

ELECTRON SPIN RESONANCE OF A GAMMA-IRRADIATED SINGLE CRYSTAL OF 3'-CYTIDYLIC ACID*

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Electron spin resonance (ESR) studies have provided valuable information concerning the effects of ionizing radiation on nucleic acids and their constituents (*see ref. 1 for a review*). This technique permits the study of free radical species which presumably are intermediates between the initial ionizing event and a later chemical lesion causing loss of biological function. In molecules as complex as DNA and RNA, radicals may be formed at several sites so that the ESR spectrum is a complicated superposition of the spectra of all these radicals. In order to understand more fully the nature of radical formation in nucleic acids, we have studied irradiated single crystals of nucleic acid constituents and related compounds.²⁻⁵ ESR analyses of single crystals provide more positive identification and a more detailed description of the radical species than can be obtained from polycrystalline samples. We report here an ESR single crystal analysis of the nucleotide 3'-cytidylic acid. The primary radical formed in this compound by irradiation at room temperature has been identified and the parameters characterizing its ESR absorption have been measured.

Description of the Crystals.—Cytidylic acid was obtained as a mixture of 2' and 3' isomers. This mixture was dissolved in dilute hydrochloric acid solution (~5%), evaporated to dryness, redissolved in distilled water, and filtered. From this solution single crystals were grown by slow evaporation at room temperature. They had well-developed faces with dimensions of several millimeters. A silver nitrate test showed no chloride ions present in a solution of redissolved crystals. Thin-layer chromatography of this solution using a 25:4:1 mixture of saturated (NH₄)₂SO₄:0.2 M NH₄OH:isoamyl alcohol as solvent showed only the 3' isomer to be present. This chromatography system was capable of separating the two isomers from the original mixture.

X-ray diffraction data taken on a small chip from one of the crystals gave orthorhombic unit cell dimensions of 8.72, 21.6, and 6.92 Å. Within experimental error, these dimensions are the same as those reported for 3'-cytidylic acid by Alver and Furberg,⁶ and we shall assume our crystals to be identical to those for which these authors have determined the crystal and molecular structure. The space group is P2₁2₁2, with four molecules in the unit cell. The crystals were usually elongated along *c*, with (010) dominating. Figure 1 shows both the habit of crystals used in this study and the crystallographic axis system. The symmetry of the crystal structure is such that not all four molecules in the unit cell are magnetically distinct. With reference to the crystallographic planes only two are magnetically distinct, and with reference to the crystallographic axes all four are magnetically equivalent.

A pale, yellow-brown color developed when the crystals were irradiated with cobalt-60 gamma rays. After prolonged storage at room temperature the color

turned to deep purple. This was accompanied by spectral changes in the ESR absorption. More careful analysis revealed that the primary radical formed at room temperature converts, with some decay, into a secondary radical upon storage. A small contribution from this secondary radical was seen even in spectra taken immediately after irradiation, but this could be minimized by irradiating at 77°K and making observations at room temperature. The analysis presented here pertains only to the primary radical species observed at room temperature; no identification of the secondary radical was made.

Procedures for making measurements of ESR parameters have been described previously³ and will not be repeated here. Measurements were made at both X-band (9.3 kMc/sec) and P-band (16.3 kMc/sec) microwave frequencies.

Analysis of Spectra.—Figure 2 shows ESR spectra observed for two orientations of the crystal in the magnetic field (H) of the spectrometer. For H along the b axis, the spectrum of the primary radical consists of four lines with approximately equal intensity. The splitting between these lines was found to be the same at two microwave frequencies, showing that it is due to hyperfine interaction. Such a hyperfine pattern can arise from unequal coupling of the unpaired electron with two protons. For H along c , the magnitudes of the two couplings are nearly equal so that the central component is only poorly resolved. Along a , the spectrum is similar to that along b , but with different values for the hyperfine couplings. Spectral lines of lesser intensity from the secondary radical can also be seen in Figure 2.

