Photochemistry of protein and nucleic acid constituents: electron spin resonance and spin-trapping with 2-methyl-2-nitrosopropane

PETER RIESZ AND IONEL ROSENTHAL[1]

Laboratory of Pathophysiology, National Cancer Institute, National Institutes of Health, Bethesda, MD 20205, U.S.A.

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Our recent studies of the direct uv-photolysis of aliphatic and aromatic peptides, DNA constituents, and their 5-haloderivatives in aqueous solution and the photo-induced reactions of benzoylperoxide with amino acids, peptides, fatty acids, and pyrimidines in dimethylsulfoxide-containing solutions are reviewed.

2-Methyl-2-nitrosopropane was used for spin-trapping and characterization of free radicals generated photochemically with light in the wavelength range of 220–313 nm in aqueous or aprotic solvents. Direct photolysis of aliphatic dipeptides and of phenylalanine peptides produced mostly decarboxylation radicals, while for tyrosine peptides both decarboxylation and deamination radicals were spin-trapped; for tryptophan di- and tripeptides, deamination radicals were predominantly produced, while for long chain polypeptides, main-chain scission was observed.

When pyrimidine bases were photolysed, radicals consistent with the addition of an H-atom or an OH-radical at the C(5) position of the 5,6-double bond could be detected. The general reaction pattern in the photolyses of 5-chloro, bromo, or iodouracil was the homolytic cleavage of the carbon–halogen bond, while for 5-fluorouracil, the α-fluoro radical was spin-trapped.

Dibenzoylperoxide was found to photoinduce the free radical generation in amino acids, peptides, and fatty acids exposed to ultraviolet light, which is not absorbed by these compounds, that is, $\lambda = 313 \pm 10$ nm. The most predominant reaction is the decarboxylation of the terminal acid moiety. This process is explained by an electron transfer from the acid to the photoexcited peroxide or its fragmentation products. Pyrimidine bases, such as cytosine and thymine, can be oxidized under these conditions to generate C(5) centered radicals.

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On passe en revue nos récentes études sur la photolyse direct uv des peptides aromatiques et aliphatiques, des constituants de l'ADN et de leurs dérivés halogénés en solution aqueuse ainsi que les réactions photo-induites du peroxyde de benzoyle avec les acides aminés, les peptides, les acides gras et les pyrimidines dans des solutions contenant du diméthylsulfoxyde.

Nous avons utilisé le méthyl-2 nitroso-2 propane comme capteur de spin et pour caractériser les radicaux libres produits photochimiquement avec une lumière dont la longueur d'onde varie de 220 à 313 nm dans des solvants aqueux ou aprotiques. La photolyse directe des dipeptides aliphatiques et des peptides contenant de la phénylalanine produit surtout des radicaux de décarboxylation, tandis que dans les cas des peptides contenant la tyrosine, on a capté des radicaux de décarboxylation et de désamination. Dans le cas des di- et des tripeptides du tryptophane, on obtient surtout des radicaux de désamination tandis que l'on observe une scission de la chaîne principale dans le cas des longues chaînes de polypeptides.

Quant on photolyse les bases du type pyrimidine, on détecte des radicaux qui correspondent à l'addition d'un atome d'hydrogène ou d'un radical OH sur le carbone en position 5 de la double liaison en position 5,6. Le schéma général des photolyses du chloro-5, bromo-5 ou iodo-5 uracile est le clivage homolytique de la liaison carbone–halogène tandis que dans le cas du fluoro-5 uracile il y a piégeage de spin du radical α fluoro.

On a trouvé que le peroxyde de dibenzoyle induit photochimiquement la formaton de radicaux libres dans les acides aminés, dans les peptides et les acides gras exposés à lumière ultraviolette que ces composés n'absorbent pas soit à $\lambda = 313 \pm 10$ nm. La décarboxylation de l'unité acide terminale constitue la réaction la plus courante. On explique cette réaction par un transfert d'électron de l'acide au peroxyde excité photochimiquement ou à ses produits de fragmentation. Les bases du type pyrimidine telles la cytosine et la thymine peuvent être oxydées dans ces conditions en produisant des radicaux centrés en C(5).

