自由基生物学与自由基医学
学术会议资料

INTERNATIONAL WORKSHOP-SYMPOSIUM ON BIOLOGICAL AND MEDICAL ASPECTS OF FREE RADICALS

中国生物物理学会
一九八八年四月·北京

The Biophysical Society of China
April, 1988 Beijing, China

目　　录

STUDIES ON VITAMIN E IN VIVO

K.U. Ingold.

Lipid peroxidation and its retardation by preventive and by chain-breaking antioxidants will be surveyed briefly. Vitamin E (α-tocopherol) is a lipid-soluble, chain breaking antioxidant and, in fact, is probably the sole such material in human blood and in animal tissue. For stereoelectronic reasons α-tocopherol is highly reactive towards peroxyl radicals but better antioxidants, if not neeessarily better vitamin E's, can be synthesized. The interactions between α-tocopherol dispersed in a phospholipid bilayer and water-soluble, biological antioxidants will be described. The position and orientation of α-tocopherol in bilayers has been determined by ^{13}C and ^{2}H NMR spectroscopy.

The successful syntheses of the natural, 2R,4'R,8'R-stereoisomer of α-tocopherol labelled with 3 and with 6 deuterium atoms, of the unnatural, 2S,4'R,8'R-stereoisomer with 3 deuterium atoms, and of the all-racemic compound labelled with 3, and with 9 deuterium atoms have enabled us to carry out the first reliable studies of the biokinetics of vitamin E in man. The results of four separate experiments will be reported. (1) A study of the relative bioavailability of 2R,4'R,8'R-α-tocopherol for subjects receiving one dose of an equimolar mixture of the free phenol and the acetate. These results will be compared with the results of similar experiments using rats, experiments which lead to the conclusion that the current protocol for bioassaying vitamin E activity in the rat yields results that are totally irrelevant not only to the rat under normal dietary conditions but also to man. (2) A three part study of the relative net uptake for subjects receiving a single dose containing an equimolar mixture of 2R,4'R,8'R-α-tocopheryl acetate with (i) 2S,4'R,8'R-α-tocopheryl acetate, (ii) all-racemic-α-tocopheryl acetate, (iii) ambo-(2RS, 4'R, 8'R)-α-tocopheryl acetate. Some of these results will be compared with the results of similar experiments using rats. It can be concluded that man exhibits a significantly greater biodiscrimination between natural and unnatural synthetic forms of vitamin E and that the presently accepted relative biopotencies of 2R, 4'R, 8'R-α-tocopheryl acetate vs. all-racemic-α-tocopheryl acetate, viz., 1.36: 1.0 is incorrect in man and is therefore irrelevant. (3) A study of the kinetics of net uptake of 2R,4'R,8'R-α-tocopherol in the plasma proteins of subjects taking a single doze of the acetate. Vitamin E appears first in the chylomicrons, then in the very low density lipoproteins and finally and at equal rates in the low and high density lipoproteins. (4) A preliminary study of the kinetics of the relative net uptake of 2R,4'R,8'R- and 2S,4'R,8'R-α-tocopherol in the plasma proteins of a subject taking a single dose of equimolar quantities of the two acetates. Both stereoisomers appear to be absorbed with equal efficiency but the unnatural stereoisomer is lost much more rapidly than is the natural vitamin E. Some of our results with rats suggest that these two stereoisomers are also absorbed with equal efficiency by these animals.

Radicals and Melanomas

by P A Riley, Department of Chemical Pathology,
University College and Middlesex School of Medicine,
Cleveland Street, London W1P 6DB, UK

The synthesis of melanin involves the oxidation of phenolic substrates by the enzyme tyrosinase. In vertebrates, tyrosinase is present only in specialised pigment cells where it catalyses the oxidation of tyrosine and certain diphenolic intermediate products to quinones, notably indole quinones, which polymerize to give rise to melanin. This specialised metabolic pathway provides a possible approach to the specific chemotherapy of malignant tumours of pigment cells (malignant melanomas). The continued increase in the reported incidence of cutaneous malignant melanoma is a source of serious concern, particularly in view of the agressive nature of the tumour and the young age group affected. It has been found that certain analogues of tyrosine are oxidised by tyrosinase, generating reactive orthoquinones with cytotoxic potential. One such analogue, 4-hydroxyanisole, has been investigated as a possible specific melanocytotoxic precursor. The parent compound inhibits DNA synthesis but exhibits little general toxicity, while the tyrosinase oxidation products are highly toxic to cells. The mechanism of toxicity may involve semiquinone radicals. Recent pulse radiolysis studies have shown that the methoxyorthobenzo-semiquinone, formed during the oxidation of 4-hydroxyanisole by tyrosinase, is relatively unreactive towards oxygen, suggesting that the mechanism of cytotoxicity does not involve the generation of reactive oxygen species. The reactivity of anisyl 3-4, quinone towards nucleophiles suggests this may be the primary mechanism of cytotoxicity. Whilst the details of the cytotoxic mechanism remain to be elucidated, encouraging initial results have been obtained from clinical pilot studies using intra-arterial infusion of 4-hydroxyanisole in patients with localised recurrences of malignant melanoma.