At other crystal orientations two magnetically distinct molecules were observed with different hyperfine couplings and different values for the spectroscopic splitting factor (g). By taking spectra at sufficiently close intervals at X- and P-band, it was possible to follow the spectral lines for each of these molecules throughout the crystallographic planes. In this manner, the variation in each hyperfine constant and in the g value was obtained. One of the hyperfine couplings was quite anisotropic, varying in magnitude from 6 to 29 gauss. The other was almost constant with a magnitude varying from 28 to 31

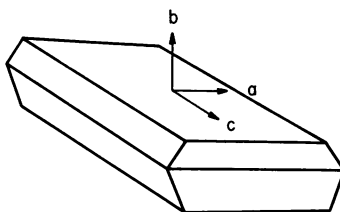
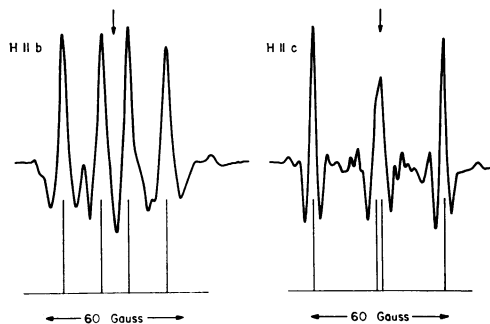


FIG. 1.—Typical habit of 3'-cytidylic acid crystals used in this study and the crystallographic axis system employed.

FIG. 2.—Second-derivative ESR spectra of a gamma-irradiated single crystal of 3'-cytidylic acid at two orientations in the magnetic field. Bars beneath the curves show the predicted pattern for the primary radical formed at room temperature. The magnetic field intensity increases from left to right, with the arrow marking the field position of DPPH for which $g = 2.0036$. The microwave frequency is 9.3 kMc/sec.



gauss. The g value varied from 2.0026 to 2.0062. Principal values for the anisotropic hyperfine coupling and for the g tensor were determined by methods outlined previously.³ These principal values and their corresponding direction cosines relating the principal axes to the crystallographic axes are presented in Table 1. In Figure 3 observed and calculated values for the hyperfine coupling

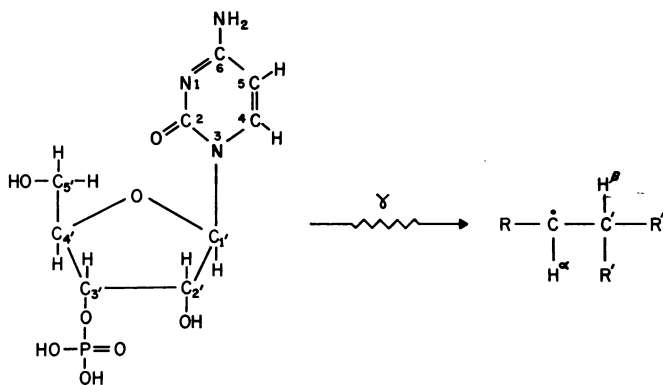
TABLE 1. *Principal values and their corresponding direction cosines relating the principal axes to the crystallographic axes.*

	Principal values	Direction Cosines		
		l	m	n
g_u	2.0063 ± 0.0002	0.77	0.42	0.47
g_v	2.0039 ± 0.0002	-0.49	-0.07	0.87
g_w	2.0026 ± 0.0002	0.40	-0.90	0.15
A_x^α	5.8 ± 1.0 gauss	-0.90	-0.44	-0.08
A_y^α	28.7 ± 1.0 gauss	0.21	-0.27	-0.94
A_z^α	16.7 ± 1.0 gauss	0.39	-0.86	0.33

Direction cosines for other molecules in the unit cell are given by $(l, -m, -n)$, $(-l, m, -n)$, and $(-l, -m, n)$.

and g are compared for the ab crystallographic plane. Similar comparisons were found for the bc and ca planes.

All our observations can be accounted for by a radical having an unpaired electron in a $2p$ -orbital on a carbon atom with hyperfine coupling to one α proton and one β proton. The primary action of gamma rays on 3'-cytidylic acid at room temperature can thus be described by



SCHEME I

where R , R' , and R'' represent groups which provide no resolvable hyperfine interaction with the unpaired electron. The spin density on the carbon atom ρ_C can be obtained from the relation

