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Release of Nitric Oxide Together with Carbon-centered Radicals from N-nitrosamines by Ultraviolet Light Irradiation

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Solutions of *N*-nitrosamines, *N*-nitrosodimethylamine, *N*-nitrosodiethylamine, *N*-nitrosomorpholine and *N*-nitrosopyrrolidine in phosphate buffer (pH 7.4) were irradiated by ultraviolet (UV) light at room temperature. The *N*-nitrosamines were extensively degraded due to irradiation for 120 min in a time-dependent fashion as monitored by UV-absorption or high performance liquid chromatographic analysis. Carbon-centered radicals were generated from four *N*-nitrosamines during the short time irradiation of 10–60 s as monitored by electron spin resonance (ESR) technique using 5,5-dimethyl-1-pyrroline *N*-oxide and *N-tert*-butyl-α-phenylnitrone as spin traps. Nitric oxide (NO) was generated during the short time irradiation as monitored by ESR technique using cysteine–Fe(II) complex, *N*-methyl-D-glucamine dithiocarbamate and 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide. Significant amounts of nitrite (4–16%) from four *N*-nitrosamines and also a significant amount of nitrate (4%) was produced from *N*-nitrosodimethylamine during the irradiation time of 120 min. Released NO from the *N*-nitrosamines must be converted into nitrite through intermediary reactive nitrogen oxide species including nitrogen dioxide and dinitrogen trioxide in contact with dissolved oxygen.

Keywords: Carbon-centered radical; Nitric oxide; *N*-nitrosamine; Nitrite; Ultraviolet irradiation

INTRODUCTION

N-nitrosamines are present in the workplace, processed meats and cigarette smoke, and are produced in the stomach by reaction of secondary amines and nitrite (NO_2^-) both taken from foods. Production of *N*-nitrosamines in the stomach has been considered to be a consequence of the reaction of dinitrogen trioxide (N_2O_3) generated from NO_2^- under acidic

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conditions.[1] On the other hand, nitric oxide (NO), an important mediator of biological functions, generated by the action of nitric oxide synthases,[2,3] readily transforms into reactive nitrogen oxide species (RNS) including nitrogen dioxide (NO_2) and N_2O_3 in aqueous solutions in the presence of dissolved oxygen,[4,5] which can nitrosate secondary amines even under neutral conditions.[6] Hence, *N*-nitrosamines may be chemically stable pools of RNS. *N*-nitrosamines are well-known carcinogenic substances that require metabolic activation before they can react with DNA to cause mutation and cancer. Irradiation of *N*-nitrosamines by ultraviolet (UV) light in a dilute aqueous solution and in the presence of phosphate ion results in the production of direct acting mutagens as detected by the Ames *Salmonella* tester strains TA100.[7,8] Treatment of DNA with *N*-nitrosomorpholine (NMOR) under UV light results in the formation of 8-oxo-deoxyguanosine,[9] and with *N*-nitrosodimethylamine (NDMA)/*N*-nitrosodiethylamine (NDEA) under UV light or natural sunlight produced O^6-methylguanine, N^7-methylguanine and 8-oxo-deoxyguanosine.[10,11] The mutation caused by the *N*-nitrosamines under UV light in the phosphate buffer was inhibited in the presence of spin trapping agents suggesting that certain carbon-centered radicals were generated.[12]

The aim of the present study was to clarify whether NO together with carbon centered radicals is released from *N*-nitrosamines in phosphate buffer under UV light, in order to find the possibilities that *N*-nitrosamines donate NO when exposed to UV light. If NO is released from *N*-nitrosamines, it may be converted into RNS causing deteriorative effects on biological molecules. It was found in the present study that *N*-nitrosamines readily released NO in phosphate buffer under UV light, which may be converted finally into nitrite through NO_2 and N_2O_3 by autooxidation in contact with dissolved oxygen.

MATERIALS AND METHODS

Chemicals

N-nitrosodimethylamine (NDMA) (purity: more than 99.5%) and *N*-nitrosodiethylamine (NDEA) were obtained from Wako Pure Chemical Industries, Osaka, Japan. *N*-nitrosomorpholine (NMOR) and *N*-nitrosopyrrolidine (NPYR) were obtained from Sigma Chemical Company, St. Louis, MO, USA. 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO) and *N-tert*-butyl-α-phenylnitrone (PBN) were obtained from Labotec, Tokyo, Japan and Aldrich Chemical Company, Milwaukee, WI, USA, respectively. 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (c-PTIO) was obtained from Dojindo Laboratories, Kumamoto, Japan. *N*-methyl-D-glucamine dithiocarbamate (MGD) sodium salt was prepared as reported previously[13] from *N*-methyl-D(−)-glucamine obtained from Wako and carbon disulfide. MGD–Fe(II) complex was prepared by dissolving MGD sodium salt and Fe(II) sulfate heptahydrate in 0.1 M phosphate buffer (pH 7.4). Nitrate reductase (from *Aspergillus* sp.) [EC: 1.6.6.2], flavin adenine dinucleotide (FAD), β-nicotinamide adenine dinucleotide phosphate reduced form (NADPH) tetrasodium salt, L-lactate dehydrogenase (LDH) [EC: 1.1.1.27] and pyruvic acid sodium salt were obtained from Sigma.