FREE RADICALS IN CHEMICALLY-INDUCED PHOTOSENSITIVITY REACTIONS

Colin F. Chignell
Laboratory of Molecular Biophysics
National Institute of Environmental Health Sciences
Research Triangle Park, NC 27709 USA

Light is known to interact with chemical agents in the skin and eyes to produce photosensitization (phototoxicity and photoallergy). The chemical may be an endogenous compound (protoporphyrin in erythropoetic porphyria), a drug (sulfonamide, tetracyclines, phenothiazines), a topical agent (4-aminobenzoic acid in sunscreens) or an environmental agent (polycyclic aromatic hydrocarbons in coal tar). While the precise mechanisms of photosensitization are unknown, the initial step must be the absorption of light by the ground state of the photosensitizer and its conversion first to the singlet excited state and then to the long-lived triplet state. Subsequent reactions can proceed by a number of different pathways, depending on the chemical nature of the photosensitizer and the substrate, as well as the reaction conditions. In Type I (free radical or redox) reactions the triplet sensitizer molecule may abstract an electron (or hydrogen atom) from the substrate molecule (R) to give a semi-oxidized (free radical) form of the substrate ($R^{\cdot+}$) and a semi-reduced form ($S^{\cdot-}$) of the sensitizer. The semi-reduced form of the sensitizer may react with molecular oxygen to give the superoxide radical ($O_2^{\cdot-}$). In Type II (energy transfer) reactions, electronic excitation energy is transferred from the triplet state sensitizer to ground state oxygen to give the highly electrophilic excited singlet state of oxygen ($^1O_2$).

In order to determine the precise mechanisms by which chemical agents produce photosensitization we have attempted to identify the reaction intermediates that are generated when photosensitizing chemicals are irradiated by light. Free radicals from Type I reactions have been detected either by direct electron spin resonance (ESR) or by the technique of spin trapping. Singlet oxygen, generated by Type II reactions, has been detected by observing its luminescence at 1268 nm.

Chlorpromazine [2-chloro-N-(3-dimethylaminopropyl)phenothiazine] (ClP) is a frequently prescribed anti-psychotic drug that causes both phototoxic and photoallergic reactions. Under UV-irradiation the aryl radical (P$^{\cdot}$) resulting from the dechlorination of ClP was trapped using 2-methyl-2-nitrosopropane (MNP). The aryl radical can react with oxygen to form a peroxy radical or may abstract a hydrogen atom from suitable donors. The reactivity of P$^{\cdot}$ is sufficient to explain why ClP is much more phototoxic than the parent drug promazine. When chlorpromazine sulfoxide, a metabolite formed in man and other mammalian species, is irradiated with near UV light it produces large amounts of the highly reactive hydroxyl radical as well as the chlorpromazine cation radical. The ocular phototoxicity of phenothiazine in calves may be due to accumulation of phenothiazine sulfoxide in the eye.

Several anti-bacterial halogenated salicylanilides eg. 3,3',4'5-tetrachlorosalicylanilide (TCSA), 3,4',5-tribromosalicylanilide (TBSA) and N-butyl-4-chlorosalicylanilide (Buclosamide) are known to cause contact photoallergy in humans. Spin trapping studies with MNP in alkaline aqueous solution provided evidence for the photogeneration of aryl radicals from all three compounds.

Irradiation of TBSA with glutathione or cysteine resulted in hydrogen abstraction from the sulfhydryl group and the generation of the corresponding thiyl radical ($RS^{\cdot}$). The aryl radical generated by the photodechlorination of TCSA was capable of extracting hydrogen atoms from the α-carbon atom (backbone) of peptides containing simple amino acids such as alanine and glycine, while for peptides containing leucine and serine hydrogen abstraction also occurred in the side chains. The reaction of these protein-derived radicals with aryl radicals derived from TCSA *in vivo* would result in covalent binding of the drug to form modified proteins that could elicit an allergic reaction.

Tetracycline antibiotics are known to be phototoxic and *in vitro* experiments indicate that this effect is oxygen-dependent. Spin trapping studies with 5,5-dimethyl-1-pyrroline-N-Oxide (DMPO) have shown that superoxide is generated when chlortetracycline, oxytetracycline and demeclocycline but not doxycycline tetracycline and minocycline are irradiated in aqueous aerated buffer. This trend is in reasonable agreement with clinical reports on the phototoxicity of these drugs.