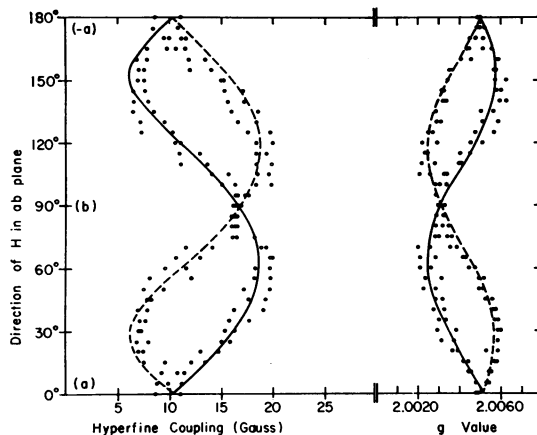
$$A_f = \rho_C Q.$$

The isotropic component of the α proton hyperfine coupling A_f is given by

$$A_f = 1/3(A_x^\alpha + A_y^\alpha + A_z^\alpha).$$

The magnitude of A_f , using values from Table 1, is 17 gauss. On theoretical

FIG. 3.—Variation of the α proton hyperfine coupling and the g value for irradiated 3'-cytidylic acid, with H in the ab plane. Solid circles represent data taken at X-band; open circles, data at P-band. Solid and dashed lines are theoretical curves for the two magnetically distinct molecules in the crystallographic plane, obtained by a least-squares fit to the data.³ From these and similar curves for the bc and ca planes, tensor elements given in Table 1 were obtained.



grounds, both A^{α} and Q are known to be negative. With $Q = 23$ gauss, the spin density on the carbon atom is 0.73. The remainder of the spin density must reside on some atom which has no nuclear magnetic moment.

Isotropic coupling to the β proton arises primarily from hyperconjugation of the p -orbital of the unpaired electron and the s -orbital of the hydrogen. The magnitude of this coupling depends on the dihedral angle ϕ which the p -orbital makes with a plane containing C, C', and H $^{\beta}$. The relation giving ϕ is approximately⁷

$$A^{\beta} = B \rho_C \cos^2 \phi,$$

where A^{β} is the β proton hyperfine coupling constant. With $B = 50$ gauss and $A^{\beta} = 29.5$ gauss, the angle calculated is $\phi = 26^{\circ}$.

Identification of the Radical.—There are a number of possible free radicals produced by covalent bond breakage in 3'-cytidylic acid that would have an α and a β proton. Nine possibilities have been considered; four of these are shown in Figure 4. Without detailed knowledge of the orientation of the

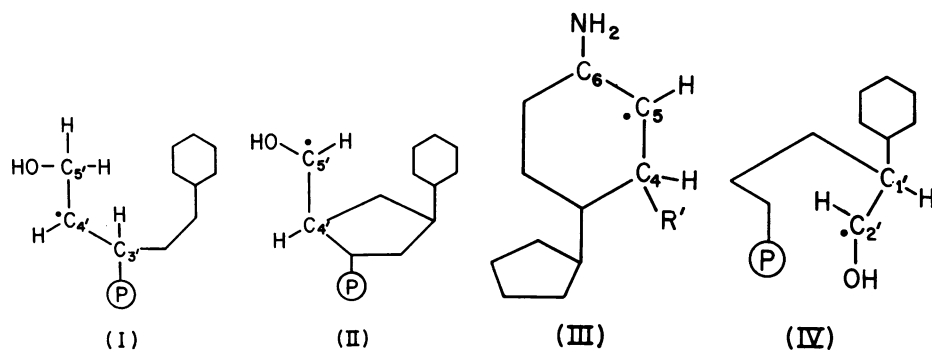


FIG. 4.—Four of the radicals which could be formed in 3'-cytidylic acid having an α proton and at least one β proton. The detailed molecular structure is only shown in the portion of radical near the unpaired electron.

molecules within the crystal lattice it would be difficult to distinguish between these various radicals. Fortunately this knowledge is available, and the observed directions of the principal axes for g and A^α can be compared with those calculated from the X-ray analysis data. This comparison relies on the assumption that radical formation is not accompanied by any major reorientation of the molecule within the crystal lattice.

The minimum g value for an unpaired electron in a carbon p -orbital is expected to occur when H is parallel to the p -orbital.⁸ This direction is normal to the plane formed by R, C, and C' (Scheme I) since C has planar sp^2 -hybrid bonding orbitals. From the atomic coordinates reported by Alver and Furberg⁶ this direction was calculated for each radical which could be produced by rupture of a single covalent bond. Only for structures (I) and (II) of Figure 4 was the calculated direction of g_w in reasonable agreement with that from Table 1. We conclude that without drastic molecular reorientation, none of the other radicals could give the experimental results obtained.