[Traduit par le journal]

Introduction

The damaging effects of ultraviolet light on biological systems have been extensively described in the literature. Correlations have been made between survival of uv-irradiated cells and the production of certain types of photochemical damage in their DNA. Vital proteins and enzymes are denatured and inactivated due to changes which could be traced to particular amino acid residues. Finally, DNA and protein could be photochemically cross-linked in cells (1, 2).

The understanding of these effects requires basic knowledge of the primary chemical events which followed the light absorption. In this respect, esr provides the experimental tool for detecting the initial transients generated in response to the specific energy input in a certain chromophore moiety of a complex biological polymer.

With the advent of the spin-trapping method (3, 4), it is now possible to gain information about free radicals generated at physiological temperatures without suppressing thermally activated processes

[1]On sabbatical leave from the Department of Technology, Agricultural Research Organization, Volcani Center, Bet-Dagan, Israel.

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and hydration interaction with the solvent. We have extensively used 2-methyl-2-nitrosopropane (MNP) for trapping and characterization of free radicals generated in protein and nucleic acid constituents over a wide range of wavelengths of (220–313 nm) by direct or sensitized photolyses.

Experimental

Aqueous solutions of MNP (1.5 mg/mL) were prepared by stirring overnight at room temperature. Dimethylsulfoxide solutions were prepared by stirring MNP (15 mg) and dibenzoylperoxide (50 mg) in 10 mL of solvent for 15 min. The photolyses were performed *in situ* at room temperature in aerated solutions, in a standard aqueous quartz cell placed in the esr cavity, using a Schoeffel 1000 W high pressure Hg–Xe lamp coupled with a grating monochromator. The irradiation times varied between 10 s up to 45 min. A Varian E-9, X-band spectrometer was used for esr measurements. The spectra from spin-trapped radicals were recorded at room temperature as first derivatives. The magnetic field modulation frequency was 100 kHz, and 10 mW of microwave power were used.

In many cases a large di-*tert*-butyl nitroxide (DTBN) signal prevented the identification of the underlying spectrum due to other radical species. Two methods were used to remove the DTBN signal: one was to adjust the pH to about 4 and allow the DTBN signal to decay, since it generally decayed faster than the other spin-adducts; the other was to extract the DTBN formed after irradiation with petroleum ether (boiling range 37–55°C). Except in some instances, where the spin-adduct was also soluble in the organic phase, good results were generally obtained. The procedure was the following: after irradiation of the esr cell outside the cavity, which was often carried out three or four times to expose the entire flat part of the cell to the uv beam, the solution contained in the cell was transferred to a small test tube and extracted three times with an equal volume of petroleum ether. The aqueous phase was then transferred back into the cell and the spectrum recorded. The spectrum before extraction was usually also recorded in order to check for possible loss of signals other than DTBN.

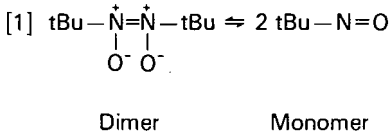
Results and discussion

A. *Direct photolyses in aqueous solutions*

I. *MNP*

Since MNP became a part of the system investigated, knowledge of its photochemical properties is required.

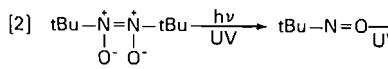
In aqueous solution, MNP exists in a monomer–dimer equilibrium (eq. [1]) (5).



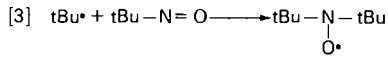
[1] $tBu-\overset{+}{N}(O^-)=\overset{+}{N}(O^-)-tBu \rightleftharpoons 2\ tBu-N=O$

Dimer Monomer

After 12 h at 30°C the monomer concentration reaches its maximum value (ca. 60%). The aqueous MNP exhibits a light absorption at 662 nm due to the monomer, and more intense absorptions at 287 and 224 nm attributed mainly to the dimer. The photochemistry of MNP in water has not been studied, but the following reactions (eqs. [2], [3]) are known to occur in organic solvents (6, 7):



[2] $tBu-\overset{+}{N}(O^-)=\overset{+}{N}(O^-)-tBu \xrightarrow[UV]{h\nu} tBu-N=O \xrightarrow[UVorVIS]{h\nu} tBu^{\bullet} + NO^{\bullet}$