Free radicals have also been detected during the irradiation of other photosensitizing chemicals including sulfanilamide, 4-aminobenzoic acid, benoxaprofen, amiodarone, fentichlor, bithionol, anthracene and musk ambrette. It therefore seems likely that these chemically reactive species may be responsible for the *in vivo* photosensitizing capabilities of these compounds.

RAPID COMMUNICATIONS

ESR DETECTION OF ENDOGENOUS ASCORBATE FREE RADICAL IN MOUSE SKIN: ENHANCEMENT OF RADICAL PRODUCTION DURING UV IRRADIATION FOLLOWING TOPICAL APPLICATION OF CHLORPROMAZINE

Garry R. Buettner*, Ann G. Motten†, Robert D. Hall and Colin F. Chignell**

Laboratory of Molecular Biophysics
National Institute of Environmental Health Sciences
PO Box 12233
Research Triangle Park, NC 27709, USA

(Received 16 April 1987; accepted 5 May 1987)

Abstract - Using electron spin resonance spectroscopy, we observed that UV radiation (330 nm) increased the endogenous ascorbate free radical concentration in hairless mouse (HRS/J) skin. When the skin was topically treated with a chlorpromazine solution prior to illumination, UV irradiation caused the ascorbate free radical concentration to increase even more. This observation suggests that there is an increased UV-induced oxidative stress in the presence of chlorpromazine, probably caused by the production of free radicals from chlorpromazine.

INTRODUCTION

The UV wavelengths of sunlight are known to produce deleterious effects on skin, ranging from sunburn to cancer. These effects have often been attributed to oxidation by free radical intermediates (Slater, 1972). Protection against UV damage by endogenous GSH§ has recently been demonstrated with in vivo human fibroblasts by Tyrrell and Pidoux (1986); the level of protection approaches that afforded by excision repair. These researchers propose that the observed protection results from the antioxidant properties of GSH.

The ascorbate anion (AH^-), an antioxidant which is naturally present in tissue, reacts rapidly with the glutathiyl free radical yielding GSH and $A^{\cdot-}$ with a rate constant of 6×10^8 $dm^3 mol^{-1} s^{-1}$ at pH 7 (Forni et al., 1983). Thus, ascorbate may serve as an important component in the "repair" of UV radiation-produced free radicals.

Chlorpromazine often induces phototoxic and photoallergic reactions (Fitzpatrick et al., 1963). This toxicity has been attributed to the formation of radicals by the UV radiation-induced dechlorination of CPZ (Jose, 1979). Using the ESR spin trapping technique, Motten et al. (1985) have clearly demonstrated the photoproduction of the dechlorination radical from CPZ. Moreover, the dechlorination radical abstracts hydrogen atoms from a variety of substrates as effectively as the hydroxyl or phenyl radicals.

The object of the present work was to determine whether ESR spectroscopy could be used to detect UV radiation-induced free radicals in skin treated with CPZ. While direct evidence for CPZ radicals was not obtained, indirect evidence for CPZ-produced radical reactions in skin was found by monitoring the UV radiation-induced increase in concentration of the ascorbate radical derived from endogenous ascorbic acid. We believe that this is the first report of $A^{\cdot-}$ being observed by ESR in skin tissue.

MATERIALS AND METHODS

Skin from HRS/J female mice 6-8 weeks old, was obtained after killing the animals by cervical dislocation. Excess fat was removed by gentle scraping. The skin was then placed in pH 7.0, 50 mM phosphate buffer with or without 2 mM CPZ for 10 minutes, rinsed with buffer solution, then kept at ice temperature prior to ESR examination. The skin was placed in an ESR tissue cell and in the ESR spectrometer as soon as possible.

---

*Present address: GSF Forschungszentrum; D-8042 Neuherberg; West Germany
†Present address: Department of Chemistry; Duke University; Durham, NC 27706
**To whom correspondence should be addressed.
§Abbreviations: $A^{\cdot-}$, ascorbate free radical; AH^-, ascorbate anion; CPZ, chlorpromazine; ESR, electron spin resonance; GSH, glutathione; TEMPO, 2,2,6,6-tetramethyl-piperidine-1-oxyl.

ESR spectra were recorded at 9.5 GHz with a Varian E-109 spectrometer equipped with a TM_{110} cavity. Skin samples were at room temperature during ESR measurements. Samples were irradiated at 330 nm (10 bandpass *nm*) in the cavity with a Schoeffel 1000 W Xe arc lamp and monochromator combination. The irradiance was approximately 15 W/m^2, assuming the cavity grid transmits 75% of the incident light, as measured with a Yellow Springs Instruments Model 65 radiometer.

