Radical Intermediates during the Oxidation of Nitropropanes. The Formation of NO_2 from 2-Nitropropane, Its Reactivity with Nucleosides, and Implications for the Genotoxicity of 2-Nitropropane

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The chemistry of the nonenzymatic oxidation of the rat liver carcinogen, 2-nitropropane, and its anionic form, propane-2-nitronate, was investigated using pulse radiolysis and EPR/spin trapping with 3,5-dibromo-4-nitrosobenzenesulfonic acid as the trapping agent. The results suggest that, following initial oxidation to a secondary alkyl radical, propane-2-nitronate is effectively degraded in a peroxidative chain reaction with the intermediary formation of peroxyl and $NO_2^{\bullet}$ radicals. The latter radical was shown to react appreciably fast with ribonucleosides, deoxyribonucleosides, and guanosine nucleotides. It is proposed that nonenzymatic formation of $NO_2^{\bullet}$ radicals after enzymatic oxidation of propane-2-nitronate to the corresponding secondary alkyl radical accounts for the induction of DNA damage observed after exposure of rats to 2-nitropropane.

Introduction

The industrial solvent 2-nitropropane (2-NP)[1] induces liver tumors in rats following chronic exposure (*1*, *2*). Treatment of the animals with the compound has been shown to result in a variety of genotoxic effects in liver, such as formation of 8-hydroxyguanine (*3–6*) and 8-aminoguanine (*7*) in DNA and RNA, induction of DNA single-strand breaks (*8*), DNA repair synthesis (*9*, *10*), and micronuclei (*10*). The mechanism underlying the production of DNA damage by 2-NP is not yet clear. Evidence has been presented that genotoxicity in rat liver cells is the consequence of an oxidation of the anionic form of 2-NP, propane-2-nitronate (P2-N; *11*), by a liver-specific cytochrome P450-dependent monooxygenase (*8*, *11*, *12*). Oxidation products have been identified as acetone (*13*, *14*), nitrite (*14*, *15*), and 2-propanol (*13*), but the precise route of the formation of these metabolites, which appears to be associated with the production of a reactive, DNA-damaging intermediate has not yet been elucidated.

Several pathways for the oxidation of 2-NP to acetone and nitrite in vitro have been suggested (*16–18*). Porter and Bright (*16*) and Kuo and Fridovich (*17*) proposed that oxidation should occur via the initial formation of the 2-NP radical with subsequent nonenzymatic oxidation in a free-radical chain reaction. In the in vitro systems used by these authors, the 2-NP radical was produced either enzymatically from P2-N using horseradish peroxidase (HRP)/H_2O_2 (*16*) or by reaction of the nitronate with superoxide (*17*). The individual reactions of the radical chains proposed by these authors differed considerably, however. Porter and Bright (*16*) suggested that P2-N was oxidized by HRP/H_2O_2 to the 2-NP radical which then reacts with oxygen to yield a peroxyl radical. This could propagate the chain by oxidizing P2-N to the 2-NP radical, being concomitantly reduced to the peroxy anion. Reaction of the protonated peroxy anion with P2-N was suggested to yield unstable intermediates which decompose to acetone and nitrite. In contrast, Kuo and Fridovich (*17*) postulated that, following the formation of the 2-NP radical and of H_2O_2 by the reaction of P2-N with superoxide, the 2-NP radical does not form a peroxy radical but is hydrolyzed to the hydroxyisopropyl radical and nitrite and that the hydroxyisopropyl radical reacts with oxygen to yield acetone and superoxide which then propagates the chain reaction.

Although various organic radicals as well as H_2O_2 may be considered as intermediates, none of these species has been identified as being involved in the production of DNA damage in cells. In the present study we have reinvestigated the mechanism of the nonenzymatic oxidation of the 2-NP radical using pulse radiolysis for the production of the individual radicals. Optical and EPR spectroscopy were used for the identification of the intermediate species and for monitoring their formation and decay. For comparative purposes, studies were also performed on 1-nitropropane (1-NP), a noncarcinogenic analog of 2-NP (*2*, *19*). The inability of 1-NP to cause tumors in experimental animals has been attributed to a nearly negligible formation of its anionic form, propane-1-nitronate (*6*, *18*).

Materials and Methods

2-NP (>99%, potential carcinogen) was a gift from Angus Chemie (Ibbenbüren-Uffeln, FRG). P2-N (pK_a = 7.43; *18*) was prepared by mixing equimolar amounts of 2-NP and KOH in water and allowing the mixture to stand at room temperature for 30 min (*11*). 1-NP (98%) and 2-bromo-2-nitropropane (irritant) were from Aldrich (Steinheim, FRG). Sodium nitrite (Merck,

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[1] Abbreviations: 1-NP, 1-nitropropane; 2-NP, 2-nitropropane; P2-N, propane-2-nitronate; HRP, horseradish peroxidase; NG, nitroguaiacol (2-methoxy-4-nitrophenol); *p*-NDA, 4-nitroso-*N*,*N*-dimethylaniline; DB-NBS, 3,5-dibromo-4-nitrosobenzenesulfonic acid; DMPO, 5,5-dimethylpyrroline *N*-oxide; MNP, 2-methyl-2-nitrosopropane.