Discriminating between structures (I) and (II) is considerably more difficult since both these radicals have their p -orbitals near the expected direction. Structure (II) has an OH group attached to C, and for this radical it would be difficult to explain the absence of any hyperfine coupling to the hydroxyl proton. On the other hand, (I) has three β protons, and with dihedral angles ϕ much different from $\pi/2$ these should all give hyperfine coupling. For one of these, H^β , $\phi = 26^\circ$ as determined above. A three-dimensional model of the unit cell was constructed from the atomic coordinates, and it was found that one of the two hydrogens on $C_{5'}$ has the proper orientation to give the observed coupling. The other hydrogen on $C_{5'}$ lies in the nodal plane of the p -orbital and thus produces no hyperfine coupling. The β proton on $C_{3'}$ lies almost, but not quite, in the nodal plane of the p -orbital. A very slight reorientation produced by a rotation of 20° about the $C_{3'}-C_{4'}$ bond would put this proton at a position to give an undetectable hyperfine coupling. It was apparent from the unit cell model that such a small rotation could easily occur with no significant change in crystal structure.

The most convincing evidence that (I) rather than (II) is the correct assignment is provided by the principal directions for the α proton hyperfine coupling. The principal value least in magnitude is expected⁹ to occur with H along the C-H $^\alpha$ bond. The intermediate value occurs with H along the p -orbital, and the largest (negative) value in a direction perpendicular to these two. These directions were calculated for (I) and (II) and compared with the observed directions. In Table 2 the angle between the observed and calculated directions for

TABLE 2. *Angles between the observed and calculated directions for g_w , A_x , A_y , and A_z for (I) and (II).*

Element	Angle (degrees)	
	(I)	(II)
g_w	5.5	7
A_x^α	13.5	51
A_y^α	5	40
A_z^α	16	14

The calculated directions are based on atomic coordinates reported by Alver and Furberg.⁶

g_w , A_x^α , A_y^α , and A_z^α are given for (I) and (II). In general, the agreement is much better for (I) than (II). Reorientation of (II) so that there is agreement with the observed directions would require significant disruption of the crystal lattice. On the basis of these arguments we conclude that the radical in irradiated 3'-cytidylic acid has an unpaired electron localized on C_4' of the sugar moiety.

Mechanism of Radical Formation.—Radical (I) is formed by breaking the covalent bond between C_4' and the oxygen atom O_1' in the ribose sugar ring. Direct cleavage of the $C_4'-O_1'$ bond would leave an additional unpaired electron on O_1' . This O_1' portion of the molecule would then have to undergo secondary reactions since no additional radical comparable in quantity to (I) is observed. As is usually the case, the exact mechanism of radical formation is not known for 3'-cytidylic acid. However, we wish to present an interesting mechanism which can lead to the formation of radical (I).

Hydrogen abstraction is one of the most frequent occurrences in gamma-irradiated organic solids. The hydrogen atoms usually escape undetected from the crystal lattice or else recombine to form nonradical species. The mechanism we suggest for radical formation in 3'-cytidylic acid is initiated by hydrogen abstraction at C_1' (Fig. 5). Subsequent C-O breakage by this mechanism

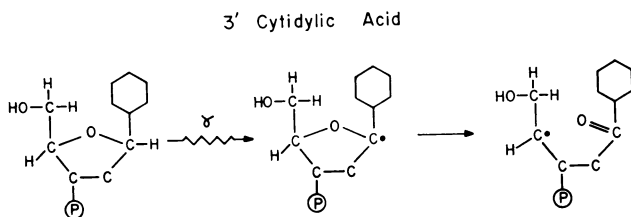


FIG. 5.—Suggested mechanisms for radical formation in 3'-cytidylic acid.

leaves an unpaired electron on C_4' with the formation of a double bond between the oxygen atom and C_1' . It is interesting to note in this regard that the intermediate is identical in form to the radical which is thought to be present in irradiated cytidine.¹⁰ The crystal structures of cytidine and cytidylic acid may be sufficiently different that the C_1' radical is stabilized in the former but undergoes subsequent molecular rearrangement in the latter.

Summary.—The primary radical formed in 3'-cytidylic acid by gamma irradiation at room temperature has been studied by ESR spectroscopy. The unpaired electron is localized on a carbon atom and has hyperfine interaction with one α proton and one β proton. A detailed comparison of the g tensor and the α proton hyperfine tensor with the known crystal structure shows that the radical most probably has the unpaired electron on C_4' of the ribose sugar moiety.

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