Ultraviolet (UV) Light Irradiation

For measurement of decomposition of *N*-nitrosamine or release of nitrite from *N*-nitrosamine, a quartz cuvette containing 10 mM *N*-nitrosamine solution in 0.1 M phosphate buffer (pH 7.4) was irradiated with a low intensity UV lamp model NY, Nikko Seiki Works (Tokyo, Japan) at a distance of 1–2 mm at room temperature for the indicated period. For ESR measurements, a quartz flat cell containing a solution of a mixture of 0.1 M *N*-nitrosamine and cysteine–Fe(II), MGD–Fe(II) or c-PTIO in the phosphate buffer

was irradiated similarly at room temperature for the indicated period.

Measurement of Decomposition of N-nitrosamines

Absorbance of a 10 mM NDMA or NDEA solution in 0.1 M phosphate buffer (pH 7.4) irradiated by UV light was measured: absorbance at 338 nm for NDMA and 332 nm for NDEA. High performance liquid chromatography (HPLC) of 10 mM NMOR or NPYR in 0.1 M phosphate buffer (pH 7.4) irradiated by UV light was performed using a Hitachi 655A-11 liquid chromatograph (Tokyo, Japan) equipped with a column (4.6 mm i.d.×250 mm) of Inertsil ODS-2 (GL Science, Tokyo, Japan) by using a mobile phase composed of 27 mM acetate/30 mM citrate buffer (pH 3.2)/5% acetonitrile at a flow rate of 1.0 ml/min. The peaks of NMOR and NPYR were detected at 340 nm with a Hitachi L-7420 UV–Vis. detector (Tokyo, Japan). NMOR and NPYR were eluted at retention times of 10.0 and 13.5 mm, respectively. The amount of NMOR or NPYR remaining in the solution was determined by comparing the peak area obtained from the sample solution with that from the standard solution of NMOR or NPYR in the phosphate buffer.

Electron Spin Resonance Studies

Electron spin resonance (ESR) spectrum was obtained on an X-band JES-RE1X spectrometer (JEOL, Tokyo, Japan) at room temperature with a quartz flat cell. Field setting was at 335.5 mT for DMPO, PBN and c-PTIO spin trapping in the presence of Mn^{2+} marker, or at 330.0 mT for cysteine–Fe(II) and MGD–Fe(II) spin trapping in the absence of Mn^{2+} marker. Scan range 10 mT, modulation frequency 100 kHz, microwave power 4 mW and modulation amplitude 0.1 mT.

Nitrite and Nitrate Determination

A 0.10 ml aliquot of 10 mM *N*-nitrosamine solution in 0.1 M phosphate buffer (pH 7.4) irradiated by UV light was made up to 10.0 ml with a Saltzman solution composed of 50 ml of glacial acetic acid, 50 mg of *N*-(1-naphthyl)ethylenediamine and 5 g of sulfanilic acid in 1 l of water, and the mixture was left at room temperature for 10 min. Nitrite standard solution (1.0 mM) was similarly treated. Absorbance at 545 nm of each of the mixtures was measured. Nitrite content in the sample solution was determined by comparing its absorbance with that of the nitrite standard solution. For determination of nitrite plus nitrate,[14] to a 1.0 ml aliquot of the solution in tube A, added 2.8 ml of water, 280 µl of 1 mM FAD solution in water, 560 µl of 10 mM NADPH in water and 200 µl of 6 U/ml nitrate reductase solution in water, and the mixture was incubated at 37°C for 30 min. In order to destroy NADPH that disturbs the pigment development, 50 µl of 5400 U/ml LDH solution in water and 560 µl of 0.1 M pyruvic acid solution in water were added to the mixture, and the mixture was further incubated at 37°C for 10 min. By this procedure, NO_3^- was quantitatively converted into NO_2^-. The solution was made up to 10.0 ml with a Saltzman solution, and the nitrite content was similarly determined.