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Darmstadt, FRG) was employed to generate $NO_2^{\bullet}$ radicals in radiolytic experiments. Nitroguaiacol (NG; 2-methoxy-4-nitrophenol, Aldrich; irritant), 4-nitroso-*N*,*N*-dimethylaniline (p-NDA, Aldrich; toxic substance), and kaempferol (3,5,7,4'-tetrahydroxyflavone, Roth, Karlsruhe, FRG) were used as reference substances during competition experiments. Nucleosides and nucleotides were from Fluka (Neu-Ulm, FRG). The spin traps 5,5-dimethylpyrroline *N*-oxide (DMPO) and 2-methyl-2-nitrosopropane (MNP) were from Sigma (Deisenhofen, FRG), while 3,5-dibromo-4-nitrosobenzenesulfonic acid (DBNBS) was synthesized according to Kaur et al. (*20*) from 3,5-dibromosulfanilic acid (Aldrich). Except for DMPO, which was purified by charcoal filtration under N_2 in the dark (*21*), all substances were used as supplied. Solutions were prepared with Milli-Q water (Millipore, Eschborn, FRG) and phosphate-buffered (5 mM) at pH 7.0–7.5, except where indicated otherwise.

The pulse radiolysis experiments were performed using a Febetron 705 (Hewlett-Packard, McMinnville, OR) as an electron source (100-ns pulses of 1.6-MeV energy). Kinetic spectroscopy (observation of time-dependent absorption changes for spectral and kinetic evaluation) was done simultaneously at 15 wavelengths, using a spectrograph arrangement. The transient absorbances were digitized in a modular transient recorder (VUKO, Wuntronic, München, FRG). Solutions were delivered to the pulse cuvette (1.6-mL volume, 2-cm light path) via a syringe-driven mixing system. For process control and data handling and storage a Wang 2200 VP computer was used (*22*).

To generate specific radical species, we used the following radiolytic systems (*23*): N_2O-saturated aqueous solutions, in which the hydrated electrons (e^-_{aq}) are converted into $^{\bullet}OH$ radicals,

$$N_2O + e^-_{aq} + H_2O \rightarrow {}^{\bullet}OH + OH^- + N_2 \quad (1)$$

thereby yielding solutions that predominantly contain this radical (except for an additional 10% of H atoms). The presence of O_2 in these solutions causes the alkyl radicals formed after the attack by $^{\bullet}OH$ radicals to be converted into peroxyl radicals:

$$^{\bullet}OH + RH \rightarrow R^{\bullet} + H_2O \quad (2)$$

$$R^{\bullet} + O_2 \rightarrow ROO^{\bullet} \quad (3)$$

Oxygen-saturated solutions containing 10 mM sodium formate convert all primary radicals ($^{\bullet}OH$, e^-_{aq}, $^{\bullet}H$) into superoxide anions ($O_2^{\bullet-}$):

$$O_2 + e^-_{aq} \rightarrow O_2^{\bullet-} \quad (4)$$

$$O_2 + {}^{\bullet}H \rightarrow HO_2^{\bullet} \rightleftharpoons O_2^{\bullet-} + H^+ \quad (5)$$

$$HCOO^- + {}^{\bullet}OH \rightarrow CO_2^{\bullet-} + H_2O \quad (6)$$

$$O_2 + CO_2^{\bullet-} \rightarrow O_2^{\bullet-} + CO_2 \quad (7)$$

Finally, but most important for our studies, $NO_2^{\bullet}$ radicals were generated in N_2O-saturated solutions containing 10 mM sodium nitrite (*24*):

$$^{\bullet}OH + NO_2^- \rightarrow NO_2^{\bullet} + H_2O \quad (8)$$

In the last system a competition exists between N_2O and NO_2^- for the hydrated electron. According to the respective rate constants of 9.1×10^9 (N_2O) and 4.1×10^9 dm^3 mol^{-1} s^{-1} (NO_2^-; *25*), we calculate that at the concentrations employed ([N_2O] = 25.5 mM, [NO_2^-] = 10 mM) maximally 15% of e^-_{aq} would react directly with NO_2^- and thus would only marginally interfere with the reaction.

Competition experiments with nucleic acid components were also done by pulse radiolysis, in which case the reaction of $NO_2^{\bullet}$ with kaempferol, leading to a strongly absorbing aroxyl radical, was used as the reference reaction. When the ratio of the peak absorption in the absence (A_0) or in the presence of a given competitor (A_c) is plotted as a function of the concentration ratio, the slope of a line intersecting the ordinate at unity yields the ratio of the rate constants k_c/k_r:

$$A_0/A_c = 1 + (k_c/k_r)[\text{competitor}]/[\text{reference substance}]$$

Knowing the rate constant of the reference substance with the radical in question (k_r), in this case $NO_2^{\bullet}$, the relative rate constants can be converted into absolute second-order rate constants (dimension dm^3 mol^{-1} s^{-1}; *26*).

EPR spectroscopy was performed on a Bruker ESP 300 instrument (Bruker, Karlsruhe, FRG) using a TM-110 cavity. Instrument settings were 0.5-G modulation amplitude and 2 G/s scan speed. Either radicals were prepared in situ by HRP/H_2O_2 (*16*) or pulse-irradiated solutions were transferred to the EPR cavity within 35–45 s.

Results

Pulse-Radiolytic Oxidation of Nitropropanes. After pulse-radiolytic oxidation of 1-NP, 2-NP, and its nitronate anion P2-N, each substrate, depending on the oxidation system, revealed quite distinct behavior with regard to absorption spectroscopy and kinetics. As shown in Figure 1, the two neutral nitropropanes behave similarly with $^{\bullet}OH$ or $^{\bullet}OH + O_2$. As one might expect for aliphatic compounds, a rapidly decaying alkyl radical is formed with $^{\bullet}OH$ alone, while under peroxidizing conditions ($^{\bullet}OH + O_2$), slow buildup of absorption peaks around 340–350 nm for 2-NP and 320–330 nm for 1-NP are seen (insert of Figure 1)—both with first-order rate constants of about 200 s^{-1}—which may be attributed to formation of peroxyl radical species. In contrast, P2-N shows no shift in the maximum wavelength in the presence of O_2 and rather similar transient spectra in both systems.

