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Source: *Radiation Research*, Vol. 61, No. 1 (Jan., 1975), pp. 47-54
Published by: Radiation Research Society
Stable URL: http://www.jstor.org/stable/3574057
Accessed: 28/11/2013 22:46

RADIATION RESEARCH 61, 47–54 (1975)

Electron Spin Resonance of Gamma- and X-Irradiated Nucleic Acid Base Pairs

1-Methylcytosine: 5-Fluorouracil Co-Crystals at 77°K[1]

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FARLEY, R. A., AND BERNHARD, W. A. Electron Spin Resonance of Gamma and X-Irradiated Nucleic Acid Base Pairs. 1-Methylcytosine: 5-Fluorouracil Co-crystals at 77°K. *Radiat. Res.* 61, 47–54 (1975).

The predominant free radical trapped in single crystals of a hydrogen-bonded complex of 1-methylcytosine and 5-fluorouracil x-irradiated between 77°K and room temperature has been identified. It is characterized by hydrogen atom extraction from N(1) of the 5-fluorouracil moiety, the unpaired electron interacting with the N(1) and F nuclei of this molecule. The principal values of the hyperfine and g-tensors are: $[A_x{}^{\mathrm{N}} = 14.4,\ A_y{}^{\mathrm{N}} = A_z{}^{\mathrm{N}} = 0.0]$; $[A_x{}^{\mathrm{F}} = 156.4,\ A_y{}^{\mathrm{F}} = 16.4,\ A_z{}^{\mathrm{F}} = 16.1\ \mathrm{G}]$; $[g_u = 2.00114,\ g_v = 2.00564,\ g_w = 2.00620]$. The radical is present at 77°K and decays upon warming to room temperature. There is at least one additional radical present at 77°K, but its structure has not been determined.

INTRODUCTION

Free radical formation in purines and pyrimidines can be influenced by the matrix in which they are irradiated. Of particular interest are the systems in which two different nucleic acid bases are complexed together, providing situations which approximate the close proximity of bases in nucleic acid polymers. Schmidt and Snipes (*1*) have studied free radical formation in polycrystalline samples of 1-methythymine: 9 methyladenine hydrogen-bonded base-pairs. Though these authors obtained good evidence for the occurrence of spin migration to 1-methylthymine on warming from 77°K to room temperature, it was not possible to determine the structure of the free radicals preceding formation of the 1-methyl-5,6-dihydrothymine-5-yl radical because of the inherent difficulties in working with polycrystalline samples. Others have also observed a preferential localization of free radical damage on pyrimidines. The work of Gregoli *et al.* (*2*)

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[1] This paper is based on work performed under contract with the U. S. Atomic Energy Commission at the University of Rochester Atomic Energy Project and has been assigned Report No. UR-3490-571.

[2] Supported in part by National Defense Education Act Fellowship.

[3] Supported by Public Health Service, Research Career Program Development Award.

indicates that spin migration is facilitated by interactions between stacked bases, whereas Singh (*3*), Cook and Wyard (*4*), and Schmidt and Snipes (*1*) have proposed that spin migration occurs across hydrogen bonds.

In an effort to determine what mechanisms contribute to the transfer of free radical damage from one base to another we have undertaken the investigation of single crystals of base-paired complexes. It is generally difficult to obtain crystals of these complexes suitable in size for ESR analysis but in the case of 1-methylcytosine:5-fluorouracil (1MeC:5FU) large crystals are readily obtained.

Gamma- or X-irradiation of 1 MeC:5FU crystals at 77°K results in the production of at least two free radicals that can be observed at that temperature. The most predominant radical is the subject of this report. It accounts for about 80% of the spectral intensity at doses between 20 and 40 MR and decays upon annealing to 300°K. Analysis of the other radical which is stable at 77°K, at these and lower doses, and which remains after annealing, has so far been unsuccessful due to considerable spectral overlap and complexity.