Because the ascorbate signal is weak and decays rapidly it is essential to minimize the time between harvesting the skin and collecting the spectra. Each tissue sample required different tuning of the ESR spectrometer and thus, without accurate knowledge of both the magnetic field strength and the operating-frequency of the spectrometer for a particular tuning condition, it was often difficult and time-consuming to find the very weak signal of the ascorbate free radical. To minimize this time, a small piece of plastic tubing with a speck of solid spin label, TEMPO (g = 2.0062), sealed in the end, was lowered into the cavity just to the point where its signal could be found with the spectrometer's field sweep option. The TEMPO signal was centered by adjusting the magnetic field strength, the TEMPO was then removed and after adjusting the instrument settings, the ascorbate free radical signal (g = 2.0052) was monitored. The identity of the ascorbate free radical was confirmed by comparing the intrinsic signal with that obtained after applying pure sodium ascorbate to the skin sample.

RESULTS AND DISCUSSION

We observed the ESR signal of the ascorbate free radical in freshly prepared hairless mouse skin. The intensity of the signal decreased with time. When the skin sample was irradiated with 330 *nm* UV light, the intensity of the ascorbate free radical signal increased by 25-35% under the experimental conditions used (Fig. 1). If the light intensity was increased, the signal intensity of $A^{\bullet-}$ also increased.

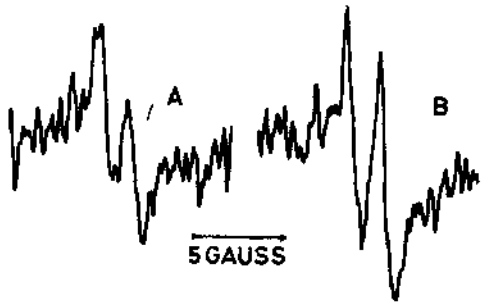


Figure 1. Ascorbate free radical observed in hairless (HRS/J) mouse skin: A. without UV radiation; B. with 330 nm UV radiation. The observed hyperfine splitting is 1.77 gauss, quite similar to other reported values for $A^{\bullet-}$ (Fisher and Hellwege, 1977-1979), and in addition, features consistent with an additional hyperfine splitting of approximately 0.2 gauss from two equivalent hydrogens can be seen, although the signal to noise is too low and the modulation amplitude too high to make a positive assignment. Instrument settings were: Mod. Amp., 1 gauss; Gain, 3.2 x 10^5; Power, 20 mW; Scan, 1.33 gauss/min; Time Constant, 4 s.

When the skin samples were pretreated with CPZ prior to ESR examination, the ascorbate free radical was also observed prior to UV exposure. However, when the pretreated samples were irradiated using the same conditions as for the untreated skin, the intensity of the $A^{\cdot}$ signal increased by a factor of two (Fig. 2).

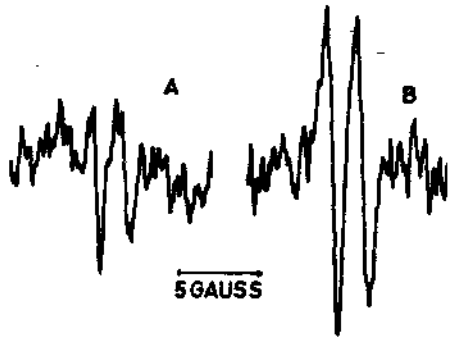


Figure 2. Ascorbate free radical in hairless (HRS/J) mouse skin treated with 2 mM CPZ aqueous solution applied topically and rinsed prior to ESR measurements: A. without UV light; B. with 330 nm UV light. Hyperfine splittings and instrument settings are as in Figure 1.

To show that 330 nm light does not affect ascorbate itself, we irradiated an aqueous solution of 1 mM ascorbate and observed no change in the ESR intensity of $A^{\cdot}$. However, when 0.3 mM CPZ was included, the $A^{\cdot}$ signal increased by approximately 250% (Fig. 3).

Although free radicals have been implicated in the damage produced by UV irradiation of skin, there are few reports of direct ESR detection of radical species produced in skin. Norris (1962) was not able to identify the radical he observed in UV irradiated human skin samples. Pathak (1967) observed free radical signals in human skin which he assigned to the various melanins. His samples were collected as many as four hours after death. Ogura's group concluded that the radical they observed on exposure of hairless mouse skin

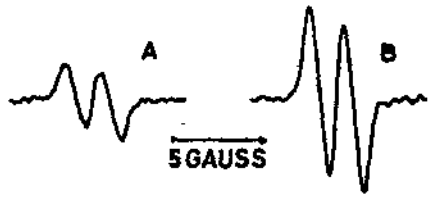


Figure 3. Ascorbate free radical in aqueous solution. Samples contained 1 mM ascorbate and 0.3 mM CPZ in pH 7.0 air-saturated 50 mM phosphate buffer: A. without UV radiation; B. with 330 nm radiation. When ascorbate was irradiated at 330 nm in the absence of CPZ, no increase in the ascorbate free radical signal was observed (not shown). Hyperfine splittings are as in Fig. 1. Instrument settings: Mod. Amp., 1 gauss; Gain 1.25×10^4; Power, 20 mW; Scan, 5 gauss/min; Time Constant, 1 s.