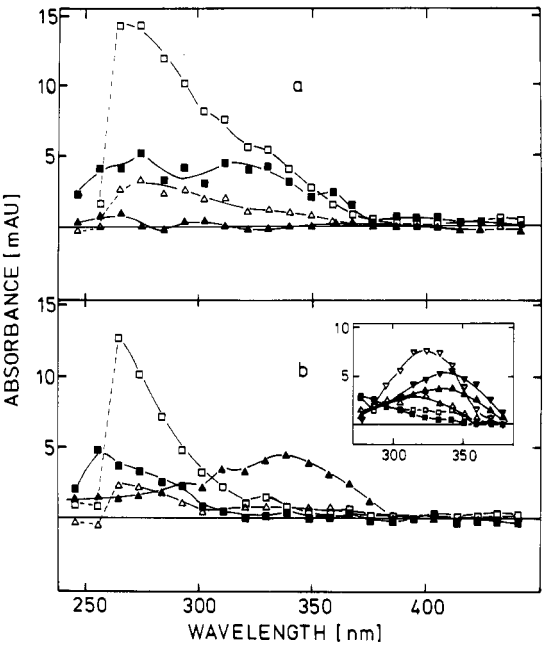


Figure 1. Dose-normalized transient spectra of 1-nitropropane, 2-nitropropane, and propane-2-nitronate. (a) After reaction of $^{\bullet}OH$ radicals with 2-NP (solid symbols) and P2-N (open symbols); squares 17.5 μs and triangles 95.6 ms after the pulse (N_2O-saturated solutions, 0.1 M phosphate buffer). [2-NP] = 15 mM, pH = 7.3, average pulse dose = 28 Gy; [P2-N] = 10 mM, pH = 7.2, pulse dose = 33 Gy. (b) After reaction of $^{\bullet}OH + O_2$ with 2-NP (solid symbols) and P2-N (open symbols; same observation periods as above; solutions saturated with N_2O/O_2 mixture, 0.1 M phosphate buffer). [2-NP] = 5 mM, pH = 7.3 (N_2O/O_2 = 2:1), pulse dose = 27 Gy; [P2-N] = 10 mM, pH = 7.2 (N_2O/O_2 = 1:1), pulse dose = 32.4 Gy. Insert: Transient spectra of 1-NP (open symbols) and 2-NP (solid symbols) under peroxidizing conditions (N_2O/O_2 mixing ratio of 1:1), [1-NP] = 10 mM, pH = 7, average pulse dose = 35 Gy; [2-NP] = 10 mM, pH = 7.3, pulse dose = 55 Gy; observation times: (■) 28 ms, (▲) 56 ms, (▼) 462 ms.

Table I. Rate Constants for the Reactions of •OH and $O_2^{\bullet-}$ with Nitropropanes and Propane-2-nitronate

	rate constant ($dm^3\ mol^{-1}\ s^{-1}$)		
radical	1-NP	2-NP	P2-N
•OH	2.5×10^8	0.8×10^8	25×10^8
$O_2^{\bullet-}$	nd	0.1×10^2	7×10^2

[a] Reactions of •OH with 1-NP and 2-NP were determined by competition with *p*-NDA ($k_{\cdot OH} = 1.25 \times 10^{10}$ $dm^3\ mol^{-1}\ s^{-1}$; ref 25, no. 603); with P2-N by competition with tert-butyl alcohol ($k_{\cdot OH} = 6.0 \times 10^8$ $dm^3\ mol^{-1}\ s^{-1}$; ref 25, no. 954). Rate constants for $O_2^{\bullet-}$ were determined from computer simulation of $O_2^{\bullet-}$ decay with superimposed pseudo-first-order reaction with substrates; values are upper limits.

In Table I the rate constants for the reactions with •OH and $O_2^{\bullet-}$ are compiled. The values for •OH were determined by competition kinetics, using *p*-NDA for the neutral nitropropanes and *tert*-butyl alcohol for P2-N (*26*). $O_2^{\bullet-}$ decayed predominantly by pH-dependent spontaneous dismutation. To obtain the second-order rate constants with the substrates, we used computer simulation to discriminate the slight deviation from the theoretical dismutation rate (*27*). Clearly, P2-N is the most susceptible substrate with respect to both oxygen radicals.

EPR Spectra after Spin Trapping. To obtain more detailed information on the structures of the individual radicals, we employed EPR spectroscopy. Direct observation after in situ generation with HRP/H_2O_2 (*16*) proved unsuccessful, and the spin traps DMPO and MNP gave mostly unspecific degradation artifacts. In contrast, DBNBS produced spin adducts whose signals could be interpreted unequivocally. This was achieved both by comparing the spectra of the individual alkyl radicals formed from the nitropropane isomers and by using the ability of DBNBS to trap primary, secondary, and tertiary alkyl radicals with different efficiencies depending on the pH (*28*, *29*). Unfortunately, DBNBS does not form stable spin adducts with O-centered radicals (*29*); thus the formation of peroxyl radicals could not be proven by this method [the structures for each postulated radical in Figures 2–4 and the hyperfine splitting constants (hfsc) are given in Table II, with the radicals listed in italic letters (*a*–*e*)].