EXPERIMENTAL METHODS

Co-Crystal of 1-Methylcytosine:5-Fluorouracil

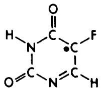
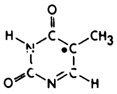
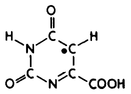
1-Methylcytosine was purchased from Cyclo Chemical Corporation and 5-fluorouracil from the Sigma Chemical Company. Single crystals of the complex were obtained by slow evaporation of an aqueous solution containing equal molar concentrations of each compound. The crystals used in this study typically weighed 0.1 g. Partially deuterated crystals were obtained from 99.8% D_2O solutions. The composition and structure of the crystals were verified by uv spectroscopy and X-ray diffraction. The space group symmetry and unit cell dimensions, determined with a Buerger precession camera, agree with those reported for the complex by Kim and Rich (*5*). The hydrogen-bond scheme between molecules and the orientation of the molecules with respect to the crystal axes are presented in Fig. 1. The molecules are arranged so that no two pyrimidine rings on adjacent levels overlap.

Electron Spin Resonance

Crystals were aligned in one of three orthogonal planes using the Buerger precession camera, transferred to an ESR sample holder, and then rechecked for alignment with the precession camera. Duco cement (Dupont) mixed with powdered copper (Fisher) was used to adhere the crystals to a copper rod for X-band ESR measurements. The irradiated Duco cement gives a background signal but this did not affect the present analysis.

X-band ESR measurements were taken with a modified version of the EPR cryostat developed by Weil *et al.* (*6*). Irradiations were performed using a 50-kV X-ray tube (Machlett) at a dose rate of about 2 MR/hr to total doses of up to 40 Mrads. ESR data were collected using a Varian E-12 spectrometer by rotating the crystal in 5° increments through each of the three orthogonal planes. Measurements of the static magnetic field were made continuously with an NMR

TABLE II
COMPARISON OF FREE RADICAL STRUCTURES OF SIMILAR HYDROGEN-ABSTRACTION RADICALS.

I	II	III
		
$\rho^{2p}_{N(1)} = 0.28$ $= 0.28$ (DULCIC & HERAK) $= 0.31$ (COOK et. al.)	$\rho^{2p}_{N(1)} = 0.24$	$\rho^{2p}_{N(1)} = 0.29$
$\rho_{F} = 0.10$	$\rho_{H(CH_3)} = 0.09$	
$\rho_{C(5)} \cong 0.75$	$\rho_{C(5)} = 0.66$	$\rho_{C(5)} = 0.64$
$\rho_{N(3)} \cong 0$	$\rho_{N(3)} \cong 0$	$\rho_{N(3)} \cong 0$
REFERENCE	10	12

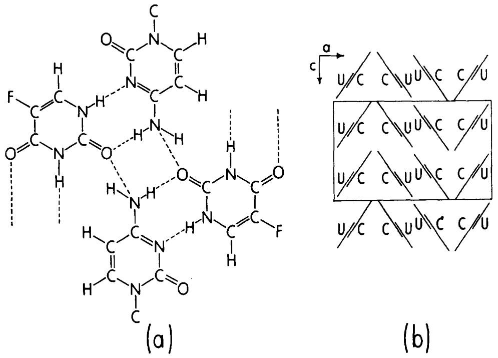


FIG. 1. Hydrogen-bond association between molecules of 1-methylcytosine and 5-fluorouracil in the (502) crystallographic plane (a), and orientation of the molecules with respect to the crystal axes (b). Each of the four partially illustrated hydrogen bonds, extending from 5-fluorouracil, go to an adjacent 5-fluorouracil forming N–H... O bonds. Redrawn from Kim and Rich (*5*).

gaussmeter (Alpha Scientific) and of microwave frequency using a frequency counter and transfer oscillator (Hewlett-Packard).