RESULTS

Solutions of *N*-nitrosamines, NDMA, NDEA, NMOR and NPYR (Fig. 1), in phosphate buffer (pH 7.4) were irradiated by UV light at room temperature for 120 min, and the decomposition of the *N*-nitrosamines was monitored (Fig. 2). NDMA and NDEA at 10 mM concentrations were degraded by UV irradiation in a time-dependent fashion, as monitored by absorbance at 338 and 332 nm, respectively ((●) and (■)). While the absorbances of NMOR and NPYR at 340 nm were unchanged, NMOR

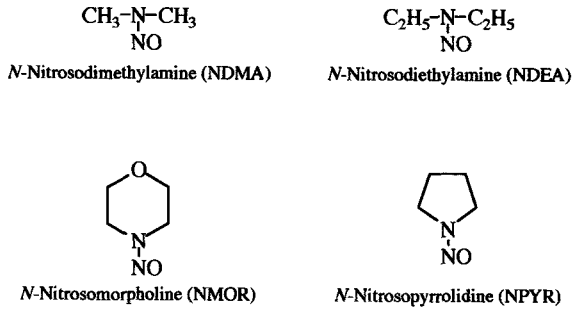

FIGURE 1 *N*-nitrosamines.

and NPYR were found to be degraded by UV irradiation in a time-dependent fashion, as monitored by HPLC analysis ((▲) and (◆)). UV-sensitivity of NDMA was the highest, and more than 95% of the *N*-nitrosamine was degraded in the 120 min irradiation. The order of the sensitivity was found to be NDMA > NDEA > NPYR > NMOR.

Generation of carbon-centered radicals from the *N*-nitrosamines during the photoirradiation was monitored by ESR technique using DMPO or PBN as spin traps,[15] which are useful for the spin trap of carbon-centered and oxygen-centered radicals. When NDMA was irradiated by UV light during the short time period of 10–60 s in the presence of DMPO, ESR signals as a doublet of triplets with hyperfine splitting constants (hfsc) of $a_N = 1.57$ mT and $a_H = 2.24$ mT appeared (Fig. 3A left panel). The signals were characteristic of those of the adducts of DMPO and carbon-centered radicals[16] and different from those of the adducts of DMPO and oxygen-centered radicals.[15] The intensity of the signals was increased in a time-dependent fashion. When NDMA was irradiated in the presence of another spin trapping agent PBN, ESR signals as a doublet of triplets with hfsc of $a_N = 1.57$ ml and $a_H = 0.58$ mT appeared (Fig. 3A right panel). The signals were characteristic of those of the adduct of PBN and carbon-centered radicals.[15,17–19] The a_N value was similar to, but the a_H value was larger than that of the adduct of PBN and oxygen-centered radicals.[15,20,21]

When NDEA was similarly treated in the presence of DMPO or PBN, six line ESR signals of DMPO adduct with hfsc of $a_N = 1.56$ mT and $a_H = 2.40$ ml, and six line ESR signals of PBN adduct with hfsc of $a_N = 1.57$ ml and $a_H = 0.47$ mT appeared (Fig. 3B), and these signals were ascribable to the adduct of DMPO or to the adduct of PBN with carbon-centered radicals, respectively. When NMOR was similarly treated in the presence of DMPO or PBN, no ESR signals were observable in the presence of DMPO (data not shown), but six line ESR signals of PBN adduct with hfsc of $a_N = 1.59$ mT and $a_H = 0.32$ ml (Fig. 3C left panel). These signals were not changed into those of the adduct of PBN with hydroxyethyl radical in the presence of ethanol (Fig. 3C right panel), indicating that the signals were not due to the adduct of PBN with hydroxyl radical. When NPYR was similarly treated in the presence of DMPO or PBN, six line ESR signals of the DMPO adduct with hfsc of $a_N = 1.55$ mT and $a_H = 2.29$ ml, and six line ESR signals of the PBN adduct with hfsc of $a_N = 1.57$ ml and $a_H = 0.48$ mT, characteristic of those of the adduct of DMPO or PBN with carbon-centered radicals, appeared (Fig. 3D). These results indicate that the *N*-nitrosamines released carbon-centered radicals in the short time UV irradiation. The intensities of the ESR signals of the DMPO and PBN adduct of the carbon-centered radicals from NDEA were the highest among those from other *N*-nitrosamines.

Generation of NO from the *N*-nitrosamines by the short time UV-irradiation was monitored by ESR technique using cysteine–Fe(II) complex,[22,23] MGD–Fe(II) complex[24,25] and c-PTIO.[26] When NDMA and NDEA in the phosphate buffer were irradiated by UV light in the presence of cysteine–Fe(II)–ascorbic acid according to the protocol previously reported,[22,23] 3 line ESR signals with hfsc of $a_N = 1.4$ mT appeared in the shorter irradiation for 30 s, which were in turn converted into single line ESR signal after irradiation for 60 s (Fig. 4A and B). The