As shown in Figure 2, 1-NP yields both primary (*a*, spectrum 3) and secondary (*b*, spectrum 4) alkyl radicals after reaction with •OH radicals. Analyzing the EPR spectra for 2-NP at different pHs, we could distinguish between two radical adducts, which may coexist simultaneously (Figure 3). The experimental spectrum (1), which was obtained with 2-NP at pH 2 in N_2O-saturated solution, could best be simulated (spectrum 2) by superimposing the adduct spectra of a primary *c* (spectrum 4) and a secondary *d* (spectrum 6) alkyl radical. Verification of radical *c* comes from spin trapping experiments at pH 7 (spectrum 3), at which pH DBNBS is only capable of trapping primary alkyl radicals (*29*). In turn, radical *d* is the only species observed at pH 2 (spectrum 5), when O_2 is added to the N_2O solution. The latter radical is quite interesting, as it could only be assigned to a secondary alkyl radical evidently devoid of a NO_2 moiety.

With P2-N, •OH attack produced adduct spectra (Figure 4), which were also obtained with 2-NP in alkaline solutions and from the model compound 2-bromo-2-nitropropane by direct UV photolysis in alkaline solution, obviously after elimination of bromine. We identified spectrum 1 from its similarity with the simulated spectrum 2 as a superposition of a primary alkyl radical adduct (*e*, spectrum 3) and a secondary alkyl radical (*f*, spectrum 4). The radicals derived from P2-N could also be generated with HRP/H_2O_2, in contrast to 2-NP where radicals could not be trapped after the enzymatic reaction—in line with the repeatedly observed preference of P2-N as the biological substrate (*11*, *16*, *17*).

Detection and Reactivity of the NO_2 Radical. Spin trapping of the secondary alkyl radical lacking the NO_2 group (radical *d*) led us to search for methods to detect the formation of $NO_2^{\bullet}$ radicals in this system. Spin trapping with DMPO after radiolytic generation of $NO_2^{\bullet}$ produced only oxidized "DMPOX" (data not shown), DBNBS did not react at all, and use of the spin label hydroxy-TEMPO as described by Shugalei and Tselinsky (*30*) proved futile under our experimental conditions.

In contrast, screening of nitroaromatic compounds which might conceivably form dinitro compounds after reaction with $NO_2^{\bullet}$ with a concomitant change of the absorption spectrum was successful in the case of nitroguaiacol (NG). As shown in Figure 5, •OH radicals attack NG with a rate constant approaching 10^{10} $dm^3\ mol^{-1}\ s^{-1}$ and produce a transient spectrum distinct from that observed with $NO_2^{\bullet}$, generated by •OH oxidation of sodium nitrite. The slower buildup at 365 nm occurs with a second-order rate constant of 1.75×10^6 $dm^3\ mol^{-1}\ s^{-1}$ and is taken as the attack of $NO_2^{\bullet}$ at NG. The value was obtained from the plot of the concentration dependence of the pseudo-first-order buildup kinetics (Figure 6, right).

The peak of NG at 365 nm was monitored, when 2-NP was oxidized in the presence of NG. The absorption increase indicative of $NO_2^{\bullet}$ formation, however, was only marginal as this absorption change was superimposed on that of the other substrates present (data not shown).

We then determined the reactivity of $NO_2^{\bullet}$ with nucleic acid components in pulse-radiolytic experiments. Since we could not observe distinct transient spectra after reaction of $NO_2^{\bullet}$ with the nucleic acid components, we used the strong absorption of the flavonoid kaempferol aroxyl radical (*31*) for competition experiments. In contrast to NG, kaempferol behaves similarly to other phenols (*24*) and forms only the respective phenoxyl radical ($k = 3.4 \times 10^8$ $dm^3\ mol^{-1}\ s^{-1}$) upon oxidation by $NO_2^{\bullet}$ (Figure 6, left). The results of the competition experiments are compiled in Table III, demonstrating a generally high reactivity of $NO_2^{\bullet}$ from 0.75×10^6 to 2.95×10^6 $dm^3\ mol^{-1}\ s^{-1}$ with the individual DNA and RNA components. Except for the pair guanosine monophosphate/deoxyguanosine monophosphate (GMP/dGMP), with a ratio for the rate constants of close to 4, the rate constants for the other nucleosides and deoxynucleosides are very similar.

Discussion

In view of the specificity, by which individual radicals can be generated by radiolytic methods (*23*) and the possibility of following their generation and decay by rapid kinetic spectroscopy, the pulse-radiolytic approach offers an appropriate means to study the chemistry of radical reactions occurring in biological systems. The combination with EPR spectroscopy and spin trapping furthermore allows structural identification of intermediary radicals, provided they form a stable adduct with the spin trapping

Table II. Hyperfine Splitting Constants of Nitropropane-Derived Radical Adducts with DBNBS[a]

substrate	radical	hfsc (G) a_N	hfsc (G) $a_{H\alpha}$	hfsc (G) $a_{H(Ph)}$	pH	spectrum
1-NP	(*a*) $^{\bullet}CH_2CH_2CH_2NO_2$	14.2	12.1/2	*	7.0	Figure 2 (3)
	(*b*) $CH_3{}^{\bullet}CHCH_2NO_2$	13.8	9.9	*	7.0	Figure 2 (4)
2-NP	(*c*) $^{\bullet}CH_2CH(NO_2)CH_3$	13.8	11.6/2	0.8/2	2.2	Figure 3 (4)
	(*d*) $CH_3{}^{\bullet}CH\text{-}CH_3$	14.0	9.3	0.6/2	2.2	Figure 3 (6)
	(*f*)[b] $CH_3{}^{\bullet}C(NO_2)CH_3$	9.2[c]			11.0	Figure 4 (4)
P2-N	(*e*) $^{\bullet}CH_2C(=NO_2^-)CH_3$	14.2	9.8/2	0.8/2	7.0	Figure 4 (3)
	(*f*)[b] $CH_3{}^{\bullet}C(NO_2)CH_3$	9.2[c]			7.0	Figure 4 (4)

[a] Hα describes the aliphatic hydrogens of the nitropropane adducts, and H(Ph), the two aromatic H atoms in the meta position of the spin trap; they were not consistently properly resolved (*). Radicals *e* and *f* were observed with P2-N both after pulse radiolysis and reaction with HRP/H_2O_2 (with slightly different hfsc); they also occurred with irradiated 2-NP and after UV photolysis of 2-bromo-2-nitropropane in alkaline solution. [b] Most likely structure of radical *f* (see text). [c] A second small N coupling of ~1 G gives the best simulation (see text).