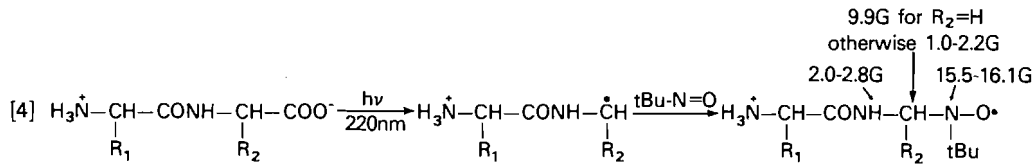
[3] $tBu^{\bullet} + tBu-N=O \longrightarrow tBu-\underset{O^{\bullet}}{N}-tBu$

We assume that the generation of this undesired di-*tert*-butyl nitroxide radical was the reason for the lack of enthusiasm in using MNP for spin-trapping in photochemical reactions (8). However, we found that frequently the formation of di-*tert*-butyl nitroxide can be entirely avoided by choosing the right experimental conditions such as reactant concentration and irradiation time. Even when produced, its very simple and characteristic esr expression (a sharp triplet of hyperfine splitting constant which is solvent dependent) has a minimal interference with the spin-adduct and can be easily subtracted. When, however, the concentration of the spin-adduct of interest is very low compared to di-*tert*-butyl nitroxide, two methods could be used to remove this undesired signal: one is to adjust the pH to about 4 and allow it to decay, since it generally decays faster than the other spin adducts; the second is to extract it with petroleum ether after photolysis. Except in instances when the spin-adduct is also soluble in the organic phase, useful results can be obtained. We note, however, that occasionally the excessive formation of di-*tert*-butyl nitroxide is symptomatic of a different process. Thus, during an attempt to detect free radicals following the photoexcitation ($\lambda_{exc} = 365$ nm) of psoralens (5-hydroxy psoralen, trioxsalen, and 8-methoxypsoralen) in a dimethylsulfoxide solution, di-*tert*-butyl nitroxide was generated exclusively in amounts much larger than in control experiments performed under identical conditions but in the absence of psoralens. We suggest that psoralens photosensitize the homolytic breakdown of MNP (unpublished results from this laboratory).

II. *Protein constituents*

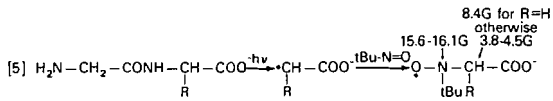
The absorption spectrum for peptide bonds shows a peak in the region of 180–190 nm which decreases essentially to zero by about 240 nm (9). It was found that the direct photolyses of aliphatic dipeptides of the types X-Gly, Gly-X, X-Ala, and Ala-X in aqueous solution with 220 ± 10 nm light, generated free radicals as a result of decarboxylation (eq. [4]) (10).

In these dipeptides no scission between the two amino acids residues was observed. The reaction was found to occur over a wide pH range from 2 to



11.5; however, the yield of decarboxylation radicals rises steeply in acid solution below pH = 3.5, suggesting an increase with the concentration of the nonionized form of the carboxyl group.

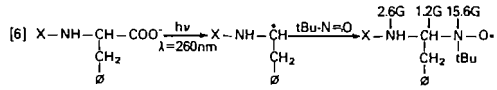
In strongly alkaline solutions (9 *M* NaOH) this reaction does not occur and only secondary deamination radicals were detected (eq. [5]).

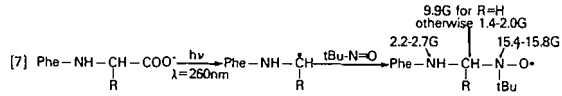


The photolyses of several polypeptides of the type $(Ala)_n$ where $n = 3-90$ was investigated in aqueous solution in order to ascertain whether the decarboxylation radical of the C-terminal residue was the only radical product or whether main-chain scission would occur. The experiments were carried out with solutions containing 10 mg/mL of the polypeptide, and all other conditions of the experiment (spin-trap concentration, pH, duration of photolysis) were kept constant. Under these conditions the concentration of peptide bonds is kept constant while that of the C-terminal carboxyl group decreases with increasing chain length. If the radical products arise only from the C-terminal carboxyl residue, the yield of the decarboxylation radical would be expected to decrease when the number of alanine residues increases from 2 to 90. However, the results indicate that main-chain scission must occur since the radical yield does not decrease appreciably when the average number of alanine residues in the polypeptide is 90.