to UV light resulted from lipid peroxide radicals (Sugiyama et al., 1984a; Ogura, 1982; Sugiyama et al., 1984b). The short lag-time between the death of the animal and the ESR observation of the skin sample in our experiments allowed us to detect the weak and transient $A^{\cdot -}$ radical. Our observation of an increase in $A^{\cdot -}$ in UV radiation-exposed skin is supporting evidence for UV radiation-induced free radical processes. The larger increase in the $A^{\cdot -}$ concentration observed in CPZ-treated skin also suggests these free radical processes are enhanced by CPZ, a drug that is well known to produce free radicals upon exposure to UV radiation (Motten et al., 1985; Buettner et al., 1986; Chignell et al., 1985).

REFERENCES

Buettner, G.R., A.G. Motten, R.D. Hall and C.F. Chignell (1986) Free radical production by chlorpromazine sulfoxide, an ESR spin-trapping and flash photolysis study. Photochem. Photobiol. 44, 5-10.

Chignell, C.F., A.G. Motten and G.R. Buettner (1985) Photoinduced free radicals from chlorpromazine and related phenothiazines: Relationship to phenothiazine-induced photosensitization. Environ. Health Pers. 64, 103-110.

Fisher, H. and K.-H. Hellwege (1977-1979) Landolt-Bornstein, New Series, Springer-Verlag, Berlin, Group II, Vol. 9.

Fitzpatrick, T.B., M.A. Pathak, I.A. Magnus and W.L. Curwen (1963) Abnormal reactions of man to light. Ann. Rev. Med. 14, 195-214.

Forni, L.G., J. Monig, V.O. Moro-Arellano and R.L. Willson (1983) Thiyl free radicals: Direct observations of electron transfer reactions with phenothiazines and ascorbate. J. Chem. Soc. Perkin Trans. II, 961-965.

Jose, J.G. (1979) Photomutagenesis by chlorinated phenothiazine tranquilizers. Proc. Natl. Acad. Sci. USA 76, 469-472.

Motten, A.G., G.R. Buettner, and C.F. Chignell (1985) Spectroscopic studies of cutaneous photosensitizing agents. VIII. A spin trapping study of light induced free radicals from chlorpromazine and promazine. Photochem. Photobiol. 42, 9-15.

Norris, A.L. (1962) Free radical formation in the skin following exposure to ultraviolet light. J. Invest. Dermatology 39, 445-448.

Ogura, R. (1982) Cellular damage of epidermis exposed to ultraviolet light. Lipid peroxide formation in mitochondria and effect of antioxidants. J. Kurume Med. Assoc. 45, 279-301.

Pathak, M.A. (1967) Photobiology of melanogenesis: Biophysical aspects. In Advances in Biology of Skin (Edited by W. Montagna and F. Hu), pp. 397-420. Pergamon Press, London.

Slater, T.F. (1972) Free Radical Mechanisms in Tissue Injury, pp. 241-263, Pion Limited, London.

Sugiyama, M., K. Kajiyama, T. Hidaka, S. Kumano and R. Ogura (1984a) Lipid peroxidation and radical formation in methyl linoleate following ultraviolet light exposure. J. Dermatology 11, 455-459.

Sugiyama, M., N. Kaneko, A. Kagiyama, S. Murakata and R. Ogura (1984b) Lipid peroxide and free radical formation in the epidermis following single exposure of ultraviolet light. J. Kurume Med. Assoc. 47, 346-350.

Tyrrell, R.M. and M. Pidoux (1986) Endogenous glutathione protects human skin fibroblasts against the cytotoxic action of UVB, UVA and near-visible radiations. Photochem. Photobiol. 44, 561-564.

Radiation-induced Radical in DNA

Giichi Yoshii
Kansai Medical School, Radiation Science, Japan

The primary phases of the physico-chemical changes of in irradiated nucleic acids characterized by the occurrence of free radocals which can be observed using ESR-spectroscopy.

Quantitative ESR-spectroscopy is used to determine the yields of radicals in DNA and its constituents. The yield of radicals increases in the order bases< nucleosides< nucleotides. The higher yields of radicals in nucleosides and nucleotides is due to the additional radicals formed in the sugar and phosphate by the irradiation.

Using qualitative ESR-measurement, attempt can be made to identify the radicals contributing to the spectra of the irradiated DNA. It is a characteristic observation that eight-line spectrum of thymine is observed with quite different intensities, and this spectrum is due to an additional product of atomic hydrogen to the C-6 atom.

The question of intra- and inter-molecular spin transfer in DNA is of special interest in this context. When a 1:1-cocrystal of adenine and thymine was irradiated, the spectrum obtained was derived almost exclusively from a radical on the thymine ring, showing preferential energy transfer to thymine from adenine. Similarly, a co-crystal of AMP and TMP or GMP and CMP gives TMP or CMP signal.