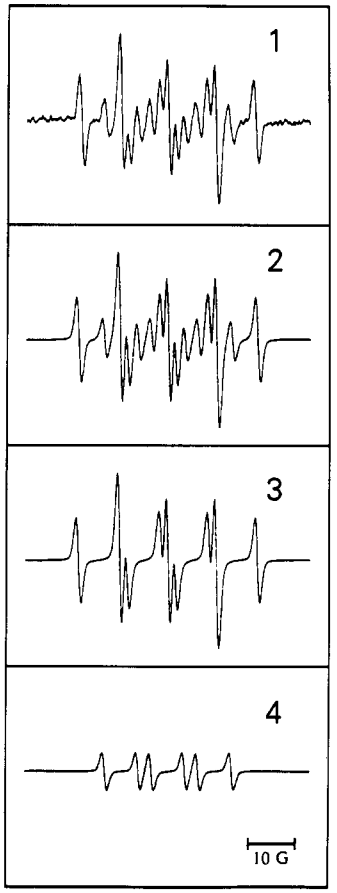


Figure 2. EPR spectra of spin adducts of 1-nitropropane-derived radicals with DBNBS. (1) Experimental spectrum after $^{\bullet}OH$ attack at pH 7 (pulse dose 60 Gy); (2) simulated composite spectrum; (3) simulation of spectrum of primary alkyl radical *a*; (4) simulated spectrum of secondary alkyl radical *b*. Experimental conditions for each spin trapping experiment: concentration of nitropropane substrates 10 mM, of DBNBS 0.625 mM; pH 2.2 adjusted with $HClO_4$, pH 7 with phosphate buffer (5–15 mM); $^{\bullet}OH$ attack in N_2O-saturated solution, $^{\bullet}OH + O_2$ with N_2O/O_2 (1:1) gas mixture.

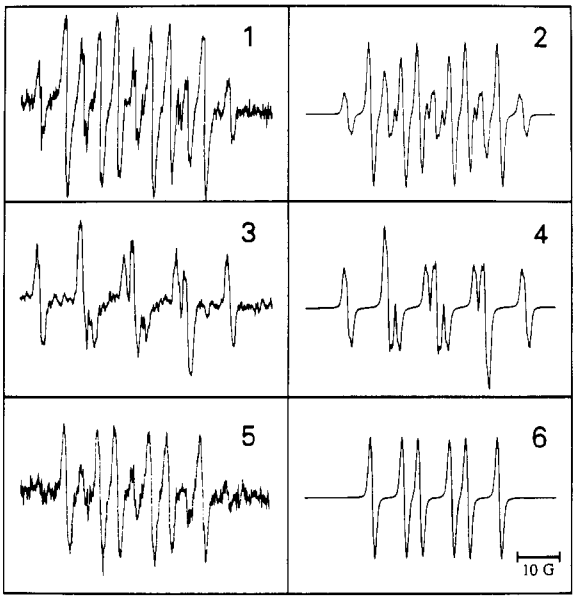


Figure 3. EPR spectra of spin adducts of 2-nitropropane-derived radicals with DBNBS. (1) Experimental spectrum after $^{\bullet}OH$ attack at pH 2.2 (pulse dose 53 Gy); (2) simulated spectrum after $^{\bullet}OH$ reaction at pH 2.2; (3) experimental spectrum after $^{\bullet}OH$ attack at pH 7, consisting exclusively of *c* (pulse dose 58 Gy); (4) simulation of radical *c*; (5) experimental spectrum after $^{\bullet}OH + O_2$ reaction at pH 2.2 (pulse dose 53 Gy), mostly consisting of radical *d*; (6) simulation of radical *d*. Experimental conditions: see Figure 2.

agent. It has to be kept in mind, however, that spin trapping is only a qualitative method and, lacking rate constants of the trapping reaction and the decay reaction of the spin adduct, it does not provide an answer to the absolute yield of each trapped radical. Unfortunately, such rate constants are difficult to determine and only few examples exist for DBNBS (*29*).

Comparing the experimental/simulated spectra in Figures 2–4 and the structures and EPR parameters in Table II for the various primary (*a*, *c*, *e*) and secondary (*b*, *d*, *f*) alkyl radicals, the resolutions of such minor structural differences by the spin trap DBNBS are quite striking. In particular, they appear to reflect in the series *a*–*c*–*e* an increasing influence of the nitro group present in γ- and β-position and, finally, as an ionized β-NO_2^- group. The similarity of spectra 4 in Figure 2 and 6 in Figure 3 (*b*, *d*) gives credence to our conclusion that *d* represents a 2-NP-derived secondary radical devoid of the nitro group. As spectrum 5 in Figure 3 was observed in acidic solution saturated with a N_2O/O_2 gas mixture, it seems likely that the *primary* alkyl radical forms a peroxyl radical much more readily as this species cannot be trapped by DBNBS (*29*). The strong deviation of $a_{H\alpha}$ of radical *e* as compared to that of the other primary alkyl radicals *a*, *c* and the line broadening in spectrum 4 of Figure 4 (*f*) were taken as evidence that both radicals (*e*, *f*) still contain the NO_2 group, the α-N contributing to the spin density distribution. The alternative explanation of spectrum 4 in Figure 4 constituting a poorly resolved triplet of triplets and representing an artifactual one-electron oxidation of DBNBS in alkaline solution (*32*) is inconsistent with our data: this signal was observed by us as well with a_N = 12.7G and $a_{H(Ph)}$ = 0.6/2 (*29*). Both this signal and the other broad three-line artifact (a_N ~12 G; *32*, *33*) are quite different to those found for radical *f* (Table II). The fact