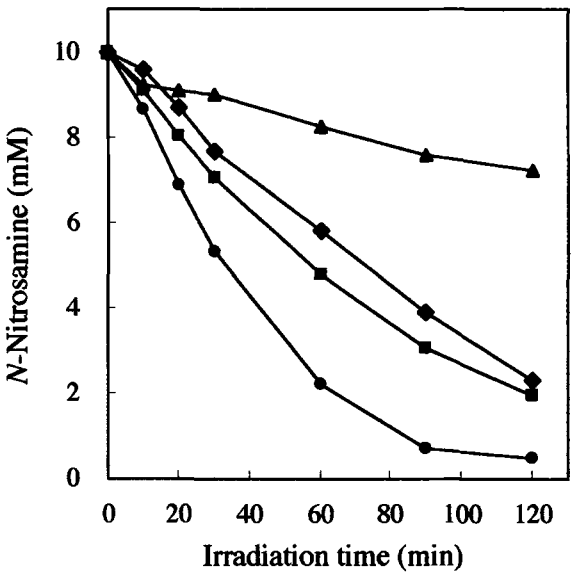

FIGURE 2 Time course of the decomposition of NDMA (●). NDEA (■), NMOR (▲) and NPYR (◆) by UV-irradiation. Each of the *N*-nitrosamines at 10 mM in 0.1 M phosphate buffer (pH 7.4) was irradiated with a low intensity UV lamp at room temperature. Decomposition of NDMA and NDEA was monitored by absorbance at 338 and 332 nm, respectively. Decomposition of NMOR and NPYR was monitored by HPLC analysis.

former signals were assignable to those due to a chelate type of complex of two cysteine groups and one NO with an iron atom, whereas the latter signal was assignable to that due to a chelate type of complex of two cysteine groups and two NO with an iron atom.[23] The results may indicate that UV-irradiation of NDMA and NDEA generated NO or nitrite because ascorbic acid in this system may have reduced a part of nitrite into NO.[22]

When NDMA, NDEA, NMOR and NPYR were irradiated by UV light in the presence of MGD–Fe(II), all the *N*-nitrosamines gave typical 3 line ESR signals of MGD–Fe(II)–NO spin adduct[24,25] with hfsc of $a_N = 1.29$ m in a time dependent fashion (Fig. 5). It is likely that NO was released from these *N*-nitrosamines by UV-irradiation, but the release of NO was not confident because NO_2 and nitrite can also produce the MGD–Fe(II)–NO adduct.[27]

It is known that nitroxyl nitroxide radical c-PTIO is reduced selectively by NO into nitroxyl radical c-PTI, and these radicals are discriminated by the ESR spectra: c-PTIO gives 5 line signals with hfsc $a_N^{1,3} = 0.82$ mT and c-PTI gives 7 line signals with hfsc $a_N^1 = 0.98$ and $a_N^3 = 0.44$ mT.[26] Formation of c-PTI in the mixtures of the *N*-nitrosamines with c-PTIO by UV-irradiation was examined (Fig. 6). It was found that all the *N*-nitrosamines produced c-PTI by the short time irradiation. Hence, all the *N*-nitrosamines generated NO by UV-irradiation.

Generation of nitrite and nitrate from the *N*-nitrosamines by UV-irradiation during the long time UV-irradiation was monitored (Fig. 7). Nitrite was generated in a time-dependent fashion in the solutions of NDMA, NDEA, NMOR and NPYR at 10 mM concentrations by UV-irradiation. The amount of nitrite generated in the 120 min UV-irradiated *N*-nitrosamine solutions was estimated to be about 4% for NDMA (●) and NMOR (▲), and about 16% for NDEA (■) and NPYR (◆). Nitrate was generated in a time-dependent fashion in the solution of NDMA (○). The amount of nitrate generated in the 120 min UV-irradiated NDMA solution was estimated to be 3.6%. The results indicate that the photodecomposition of the *N*-nitrosamines accompanied nitrite and nitrate generation. Nitrogen oxide species other than nitrite and nitrate must have been generated during the photodecomposition, because the amount of nitrite or nitrite plus nitrate in the photodegradation was much smaller than that of lost *N*-nitrosamines.

DISCUSSION

N-nitrosamines are well-known carcinogenic substances that require metabolic activation before they can react with DNA to cause mutation and cancer. The present study demonstrated the non-enzymatic complex degradation of the *N*-nitrosamines by UV-light exposure,