As a model compound for proteins, poly (Gly-LAla-LPro)$_{12}$ was photolysed. This amino acid sequence frequently occurs in collagen which is the major component of the cornea of the eye. The major radical formed with this compound is the same as the one observed from polyalanine. This indicates that the chain scission occurs predominantly between the alanine and proline residues.

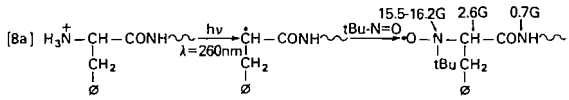
Photodecarboxylation is not limited to aliphatic peptides. It was also found to occur in phenylalanine-containing peptides (11). In this case, the reaction can also be induced by uv light of longer wave-length, that is 260 nm which is absorbed by the aromatic chromophore (eqs. [6], [7]).



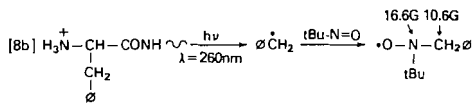


However, when the aromatic chromophore is excited the yield of decarboxylation is not sensitive to pH, and is independent of the state of ionization of the carboxyl group.

In Phe-X type dipeptides the primary deamination radicals were also detected (eq. [8a]).

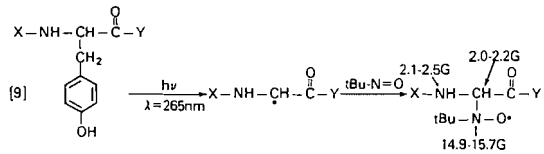


Occasionally the spectrum of spin-trapped benzyl radical generated by photo-dissociation of the Phe residue overlapped the main signal (eq. [8b]).



When phenylalanine was embedded in a larger peptide such as ribonuclease-*S*-peptide, following photolyses, chain scission occurred to give a small fragment attached to the decarboxylation radical of the phenylalanine.

In tyrosine-containing peptides several reaction patterns could also be detected (12). While the direct excitation of the peptide bond with $\lambda = 220$ nm consistently generated the decarboxylation radicals, excitation of the aromatic chromophore with $\lambda = 265$ nm in neutral solution yielded a spin adduct formed by trapping of the fragment resulting from the scission between the α carbon atom and the methylene group attached to the aromatic ring (eq. [9]).



In addition, deamination radicals could also be detected. The relative yield of deamination adducts was found to be pH dependent, increasing with the increase in pH. As a model for longer proteins, the oxidized insulin A chain was uv-photolysed. This compound was chosen because it is a relatively short polypeptide (21 amino acids) and contains only two tyrosine residues at positions 14 and 19. In this case the only fragment which could be identified was tentatively attributed to the photoscission of the side chain of the tyrosine moiety. In alkaline medium an additional type of deamination spin adduct was observed which could be a deamination radical from any residue of the chain.

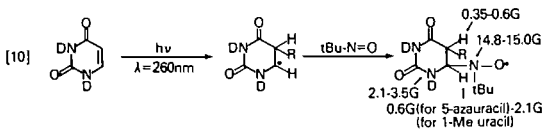
Finally, the photolyses of tryptophan-containing dipeptides ($\lambda = 280 \pm 10$ nm) was investigated (13). Consistently, these compounds generated the deamination radical of the N-terminal amino acid. These results were explained in terms of hydrated electrons photoejected from the tryptophan residue (14) reacting with the carbonyl group followed by deamination of the N-terminus. This mechanism was proved by a sensitization experiment using tryptophan as a sensitizer and glycine as substrate. The ultraviolet irradiation of a mixture of these two amino acids, with light of 280 nm absorbed exclusively by tryptophan, generated the deamination radical spin-adduct of glycine which has very characteristic hyperfine coupling constants (two equivalent β-protons, $a_H^\beta = 9.9$ G).