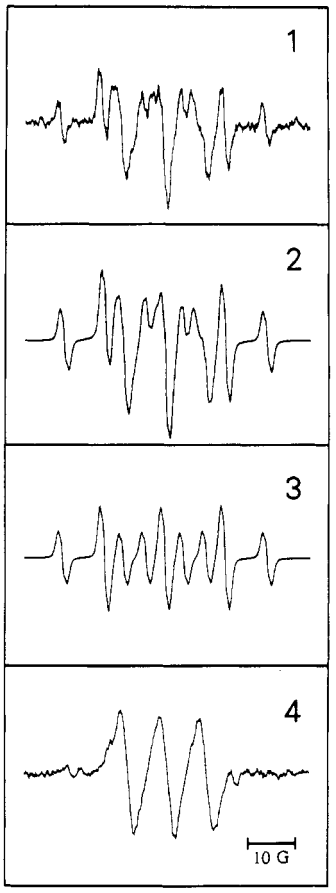


Figure 4. EPR spectra of spin adducts of propane-2-nitronate-derived radicals with DBNBS. (1) Experimental spectrum after $^{\bullet}OH + O_2$ reaction at pH 7 (pulse dose 32 Gy); (2) simulation of spectrum 1; (3) simulation of radical *e*; (4) exclusive observation of radical *f* after $^{\bullet}OH$ attack (pulse dose 60 Gy). *e* (spectrum 3) and *f* (spectrum 4) together represent the composite spectra 1 and 2. Experimental conditions: see Figure 2.

that this spectrum after 20 min reverts to the one observed with P2-N in N_2O/O_2-saturated solutions (spectrum 1, Figure 4), which is a composite of spectra 3 and 4 (radicals *e* + *f*), also favors our interpretation.

Several earlier studies on the oxidation of 2-NP already invoked radical intermediates (*16*, *17*, *34*), although none of them was directly observed. In contrast, the pulse-radiolysis and EPR/spin trapping studies reported here enable us to propose a mechanism (outlined in Scheme I) for the oxidative degradation of 2-NP and P2-N based on observed intermediates. It must be emphasized that Scheme I reflects processes initiated by the attack of $^{\bullet}OH$ radicals which are not necessarily precursors in biological systems. Yet, a one-electron oxidation of P2-N to the corresponding radical was suggested to be the initial step in the oxidation of 2-NP in cells (*16*, *17*):

$$C(CH_3)_2NO_2^- \rightarrow {}^{\bullet}C(CH_3)_2NO_2 \qquad (9)$$

This reaction has been shown to occur with HRP/H_2O_2 (*16*), but may also be catalyzed by other enzymes, such as certain cytochrome P450-dependent monooxygenases (*8*, *11*, *12*). A radical with this structure has most probably been trapped by DBNBS with P2-N and with both 2-NP and 2-bromo-2-nitropropane in alkaline solution (*f*). One other secondary alkyl radical derived from 2-NP, radical *d* devoid of the NO_2 group, was observed in oxygenated acidic solution (spectrum 5, Figure 3). Some secondary alkyl radicals, e.g., those derived from ethanol or 2-propanol, can only be trapped by DBNBS in acidic solution (*29*), yet are the dominant species after $^{\bullet}OH$ attack (*35*). Thus, while radical *d* could be both a product of radical attack or acid hydrolysis in the spin trapping reaction, the latter possibility is considered less likely.

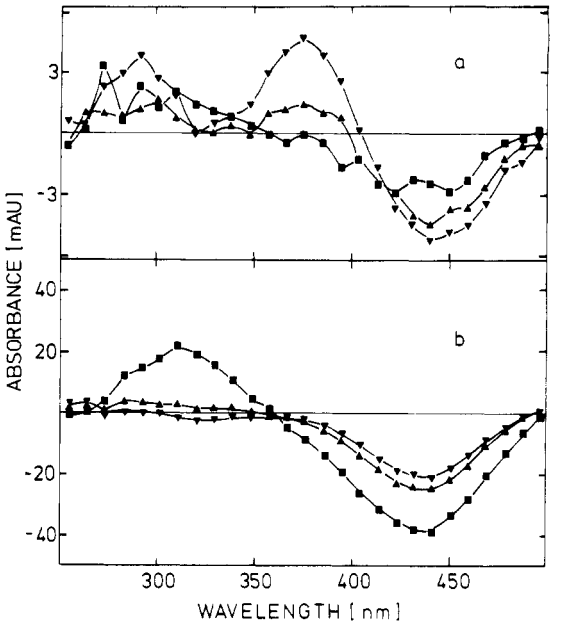


Figure 5. Dose-normalized transient spectra of nitroguaiacol. (a) After reaction with $^{\bullet}OH$ radicals ([NG] = 45 μM, pH 7 (5 mM phosphate buffer), N_2O-saturated solutions, pulse dose = 24 Gy); observation times: (■) 17.5 μs, (▲) 200 μs, (▼) 95.6 ms. (b) After reaction with $NO_2^{\bullet}$ radicals ([NO_2^-] = 0.76 mM, [NG] = 45 μM, pH 7 (5 mM phosphate buffer), N_2O-saturated solution, pulse dose = 26.7 Gy); same observation times as above.