RESULTS AND ANALYSIS

Spectra of the irradiated crystal complex of 1MeC:5FU observed with the magnetic field (**H**) parallel to the ⟨b⟩ and ⟨c⟩ crystallographic axes are shown in Fig. 2. With **H** parallel to ⟨c⟩ or ⟨a⟩ the dominant feature is a doublet of triplets. The extreme high field line in both spectra is due to free radicals in the cement used to mount the crystal, and the remaining small lines are attributed to additional unknown free radicals. On rotating the magnetic field into ⟨b⟩ the triplet hyperfine splitting collapses to zero and the doublet reaches a minimum. The angular dependence of these hyperfine splittings, along with the *g*-value variation, is shown in Fig. 3. The doublet interaction is characteristic of fluorine coupling and the triplet of nitrogen coupling.

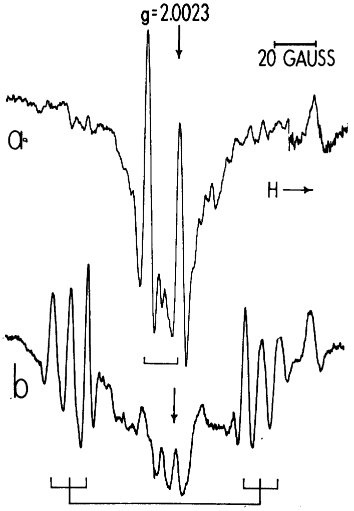


FIG. 2. Second-derivative ESR spectra obtained with the magnetic field **H** parallel to the ⟨b⟩ crystallographic axis (a) and the ⟨c⟩ crystallographic axis (b). The arrows mark the position of the free spin g value. Resonance lines of the radical reported here are noted by solid lines under the spectra. Modulation amplitude = 1.25 G, microwave power = 0.1 mW, microwave frequency = 9.5 GHz.

Analysis of the hyperfine interactions was performed following the method of Lund and Vänngård (7), and of the g tensor using the method of Schonland (8). The principal value of the hyperfine and g tensors are presented in Table I. These values along with the given direction cosines were used to obtain the solid lines which fit the data in Fig. 3. All interactions are axially symmetric, within experimental error. The maxima of both hyperfine interactions and the minimum g value occur in a direction perpendicular to the molecular plane, with the remaining principal values lying in the molecular plane. Data obtained in the (502)

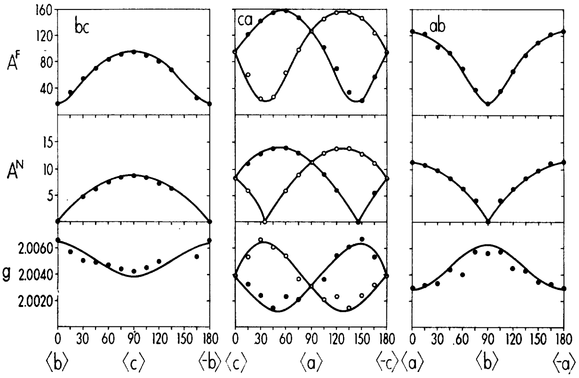


FIG. 3. Variation of the hyperfine and g interactions with orientation of the crystal in the magnetic field. Open and closed circles denote the two magnetically distinct sites in the unit cell. The solid curves were obtained from the tensor parameters reported in Table I. The data points, illustrated for every 15°, are only part of the data which were actually collected every 5°.

TABLE I

PRINCIPAL VALUES OF HYPERFINE AND g INTERACTIONS OF THE HYDROGEN ABSTRACTION RADICAL OF THE FLUOROURACIL MOLECULE, AND DIRECTION COSINES RELATING THE PRINCIPAL AXES TO THE CRYSTALLOGRAPHIC AXES