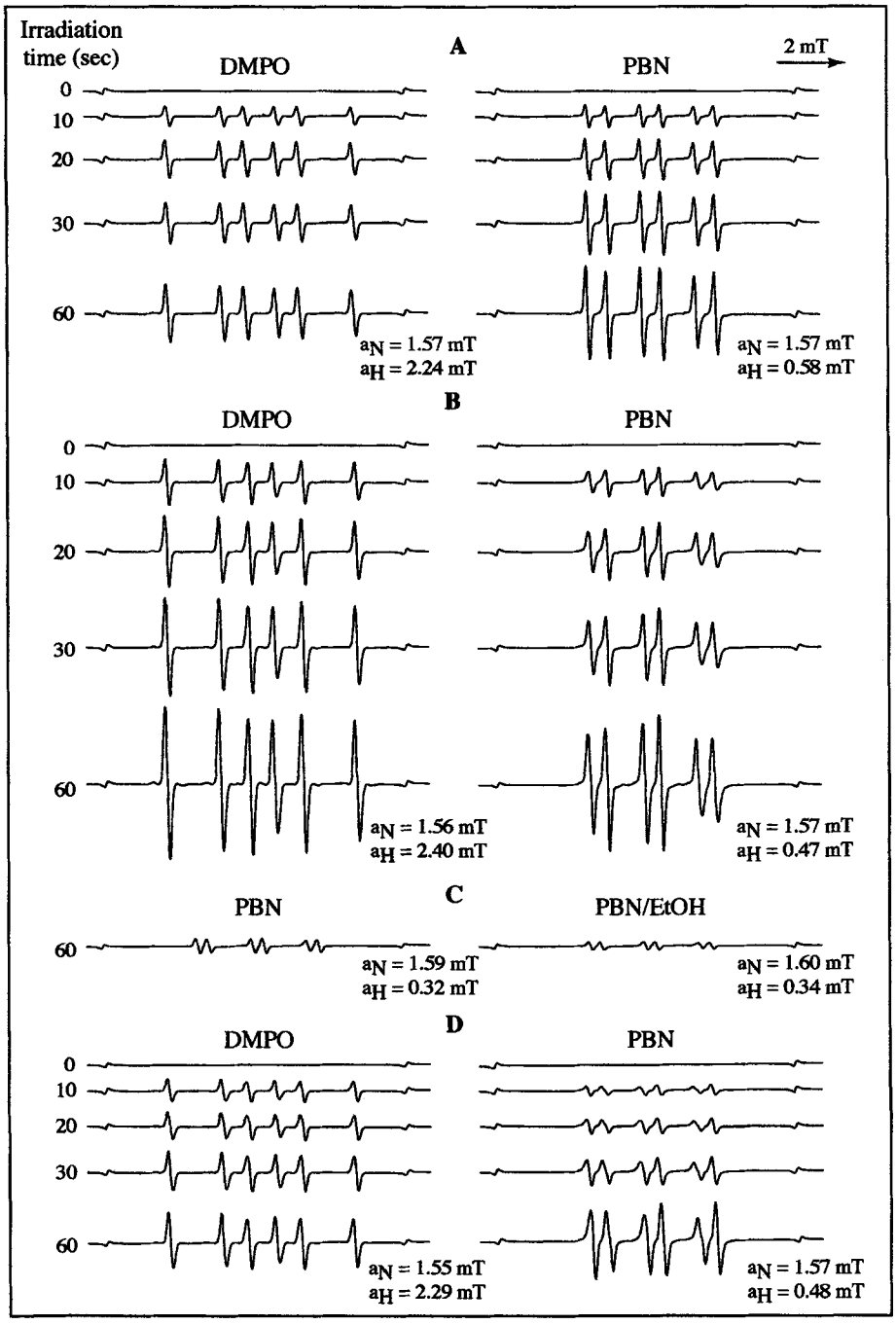

FIGURE 3 ESR spectra of the spin adducts formed by UV-irradiation of the mixtures of NDMA (A), NDEA (B), NMOR (C) or NPYR (D) with DMPO or PBN. A mixture of each of the *N*-nitrosamines at 0.1 M and 0.1 M DMPO or PBN in 0.1 M phosphate buffer (pH 7.4) was irradiated with a low intensity UV lamp at room temperature for the indicated period. ESR spectra were obtained at a receiver gain of 20. The signals appeared at the extremely right ($g = 2.034$) and left ($g = 1.981$) sides are those of Mn^{2+} marker.