The excitation energy transfer in DNA and its constituents has been demonstrated from the fluorescence and phosphorescence decay spectra after an electron-pulse irradiation to samples. Also, ESR and luminescence studies include evidence for the transfer of electron or energy from protein to DNA.

It is generally known that studies of organic free radical by the ESR methods frequently face some difficulty because of the shortness fo their life times. A spin-trapping methods combined with ESR spectroscopy was utilized to obtain evidence for the precursor radicals leading to damage in irradiated DNA

Lethal damage to the living cell by ionizing radiation is mainly due to OH-induced DNA strand break. For ribose-phosphate, ESR spectra observed consisted of signals due to $-C-5'-H_2$ radicals which were initiated by H-abstraction from the sugar at the C-4′ position and formed by the radical transfer to the C-5′ position. It is possible to propose that strand breaks of DNA, in the presence of oxygen, mainly start from the radical at the C-5′ position of the sugar moiety.

Characterization of OH-induced free radical using UMP and poly U was performed by a method combined with spin-trapping and radical chromatography. The 4-yl radical and the 5-yl radical, formed by H-abstraction at the C-4′ and C-5′ positions of sugar moieties, respectively, were identified as precursors of strand break. The 5-yl radical and the 6-yl radical were identified as precursors of various oxidized products of the base moiety. From the similarity of the free radicals of UMP and poly U, it is suggested that the reactivities of OH radicals with nucleotides are identical to those in poly-nucleides.

Informational damages to the living cell by ionizing radiation is due to OH-induced thymine glycol and dihydrothymine. The ESR spectra obtained after irradiation of DNA were identified as the spin aducts of radicals produced at the thymine base moiety of DNA. The 6-yl radical was identified as a precursor of thymine glycol and the 5-yl radical as a precursor of dihydro-thymine.

Reaction of the hydrated electron with histone H1 and related compounds studied by e.s.r. and spin-trapping

O. INANAMI, M. KUWABARA, M. HAYASHI, G. YOSHII†, B. SYUTO‡ and F. SATO

Department of Radiation Biology and ‡Department of Biochemistry, Faculty of Veterinary Medicine, Hokkaido University, Sapporo 060, †Department of Radiology, Kansai Medical University, Moriguchi 570, Osaka, Japan

(*Received 21 February 1985; revision received 15 May 1985; accepted 24 June 1985*)

The reactions of the hydrated electron with histone H1, protamine and related compounds (poly-L-lysine, poly-L-arginine and poly-D,L-alanine) were investigated by the spin-trapping technique. In order to identify the radical structure of the spin-adducts originating from macromolecules, the usual spin-trapping technique was developed as follows: N_2-saturated aqueous solutions of proteins containing sodium formate were X-irradiated (4·5 kGy) in the presence of 2-methyl-2-nitrosopropane (MNP) as a spin-trap. The side-products due to the self trapping of MNP radicals were then removed from the spin-adducts of the proteins by a Sephadex G-25 column. Finally the spin-adducts were enzymatically digested to transform the broad e.s.r. signals due to slow tumbling of nitroxyl radicals to identifiable ones. The e.s.r. spectra obtained for all samples showed thet the deaminated radical, R—ĊH—CO—NH— (R: amino acid side chain), was produced.

Furthermore, polyacrylamide gel electrophoresis of the irradiated protamine and histone H1 indicated reduction of molecular size. These results confirm that hydrated electrons react with proteins and induce the deamination reaction which leads to main-chain scission.

Indexing terms: e.s.r., spin-trapping, hydrated electron, histone H1,

1. Introduction

The spin-trapping technique has been demonstrated to be useful for the identification of radicals produced by radiolysis in aqueous solution of nucleic acid constituents (Riesz and Rustgi 1976, Joshi *et al.* 1976, Kuwabara *et al.* 1981, 1983) and protein constituents (Rustgi and Riesz 1978, Joshi *et al.* 1978, Makino *et al.* 1984, Lion *et al.* 1980, 1982). In this method, 2-methyl-2-nitrosopropane (MNP) is used as a reagent which converts short-lived radicals (R˙) into long-lived nitroxide radicals (the spin-adducts) (Janzen 1971, Lagercrantz 1971).

$$R^{\cdot} + tBu\text{–}N{=}O \longrightarrow R\text{–}\underset{tBu}{N}\text{–}O^{\cdot} \longleftrightarrow R\text{–}\overset{+}{\underset{tBu}{N}}\text{–}O^{-}$$

The stable nitroxide radicals are detected by e.s.r. and identified from hyperfine coupling constants. Although spin-trapping is a useful method of studying radiation-induced short-lived free radicals, the e.s.r. spectra obtained from macromolecules such as RNase-S-peptide (Riesz and Rustgi 1979), insulin-A-chain (Lion

et al. 1982), poly(L-proline)$_{95}$ (Lion *et al.* 1980), poly U and calf thymus DNA (Joshi *et al.* 1976) sometimes exhibit broad line widths due to the slow tumbling of nitroxide radicals, and the exact assignment of the radical structures is uncertain.