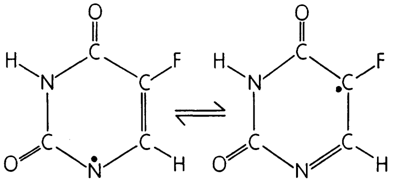
Tensor component	*Principal value*[a]	*Direction cosines*[b]		
		$\langle a \rangle$	$\langle b \rangle$	$\langle c \rangle$
$\lvert A_x^{\mathrm{F}} \rvert$	156.4 ± 0.4	0.79	0.02	±0.62
$\lvert A_y^{\mathrm{F}} \rvert$	16.4 ± 0.5	0.00	1.00	−0.03
$\lvert A_z^{\mathrm{F}} \rvert$	16.1 ± 0.5	±0.62	0.02	0.79
$\lvert A_x^{\mathrm{N}} \rvert$	14.4 ± 0.5	0.78	0.00	±0.63
$\lvert A_y^{\mathrm{N}} \rvert$	0.0 ± 0.5	0.00	1.00	0.00
$\lvert A_z^{\mathrm{N}} \rvert$	0.0 ± 0.5	±0.63	0.00	0.78
g_u	2.0011 ± 0.0006	0.77	0.00	±0.64
g_v	2.0056 ± 0.0006	0.00	1.00	0.00
g_w	2.0062 ± 0.0006	±0.57	0.00	0.82

[a] Values are not Breit-Rabi corrected.

[b] Estimated error in principal axes ±5°.

crystallographic plane, which contains the pyrimidine plane of one of the two magnetically distinct sites, confirm that the hyperfine and g interactions are axially symmetric. There is no observable hydrogen hyperfine coupling, and deuterium substitution of exchangeable hydrogens does not affect the spectra beyond a slight narrowing of line widths.

The free radical which bests fits the above data is given below as structure I.



I

The two resonance structures emphasize the positions expected to have high unpaired electron densities. Additional resonance structures are readily constructed which suggest further delocalization of the unpaired electron to the carbonyl oxygens. Other alternative free-radical assignments are discussed in the next section.

An estimate of the spin populations of fluorine s and p orbitals can be made from the relations

$$\rho_{2p}{}^{\mathrm{F}} = \lvert A_{\mathrm{dip}}{}^{\mathrm{F}}/A_{2p} \rvert, \qquad \rho_{1s}{}^{\mathrm{F}} = \lvert A_{\mathrm{iso}}{}^{\mathrm{F}}/A_{1s} \rvert, \qquad \rho_{2s}{}^{\mathrm{F}} = \lvert A_{\mathrm{iso}}{}^{\mathrm{F}}/A_{2s} \rvert .$$

The values of the isotropic and dipolar couplings, $A_{\mathrm{iso}}{}^{\mathrm{F}} = 63.0$ G and $A_{\mathrm{dip}}{}^{\mathrm{F}} = 46.7$ G, are readily determined from $A_x{}^{\mathrm{F}}$ and $A_y{}^{\mathrm{F}} = A_z{}^{\mathrm{F}}$ given in Table I. Assuming $A_{2p} = 470$ G (9), $A_{1s} = 321{,}000$ G (9), and $A_{2s} = 16{,}400$ G (9), it is found that $\rho_{2p}{}^{\mathrm{F}} = 0.099$, $\rho_{1s}{}^{\mathrm{F}} = 0.00019$, and $\rho_{2s}{}^{\mathrm{F}} = 0.0038$. The estimates of *s*-orbital density are upper limits since it is assumed that either the 1*s* or 2*s* orbitals are the sole source of isotropic hyperfine coupling. A similar calculation for nitrogen gives $\rho_{2p}{}^{\mathrm{N}} = 0.28$ and $\rho_{2s}{}^{\mathrm{N}} = 0.0087$. Comparable values for ρ^{N} are obtained using either the method employed by Dulcic and Herak (*10*) or that used by Cook, Elliot, and Wyard (*11*).