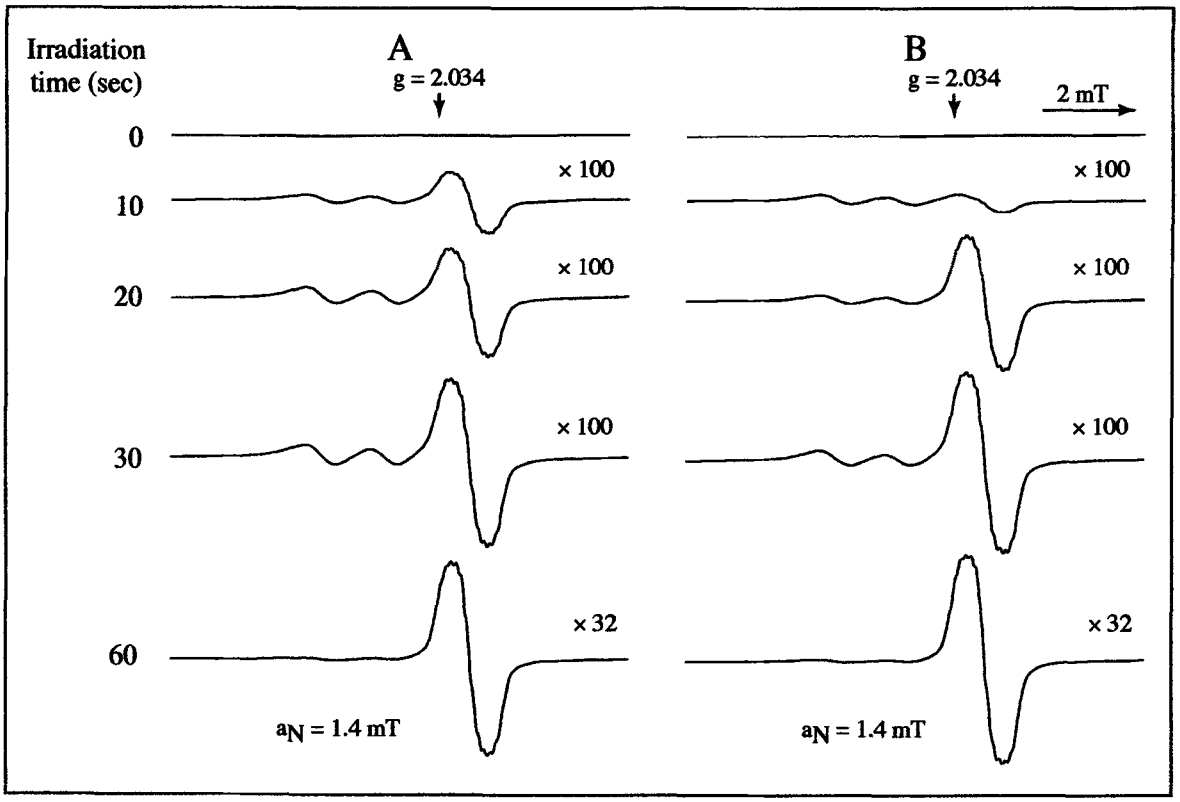

FIGURE 4 ESR spectra of the spin adducts formed by UV-irradiation of the mixtures of NDMA (A) or NDEA (B) with cysteine–Fe(II). A mixture of 0.1 M NDMA or NDEA, 30 mg/ml cysteine, 1 mM Fe(II) and 20 mM ascorbic acid in 0.1 M phosphate buffer (pH 7.4) was irradiated with a low intensity UV lamp at room temperature for the indicated period. Receiver gain of the ESR spectrum was indicated in the figure. The *g* value determined by Mn^{2+} was indicated.

accompanying release of carbon-centered radicals and NO. Generation of carbon-centered radicals from *N*-nitrosamines by UV-irradiation was confirmed by ESR technique using spin trapping agents DMPO and PBN. The result was consistent with the earlier observation showing that NDMA methylates guanine base moiety in DNA molecules by UV-irradiation.[10] Moreover, generation of carbon-centered radicals from NMOR by UV-irradiation was consistent with the earlier observations.[12] In the course of the known enzymatic activation of *N*-nitrosamines during metabolic activation, it has been shown that a *N*-nitrosamine undergoes enzymatic hydroxylation of one of two α-carbon atoms in the presence of oxygen, and subsequent non-enzymatic splitting into an aldehyde and an alkyldiazonium salt.[28] An alkyldiazonium salt is readily converted into a reactive alkyl cation by liberation of nitrogen molecules. Mechanisms for the non-enzymatic UV-induced liberation of the carbon-centered radicals from *N*-nitrosamines observed in the present study may be similar to those of the enzymatic activation. Thus, activation of one of the α-carbon atoms of the *N*-nitrosamines may be caused due to irradiation, which results in the formation of alkyldiazonium salts. The carbon-centered radicals may be generated from the alkyldiazonium salts. Because generation of the carbon-centered radicals from non-cyclic *N*-nitrosamines, NDMA and NDEA, was greater than that from cyclic *N*-nitrosamines, NMOR and NPYR (Fig. 4), the α-carbon atoms of the non-cyclic