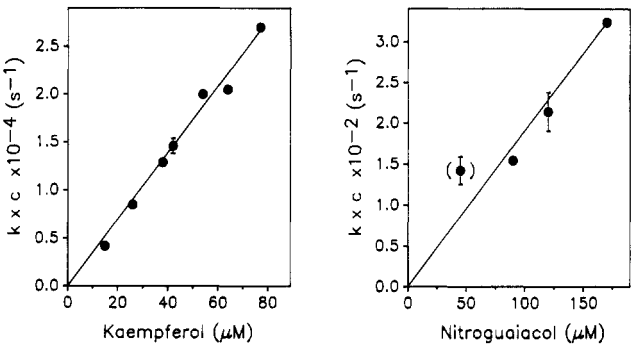


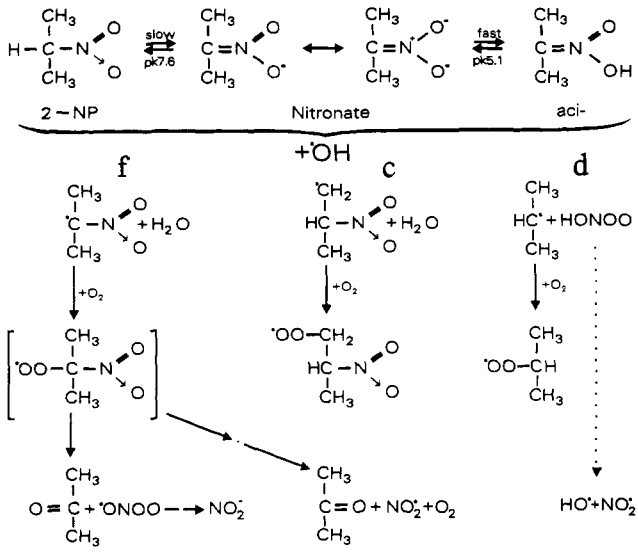
Figure 6. Determination of the rate constants of kaempferol and nitroguaiacol with $NO_2^{\bullet}$ radicals (pseudo-first-order plot). Kaempferol: kinetic evaluation of the buildup of the 550-nm absorption of the aroxyl radical, [NO_2^-] = 10 mM, pH = 8.5 (5 mM phosphate buffer). Nitroguaiacol: kinetic evaluation of the slow buildup of the 365–380-nm absorption, [NO_2^-] = 10 mM, pH = 8.5 (5 mM phosphate buffer).

Table III. Rate Constants for the Reaction of $NO_2^{\bullet}$ with Nucleosides and Nucleotides[a]

substrate	rate constant ($\times 10^6$ dm^3 mol^{-1} s^{-1})	substrate	rate constant ($\times 10^6$ dm^3 mol^{-1} s^{-1})
adenosine	1.05	deoxyadenosine	1.07
guanosine	1.95	deoxyguanosine	1.70
cytidine	0.95	deoxycytidine	1.50
uridine	1.43	thymidine	1.36
guanosine monophosphate	2.95	deoxyguanosine monophosphate	0.77

[a] All values were determined by competition with kaempferol ($k_{NO_2^{\bullet}}$ = 3.4 × 10^8 dm^3 mol^{-1} s^{-1}, see text).

Scheme I



The most likely fate of the alkyl radicals, both in pulse-radiolysis experiments and after initiation with HRP/H_2O_2 in the presence of oxygen—as is the case in the N_2O/O_2 mixtures and in the cellular environment—is conversion into peroxyl radicals. With this reaction, the nonenzymatic sequence of the metabolic degradation would become identical to the one taking place in radiolytic systems. Observation of peroxyl radicals was not possible in EPR experiments due to the failure of DBNBS to trap oxygen-centered radicals (*28*), and we have to refer to the pulse-radiolysis results. While it is possible that the transient absorption around 340–350 nm for 2-NP (and 320–330 nm for 1-NP) may be due to alkyl peroxyl radicals—assuming a strong bathochromic shift by the nitro group in β-position from the normal peroxyl radical absorption around 250 nm (*36*)—the fact that the buildup of the absorption follows first-order kinetics rather argues for an intramolecular rearrangement or degradation to some presently unknown species.

Kuo and Fridovich (*17*) did not consider peroxyl radical formation from P2-N-derived alkyl radicals a relevant reaction, contrary to the earlier mechanistic proposal of Porter and Bright (*16*). Superficially, the similarity of the transient spectra observed after radiolysis of P2-N with and without oxygen (Figure 1) supports the absence of peroxyl radical intermediates. We believe, however, that this is due to the highly unstable nature of the peroxyl radical derived from radical *f*. In pulse-radiolytic experiments nitrite was formed dose-dependently only under peroxidizing conditions (N_2O/O_2-saturated solutions; *37*). Since the yield exceeded the stoichiometric amount of primary radicals, participation of chain reaction(s) had to be assumed.