DISCUSSION

Hydrogen abstraction from nitrogen, resulting in a molecular π radical, has been postulated to occur in four other pyrimidines: thymine (*10*), orotic acid (*12*), cytosine (*11*), and 5-bromouracil (*13*). The assigned structures for the first two of these free radicals are compared to that of structure I in Table II. The latter two are not included in Table II because there is some question about the structure assignment to this cytosine radical (*18*), and the analysis was not sufficiently complete to allow the spin densities to be determined for 5-bromouracil. The method used to determine the nitrogen spin densities shown in Table II were different for thymine and orotic acid, and are also different from the calculation used here for 5-fluorouracil. All of the methods used, however, yield a similar range of values, none being changed by more than 15%. $\rho_{\mathrm{C}(5)}$ for thymine was determined from the methyl splitting of 19.5 G, observed by Dulcic and Herak (*10*), using $A(\mathrm{CH_3}) = \rho_{\mathrm{C}(15)}Q$, with $Q = 29.25$ G (*14*). In 5-fluorouracil $\rho_{\mathrm{C}(5)}$ was estimated from the isotropic α-fluorine coupling published in Refs. *15–17*. From these published values a plot of α-fluorine coupling as a function of carbon spin density is found to be nearly linear within the range of reported values. $\rho_{\mathrm{C}(5)}$ for 5-fluorouracil is found by interpolation to be approximately 0.7. It is seen in Table II that these three hydrogen abstraction radicals are characterized by comparable spin densities at the nitrogen and C(5) loci.

It is not possible to distinguish by ESR methods alone which nitrogen, N(1) or N(3), is the source of the nitrogen hyperfine coupling in any of the above radical assignments. From calculations of unpaired electron distributions (*10*) or by inspection of simple resonance structures, however, N(1) is found to be the more likely site of high spin density. For thymine, for example, Dulcic and Herak (*10*) concluded that H abstraction from N(3) should result in an observable hyperfine interaction with $\mathrm{H}_{\mathrm{N}(1)}$, whereas H abstraction from N(1) would not result in an observable splitting due to interaction with $\mathrm{H}_{\mathrm{N}(3)}$. Since deutration at N(1) and N(3) does not affect the hyperfine pattern, they considered H abstraction at N(1) of thymine to be the more tenable assignment. Deuteration of 5-fluorouracil gives the same result and it is similarly concluded that hydrogen abstraction most likely occurs at N(1).

Since hydrogen abstraction from 5-fluorouracil accounts for about 80% of the free radical population at 77°K, it is concluded that 5-fluorouracil is the preferred site of free radical stabilization in the co-crystal. Preliminary ESR observations on single crystals of 5-fluorouracil indicate that the minor free radical product in the co-crystal is also formed on the 5-fluorouracil moiety. Though this free radical could not be identified in the co-crystal, its spectra show a marked similarity to those recorded for 5-fluorouracil at 77°K.[4]

It is of interest to consider the possibility that spin migration occurs from 1-methylcytosine to 5-fluorouracil thereby resulting in a disproportionately large population of radicals characterized by hydrogen abstraction from 5-fluorouracil. Though we have no direct information about the mechanism of hydrogen abstraction from 5-fluorouracil, spin migration from 1-methylcytosine does not seem a likely explanation. Hydrogen abstraction, as observed here, occurs in at least three other pyrimidines implying that this class of free radical evolves from the same type of precursor. A likely precursor, suggested by Dulcic and Herak, is the radical cation. Subsequent deprotonation would result in the hydrogen abstraction radical. If this mechanism is correct, it indicates that spin migration from 1-methylcytosine is not a likely source of this radical. It remains, then, to account for the lack of 1-methylcytosine-centered free radicals. Assuming that the initial ionizations are randomly distributed between the two different pyrimidines, the above argument leads to the additional conclusion that the 1-methylcytosine cations decay by electron transfer to or from another 1-methylcytosine radical or a 5-fluorouracil anion.

ACKNOWLEDGMENTS

The authors thank Mr. Kermit Mercer for his assistance with the performance of these experiments, Dr. Fouad Ezra for many helpful discussions during the course of this investigation, and Dr. John Corelli for providing some of the crystals used in this work, and for his encouragement while this work was in progress.

Received: July 5, 1974

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