III. *Nucleic acid constituents*

The photochemical reactions of nucleic acids and their constituents have been the subject of extensive investigations in recent years in attempts to clarify, at the molecular level, the ultraviolet induced damage of living systems. Thus, major photochemical products of pyrimidines such as cyclobutane-type dimers and photohydrates have been correlated to biological effects (15).

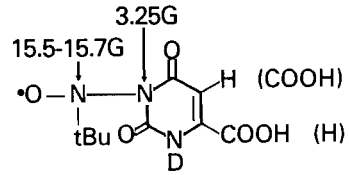
We found that direct photolyses of several pyrimidine bases (uracil and substituted derivatives) in aqueous solution (D_2O), at room temperature generated free radicals which could be trapped by MNP and characterized (16).

The general reaction pattern is exemplified for uracil in eq. [10] and is consistent with the addition of an unidentified radical (D or OD) at the C(5) position of the uracil ring.



The added radical was unambiguously identified as H when 5-azauracil was photolysed in H_2O.

Orotic and isoorotic acid behaved differently. Both these compounds afforded identical 3 × 3 lines spectra suggestive of a radical localized at N(3).

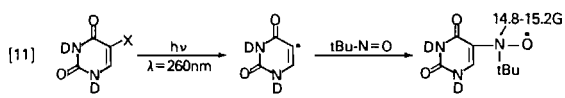


It is, however, noted that in the nucleosides tested, uridine and 2-deoxyuridine, the addition of the D-atom took place at C(6) to yield a paramagnetic center localized at C(5).

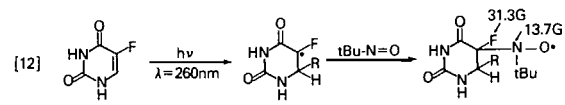
All these results indicate that the photochemical excitation of pyrimidine bases yields free radicals as a general phenomenon. While these species cannot lead to the formation of the major photoproducts in irradiated nucleic acids, such as cyclobutane-type dimers and photohydrates, they may precede the formation of dihydropyrimidines and other pyrimidine bimolecular photoproducts.

It is well known that the incorporation of 5-halogenated derivates of uracil in DNA causes an increased sensitivity to the lethal effect of uv-irradiation (17). In order to obtain information concerning the mechanism of photosensitization, the free radicals produced in 5-halopyrimidines by direct uv photolyses in aqueous solution were studied (18).

The general reaction pattern in photolyses of 5-chloro, bromo, iodo uracil was the homolytic cleavage of the carbon–halogen bond (eq. [11]).

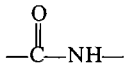


5-Fluorouracil reacted in a different manner; this was the addition of an unidentified radical (H· or OH) to the C(6) position (eq. [12]).

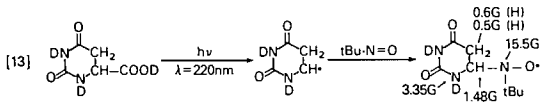


The same basic reactions could be detected when the corresponding nucleosides were photolysed. However, in these cases additional free radicals could be detected, which were tentatively attributed to the abstraction from the C(5′) position of the sugar moiety.

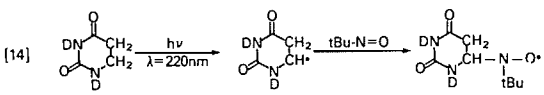
Finally, we investigated the photolyses of 5,6-dihydropyrimidines (19). The chromophoric group in these compounds is the amido moiety



and consequently their photolysis was expected to be induced by light of 220 nm which was required in the photolysis of aliphatic dipeptides. When 5,6-dihydroorotic acid was photolysed under these conditions, decarboxylation occurred at C(6), entirely analogous to the reaction of peptides (eq. [13]).



The same type of C(6) centered radical was detected when several alkyl derivatives of 5,6-dihydrouracils, dihydrothymines, and dihydrocytosines were photolysed. The reaction is exemplified for 5,6-dihydrouracil in eq. [14].

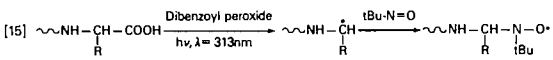


It was noted that the same radicals were trapped when polycrystalline samples of the parent compounds were γ-irradiated and subsequently dissolved in the spin-trap solution.