In this study, the reaction of hydrated electrons with histone H1 (calf thymus, mol. wt = 22000), protamine (salmon roe, mol. wt = 4400) and their related three synthetic homopolymers were investigated by the spin-trapping method. Polyacrylamide gel electrophoresis was also carried out in order to clarify the molecular reduction of the proteins. In spin-trapping experiments, to overcome the experimental difficulties described above, the spin-adducts originating from protein radicals were enzymatically digested to oligopeptide-radicals to produce e.s.r. spectra consisting of well-resolved hyperfine structures. This method made possible the identification of radical structures of damaged sites produced by X-irradiation of aqueous solutions containing intact proteins.

2. Materials and methods

2.1. *Chemicals*

The sources of chemicals used in this work were as follows: Poly-L-lysine and poly-L-arginine from the Protein Research Foundation, Osaka; poly-D,L-alanine and trypsin (EC 3.4.21.4) from Sigma Chemical Company; nagarse (i.e. subtilisin, EC 3.4.21.14) from Nagase Industries Ltd; and protamine sulphate (from salmon roe) from Wako Pure Chemical Company Ltd. Before use, the protamine was purified by gel filtration on Sephadex G-25. The spin-trapping reagent, 2-methyl-2-nitroso-propane (MNP) was acquired from Aldrich Chemical Company.

2.2. *Preparation of histone H1*

Calf thymus histone (whole histone) was extracted by the procedure described by Murray (1966). Histone H1 was isolated from whole histone by Bio-Gel P-60 column chromatography (Van der Westhyuyzen *et al.* 1974). The purity of histone H1 was examined by acid-urea polyacrylamide gel electrophoresis (Panyim and Chalkley 1969).

2.3. *Spin-trapping and e.s.r.*

Protein (3 mg) was dissolved in 1 cm^3 of an aqueous solution containing of 0·5 mol dm^{-3} sodium formate. Sodium formate was added as a scavenger of $\dot{O}H$ and H radicals. MNP powder (1·5 mg) was added to 1 cm^3 of the solution and dissolved by stirring the solution overnight at 28°C. The sample was then bubbled with N_2-gas for 20 min and sealed in a Pyrex tube. The solution was then exposed to 4·5 kGy of X-rays with an X-ray generator (KXC-18, Toshiba) operating at 170 kVp and 25 mA at a dose rate of 30 Gy min^{-1}.

Immediately after irradiation the solution was applied on a Sephadex G-25 column (1·5 × 25 cm) equilibrated with distilled water. Distilled water was used as the eluent for chromatography, 0·8 cm^3 fractions being collected at a flow rate of 15 cm^3 h^{-1}. Each fraction was freeze-dried, and the sample dissolved again in 0·2 cm^3 of 10 mol dm^{-3} Tris–HCl buffer (pH 8·0). Enzymatic digestion of the proteins was carried out by adding trypsin or nagarse solution to a ratio of 1 : 50 (w/w) with respect to the protein content and incubating at 37°C for 30 min.

E.s.r. measurements of the digested spin-adducts were made on a JEOL ME X-band spectrometer. The e.s.r. spectra from spin-trapped radicals were recorded as first derivatives at room temperature unless otherwise specified. E.s.r. scans were

traced with 100 kHz field modulation of 0·02 mT amplitude, and the microwave power level was usually 10 mW to avoid saturation of the e.s.r. signals.

2.4. *Polyacrylamide gel electrophoresis*

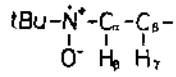
Protamine (0·4 mg cm^{-3}) and histone H1 (0·5 mg cm^{-3}) were dissolved in H_2O containing 0·5 mol dm^{-3} sodium formate (pH 6·0–6·7), respectively. The solution was bubbled with N_2-gas for 20 min, sealed in a Pyrex tube and X-irradiated.

Electrophoresis of X-irradiated protamine was performed in 15 per cent acrylamide gels in the presence of 2·5 mol dm^{-3} urea according to the method of Panyim and Chalkley (1969). In the case of X-irradiated histone H1, electrophoresis was carried out in 18 per cent polyacrylamide gels in the presence of 0·15 per cent SDS essentially according to the procedure of Laemmli (1970), as modified by Thomas and Kornberg (1975). Proteins in the gels were stained with 0·1 per cent Coomassie Brilliant blue R-250, 10 per cent (v/v) acetic acid and 40 per cent (v/v) ethanol.