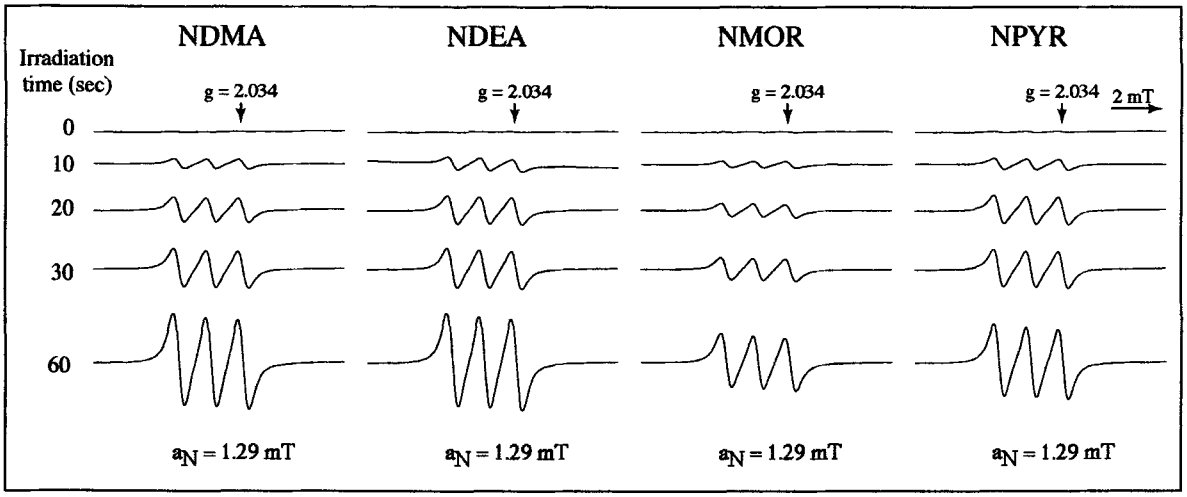

FIGURE 5 ESR spectra of the spin adducts formed by UV-irradiation of the mixtures of NDMA, NDEA, NMOR or NPYR, with MGD–Fe(II). A mixture of 0.1 M NDMA, NDEA, NMOR or NPYR, and 0.1 M MGD/0.02 M Fe(II) in 0.1 M phosphate buffer (pH 7.4) was irradiated with a low intensity UV lamp at room temperature for the indicated period. ESR spectra were obtained at a receiver gain of 100. The g value determined by Mn^{2+} was indicated.

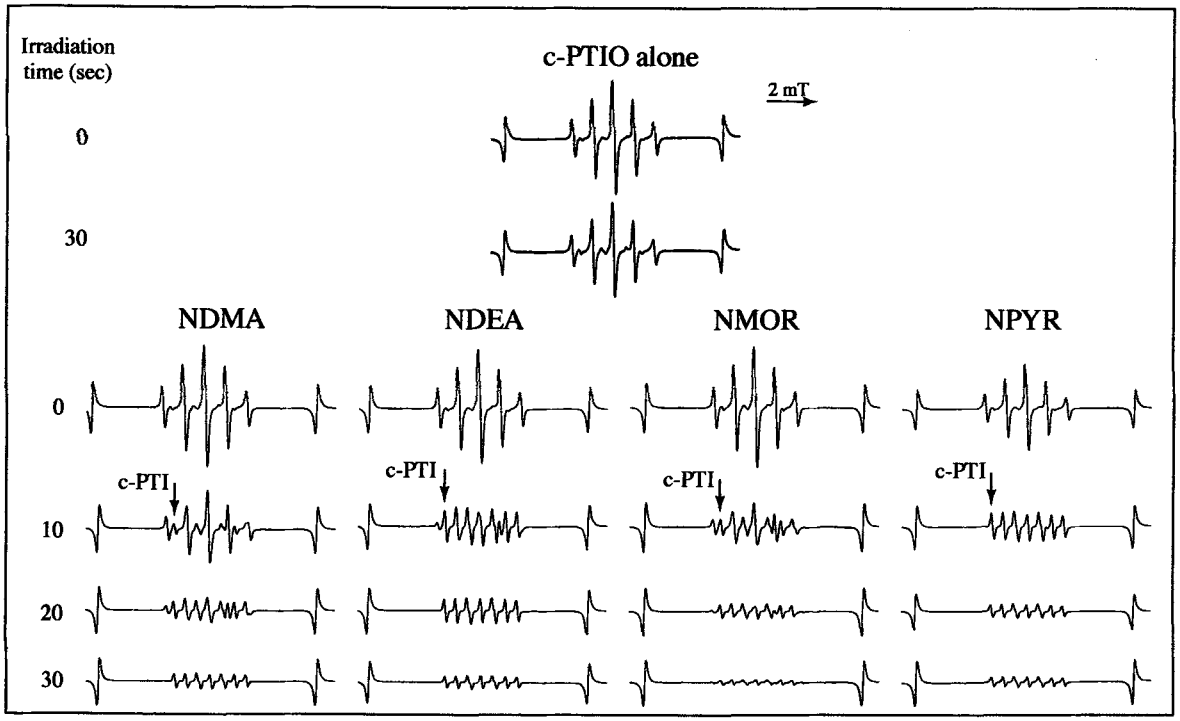

FIGURE 6 ESR spectra of the mixtures of NDMA, NDEA, NMOR or NPYR, and cPTIO irradiated by UV light. A mixture of 0.1 M NDMA, NDEA, NMOR or NPYR, and 10 mM c-PTIO in 0.1 M phosphate buffer (pH 7.4) was irradiated with a low intensity UV lamp at room temperature for the indicated period. ESR spectra were obtained at a receiver gain of 200. The signals appeared at the extremely right (g = 2.034) and left (g = 1.981) sides are those of Mn^{2+} marker. The left side non-overlapped signals of c-PTI are indicated by arrows.