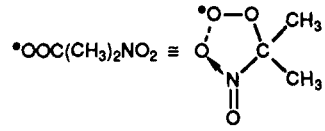
We propose that radical *f and* its corresponding peroxyl radical, $^{\cdot}OOC(CH_3)_2NO_2$, are formed both in radiolysis and metabolically (left route of Scheme I). The most common decay process of peroxyl radicals would be dimerization and immediate disintegration of the transient tetroxide into two alkoxyl radicals and oxygen, possibly in the singlet state (*38*, *39*):

$$2^{\cdot}OOC(CH_3)_2NO_2 \rightarrow 2^{\cdot}OC(CH_3)_2NO_2 + O_2 \quad (10)$$

This alkoxyl radical is probably rather unstable and decays into acetone and $NO_2^{\cdot}$ radicals:

$$^{\cdot}OC(CH_3)_2NO_2 \rightarrow O{=}C(CH_3)_2 + NO_2^{\cdot} \quad (11)$$

Owing to the close proximity of a peroxy and a nitro group at the same carbon atom, this peroxyl radical could also decay via a cyclic intermediate:



Such a first-order reaction—in addition to the regular bimolecular decay—could explain the proposed extreme instability of the peroxyl radical derived from radical *f*. The peroxonitrite radical formed in reaction 12 is a purely *hypothetical* intermediate postulated to occur during the slow autoxidation of NO (*40*), but it has recently been speculated that $^{\cdot}OONO$ may cause deamination of DNA and that it ought to be very reactive with nucleophiles (*40*). Its reaction with the most abundant nucleophile, water, would lead to nitrite and superoxide:

$$ONOO^{\cdot} + OH^- \rightarrow NO_2^- + O_2^{\cdot -} + H^+ \quad (13)$$

The formation of the latter species was neglected by Wink et al. (*40*). Whether $O_2^{\cdot -}$, formed in such a reaction sequence, may contribute to the chain reactions leading to a higher than stoichiometric yield of nitrite (*17*, *37*) remains open to debate as the rate constant for the reaction of $O_2^{\cdot -}$ with P2-N as opposed to 2-NP is only marginally higher (Table I).

Aside from the metabolic end products acetone and nitrite, reactive intermediates capable of reacting with DNA in intact cells must also be formed. For this to take place, we have to stipulate certain conditions: (a) the active agent must be neutral and stable enough to penetrate the nuclear membrane; (b) the species can be formed not only from 2-nitropropane but also from other secondary nitroalkanes, as treatment of rats with different secondary nitroalkanes results in identical patterns of DNA and RNA products (*6*); (c) the species must have oxidizing rather than reducing capacity to explain the formation of 8-hydroxyguanine in hepatic DNA and RNA from 2-NP-treated rats (*3–6*). Consequently, the proposed alkyl, alkoxyl, and peroxyl radical intermediates, which are likely to participate in the observed radical chain processes [as suggested by the higher than stoichiometric yield of nitrite (*37*)], are unlikely genotoxic candidates as they are too unstable, too reactive, and—in the case of the alkyl radicals—reducing instead of oxidizing species. The hypothetical peroxonitrite radical, $^{\cdot}OONO$, is another poor candidate as deamination products (*40*) have *not* been observed in liver RNA or DNA from 2-NP-treated rats (*3–6*).

We believe that the $NO_2^{\cdot}$ radical is the most likely species to conform to the above preconditions. Aside from its postulated formation in reaction 11, we have to consider the spin trapping evidence of a secondary alkyl radical lacking the NO_2 group (*d*). This alternative route could also be envisaged by an attack of $^{\cdot}OH$ at 2-NP:

$$CH(CH_3)_2NO_2 + {}^{\cdot}OH \rightarrow {}^{\cdot}CH(CH_3)_2 + HONOO \quad (14)$$

Whether this hypothetical peroxynitrous acid rearranges intramolecularly to nitric acid (HNO_3) or fragments to

•OH and NO_2• as suggested by Beckman and co-workers in a different context (*41*) is presently impossible to ascertain.

Corroboration of the generation of NO_2• from P2-N will have to await future methodological developments (*42*). use of the aci-anion of nitromethane to trap NO_2• (*43*) would probably prove futile since we observed radical *d* only in acidic solutions and the conditions for the trapping reactions require very high pH and very high nitromethane concentrations (*44*).

Nevertheless, the evidence for the formation of NO_2• radicals from P2-N and 2-NP, in combination with the rate constants determined from the reaction of NO_2• with the various nucleosides and nucleotides (Table III), argues for NO_2• as the ultimate DNA-damaging species derived from 2-NP in rat liver. Supportive evidence comes from the work of Fiala and co-workers (*3–6*) who, using HPLC with electrochemical detection, observed increased levels of 8-hydroxyguanine (8-OH-Gua) in both DNA and RNA following exposure of rats to 2-NP. Formation of 8-OH-Gua was attributed to the generation of oxygen radicals by 2-NP. However, alternative pathways also appear feasible. Kohda et al. showed that 8-OH-Gua is produced during treatment of guanosine with 2,4-dinitrophenoxyamine (*45*) or 4-nitroquinoline 1-oxide (*46*) and presented evidence that it was the result of a hydrolytic rearrangement of 7-aminoguanosine formed initially. It appears possible that a similar mechanism may yield 8-OH-Gua following attack of NO_2• on the N_7 of guanine. Metabolic formation of NO_2• would also be in line with the occurrence of 8-aminoguanosine in hepatic DNA and RNA of 2-NP-treated animals (*7*) and the observation of identical patterns of DNA and RNA modifications in rat liver after treatment of the animals with other secondary nitroalkanes (*6*).

To our knowledge, the present results provide the first direct evidence for a reactivity of NO_2• radicals with deoxyribonucleosides and ribonucleosides. Our finding that the rate constants for the reactions with the individual nucleic acid components are quite similar is no contradiction to the observation of only two DNA and three RNA modifications in the livers of 2-NP-treated rats (*3–6*) as the reactivity of nucleosides in aqueous solution with NO_2• is likely to be different from their reactivity in double-stranded DNA or in RNA molecules. Moreover, it cannot be excluded that additional modifications are formed following attack of NO_2• which are electrochemically *in*active and which have therefore escaped detection by the presently favored analytical method, i.e., HPLC with electrochemical detection.

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