pm 55	337 \pm 12
DNA/retinal	1733 \pm 42 ^a	1157 \pm 61 ^a
DNA/ β -apo-8'-carotenol	1859 \pm 10 ^a	1697 \pm 210 ^a
DNA/ βC	1081 \pm 57 ^a	700 \pm 71 ^a

LC/EC analysis of 35 μg DNA/injection. Adduct level (mean \pm S.D., $n=3$). The statistical comparisons were carried out using independent two population Student's t -test.

^aSignificantly different from DNA/incubation only control samples ($P < 0.05$) for both pHs.



DNA samples exposed to carotenoid oxidation products were also evaluated here. In agreement with these previous reports, the levels of 8-oxodGuo were significantly increased in DNA incubated with carotenoids at pH 7.4 and 9.4 (Table 2).

Recent evidence suggests etheno adducts as possible mediators of carcinogenesis [36]. We report herein that 1,N²-εdGuo and 8-oxodGuo are formed in the reactions of DNA with βC and with two important βC oxidation products (retinal and β-apo-8'-carotenal). Considering that (i) smokers probably have higher levels of βC oxidation products in their lung fluids than non-smokers [11] and (ii) 1,N²-εdGuo has been proven to be mutagenic in *E. coli* uvrA⁻ [18] and in mammalian cells [19], the results presented here suggest that these two DNA lesions may play a role when βC-supplemented tissues are exposed to oxidants (Scheme 2).

Acknowledgements: This work was supported by the Fundação de Amparo à Pesquisa do Estado de São Paulo, FAPESP (Brazil), the Conselho Nacional para o Desenvolvimento Científico e Tecnológico, CNPq (Brazil), Pró-Reitoria de Pesquisa da Universidade de São Paulo (Brazil), and Programa de Apoio aos Núcleos de Excelência, PRONEX/FINEP (Brazil). S.A.M. and A.P.M.L. were recipients of a FAPESP fellowship. C.C.M.G. is the recipient of a CNPq fellowship. We thank Edson A. Gomes for technical assistance.

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