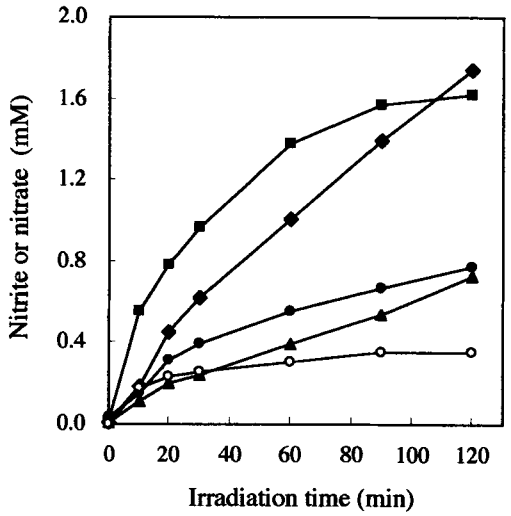

FIGURE 7 Time course of the formation of nitrite from NDMA (●). NDEA (■), NMOR (▲), NPYR (◆) and the formation of nitrate from NDMA (○) by UV irradiation. Each of the *N*-nitrosamines at 10 mM in 0.1 M phosphate buffer (pH 7.4) was irradiated with a low intensity UV lamp at room temperature.

N-nitrosamines may be more readily activated than that of the cyclic *N*-nitrosamines under the irradiation conditions. Carbon-centered radicals thus generated would modify guanine base moiety in DNA molecules as illustrated in Fig. 8.

An important finding in the present study was the release of NO from *N*-nitrosamines by UV-irradiation. Four *N*-nitrosamines examined equally liberated NO as assessed by ESR studies using cysteine–Fe(II), MGD–Fe(II) and c-PTIO. The mechanisms for NO liberation may be different from those for carbon-centered radical generation. Liberation of NO may proceed *via* the direct homolytic cleavage of N–N bond of the *N*-nitrosamine molecules. Formation of a significant amount of nitrite and nitrate in the photodegradation of the *N*-nitrosamines was observed, but the amounts of nitrite or nitrite plus nitrate did not exceed 20% of the *N*-nitrosamines. Nitrite and nitrate may be produced from released NO by reaction of dissolved oxygen.

NO is an important mediator of biological functions generated by the action of nitric oxide synthases.[2,3] However, it readily transforms into RNS including NO_2 and N_2O_3 in aqueous solutions in the presence of dissolved oxygen[4,5] and another RNS, peroxynitrite ($ONOO^-$), by reaction with superoxide which is in turn transformed into peroxynitrous acid (ONOOH) to be rapidly decomposed into the reactive NO_2-like and hydroxyl radical-like oxidants.[29,30] Formation of a significant amount of nitrite during the photodegradation of the *N*-nitrosamines indicates that NO generated was readily converted into nitrite through the formation of intermediary RNS including NO_2 and N_2O_3 by contact with dissolved oxygen.

It was found that *N*-nitrosamines donated NO and RNS, which would cause damages to DNA and protein molecules as illustrated in Fig. 8. The *in vitro* reaction of RNS with the components of DNA and proteins has been known. For instance, NO in the presence of dissolved oxygen converts

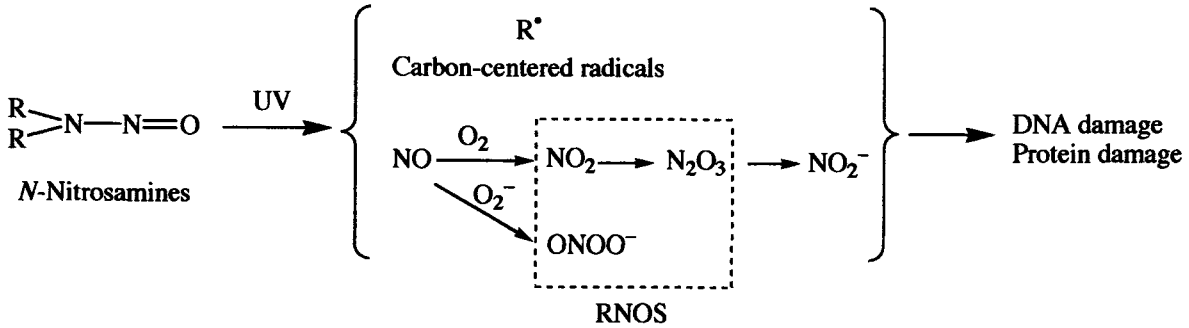

FIGURE 8 Degradation of *N*-nitrosamines induced by UV light.

guanosine into xanthosine and oxanosine,[31] and ONOOH[32] modifies guanine base moiety of DNA molecules into 8-nitroguanine moiety. NO_2[33,34] and ONOOH[35–41] react with tyrosine and tryptophan residues in proteins to produce 3-nitrotyrosine and degradation products. Moreover, ONOOH causes protein fragmentation.[36,42] In conclusion, RNS as well as carbon-centered radicals, generated in the solution of *N*-nitrosamines by UV-irradiation may exert deteriorating effects on DNA and protein molecules.

Acknowledgements

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