3. Results and discussion

3.1. *Spin-trapping and e.s.r.*

To elucidate the reactions which occur in the proteins as described above, spin-trapping-e.s.r. experiments were carried out. The e.s.r. spectra of the spin-trapped radicals generally show a primary triplet structure due to the ^{14}N of the nitroxide group which is accompanied by secondary splitting. Secondary splitting is characteristic of the trapped radical and is useful for its identification. The α, β and γ positions of the magnetic nuclei are defined with respect to the unpaired electron on the nigrogen of the nitroxide group (Riesz and Rustgi 1979). This is illustrated below.



3.1.1. *Protamine*

When an aqueous solution of protamine in the presence of MNP and sodium formate was X-irradiated, the spectrum shown in figure 1 (*a*) was obtained. This spectrum has three sets of lines labelled (S), (L) and (D). The lines labelled (S), which consist of a primary nitrogen splitting of 1·72 mT, are due to the di-*t*-butylnitroxide radical (Lagercranz 1971). The lines labelled (L) have a primary triplet of 1·58 mT which further splits into a 1:2:1 intensity triplet arising from two equivalent β-protons (0·86 mT). This signal was assigned to the spin adduct of MNP itself, although the hyperfine couplings were different from those reported by Makino (1980). This conclusion was confirmed by the fact that this signal could be observed even in the absence of proteins. In previous papers by Kuwabara *et al.* (1981, 1983), a similar signal has already been presented. The lines labelled (D) seem to consist of a secondary doublet due to a β-proton, but the lines labelled (S) and (L) due to side products prevent the estimation of hyperfine coupling constants of the spectrum (D). In order to obtain accurate hyperfine coupling constants, these spin-adducts were separated by Sephadex G-25 column chromatography. The spin-adducts showing a secondary doublet labelled (D) were eluted in the void volume and corresponded to protein fractions (confirmed by the procedure of Lowry *et al.*

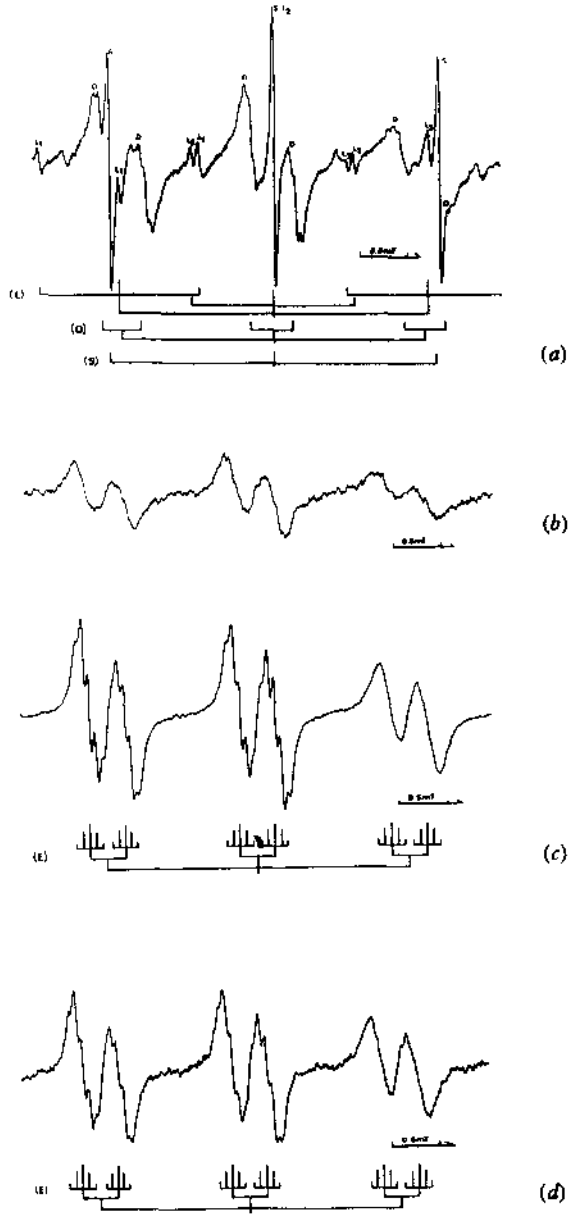


Figure 1. (*a*) E.s.r. spectrum of spin-trapped radicals from the X-radiolysis of N_2-saturated aqueous solutions of protamine containing MNP and $NaHCO_2$. (*b*) E.s.r. spectrum obtained from the chromatographic fractions, which corresponds to protein fractions. (*c*) E.s.r. spectrum separated by chromatography and followed by tryptic digestion of the spin-adducts. (*d*) E.s.r. spectrum separated by chromatography and recorded at 45°C.

1951), and the side products were eluted more slowly and found to contain no protein. Fractions containing the spin-adducts from the spin-trapping of protein radicals were concentrated by freeze-drying. The e.s.r. spectrum (figure 1 (*b*)) from the concentrated protein radicals showed a broad secondary douhlet due to a β-