B. *Photolyses in organic solvents*

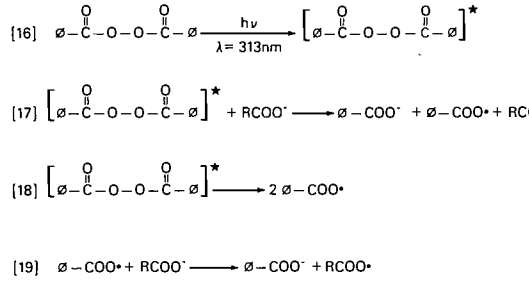
I. *Dibenzoylperoxide photo-induced reactions*

Dibenzoylperoxide can photoinitiate the free radical generation in amino acids and peptides by uv-light which is not absorbed by these compounds, that is $\lambda = 313$ nm. The most predominant reaction is the decarboxylation of the amino acids or of the carboxyl-terminal residue in peptides (eq. [15]) (20).

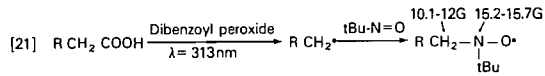


These reactions were performed in dimethylsulfoxide solutions without any interference from the solvent. Over 50 amino acids and dipeptides were tested and followed this reaction pattern. The hyperfine splittings varied between 14.55–15.00 G for nitroxide nitrogen, 2.5–3.0 G for the peptide nitrogen, and 1.0–1.8 G for the β nitrogen. When the glycine residue was terminal and consequently decarboxylated, the two equivalent β-hydrogens had the characteristic large splitting of 7.3 G.

Two mechanistic schemes could explain these results. Both are initiated by the light absorption by dibenzoylperoxide (eq. [16]). Dibenzoylperoxide has a broad adsorption band at $\lambda_{max} = 275$ nm (21) whose end absorption extends beyond 300 nm under our experimental conditions, thus acting as the exclusive light absorption system in the reaction mixture. Subsequently the nucleophilic attack of the carboxyl group cleaves the excited molecule (eq. [17]). Alternatively, the excited dibenzoylperoxide homolyses to benzoyloxy radicals (eq. [18]) which, by one-electron oxidation of the amino acid carboxyl group, generate the corresponding neutral radical (eq. [19]). This species dissociates spontaneously to CO_2 and to the trapped decarboxylation radical (eq. [20]).



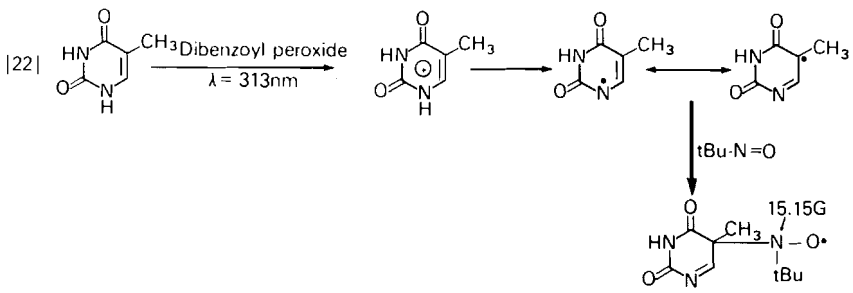
This reaction is not limited to amino acids. Carboxylic acids of different chain-lengths, from formic to linolenic, were tested and found to be decarboxylated by the photoexcited dibenzoylperoxide (22) (eq. [21]).



Even pyrimidine bases such as cytosine and thymine can be oxidized by a one-electron transfer under these reaction conditions to generate C(5) centered radicals (eq. [22]).

These results are of interest since dibenzoyl peroxide is extensively used as a topical medication for the treatment of facial acne (23) and was recently discovered to be a potent promoter of carcinogenesis (24).

The present review indicates that MNP is a useful spin-trap for identifying intermediate radical species in the photochemistry of protein and nucleic acid constituents. Since radicals produced in the photolysis of high molecular weight polypeptides (10–12) can be spin-trapped with MNP, it is reasonable to expect that in the future enzymatic hydrolysis followed by high performance liquid chromatography, which allows the separation of nitroxide radicals, will lead to the identification of



radicals in the photolysis of proteins and nucleic acids.

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