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FREE RADICALS FORMED BY HYDROGEN ATOM BOMBARDMENT OF THE NUCLEIC-ACID BASES*

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Independent and entirely different investigations in this laboratory¹ and in the Bell Telephone Laboratories² have shown that hydrogen addition on the $C_{(6)}$ of the thymidine ring produces the free radical observed with ESR in irradiated thymidine³ and in DNA irradiated under special conditions.^{4, 5} The present study provides objective proof that free hydrogen atoms combine directly at room temperature with ring carbons not only of thymine but also of uracil and of the purine bases adenine and guanine. In contrast, no corresponding evidence could be found for H-addition on the ring carbons of cytosine. These results are in agreement with work in progress in this laboratory which shows that the electron spin resonance (ESR) patterns of irradiated single crystals of cytoline do not resemble those expected for free radicals formed by such H-addition on $C_{(6)}$ or $C_{(5)}$.

The samples under present study are evacuated, are subjected to a beam of lowvelocity hydrogen atoms produced by dissociation of molecular hydrogen by an electric discharge, and are observed in the ESR spectrometer at room temperature while still under vacuum. The resulting free radicals are observed and identified by their ESR patterns. Because all proton couplings in the radicals thus formed are nearly isotropic, the proton hyperfine structure was easily resolved for the powdered samples used. Proof that the H-addition reaction occurred directly with H atoms from the beam came from observation of the expected changes in the ESR patterns which took place when deuterium atoms were substituted for bombarding hydrogen atoms.

Prior to our experiments, atomic hydrogen addition on the aromatic rings in polystyrene had been achieved with atomic hydrogen bombardment by Wall and Ingalls,⁶ who observed the resulting free radicals with ESR. Free radicals have also been produced by atomic H bombardment of malonic acid and similar organic compounds by Cole and Heller.⁷ In the latter experiments, H-addition reactions apparently did not occur, but rather H-abstraction and the consequent formation of H_2 and the observed free radicals.

In our experiments, care was required to prevent strong ultraviolet light and free electrons, also produced in the discharge, from impinging on the sample. Such radiations produce radicals giving ESR signals which tend to obscure those produced by the atomic bombardment. However, by use of strong magnetic field to deflect the electrons and by design of the discharge so that direct radiation of UV from the discharge could not impinge on the sample, the signal produced by the atomic hydrogen could be made to predominate.

Figures 1-4 illustrate the various results obtained. The top curve of Figure 1



FIG. 1.-Electron spin resonance spectra (second derivative curves) induced in thymine through bombardment with H or D atoms, under vacuum at room temperature. The bars give the theoretical hyperfine structure for the free radicals described in the text. The arrows indicate the position of the DPPH marker which corresponds to a =The extra line at the center, 2.0036.more evident for the D-bombarded sample, arises from unknown radicals probably produced by heating effects of the bombardment or from secondary radiations from the atomic source.

is the ESR pattern of the thymine powder after bombardment with H atoms. The bars represent the theoretical pattern expected for the free radical (I) obtained upon H-bombardment of thymine.



The lower curve of Figure 1 gives the corresponding observed pattern obtained by deuterium bombardment with the theoretically expected pattern for radical (II). The difference in pattern results from the difference in nuclear spins (I = 1 for deuterium as compared with I = 1/2 for hydrogen) and from the smaller nuclear magnetic moment of deuterium ($\mu = 0.857$ as compared with $\mu = 2.79$ for hydrogen). The thymine pattern in the top curve is like that observed for X-irradiated thymine or thymidine³ and consists of a triplet of quartets. The triplet splitting arises from an equivalent and isotropic coupling of the C₍₆₎H₂ hydrogens of 38 gauss; the quartet splitting, from the equivalent isotropic splitting of 20.5 gauss by the CH₃ protons of radical (I). These coupling constants are in good agreement with those found for γ -irradiated single crystals of thymidine.^{1b}

Figure 2 shows a similar comparison of the observed curves for uracil with bars giving the expected patterns for a radical formed by H (or D) addition on $C_{(6)}$. Although addition on the $C_{(5)}$ cannot be eliminated by the hyperfine pattern, the $C_{(6)}$ position is considered the more probable because the resulting radical would be stabilized by conjugation of structure (III) with structure (IV).



From the C₍₅₎H coupling of 18.5 gauss (see Table 1), we can obtain the π spin density on C₍₅₎ with the relation:⁸

$$\rho_{(5)} = A/Q = 18.5/26.2 = 0.71,$$

in which we have used Q = 26.2, as given by Fessenden and Schuler⁹ for >C—H fragments bonded to other carbons. Interestingly, this value of $\rho_{(5)}$ is within





FIG. 3.—ESR spectra of H- and D-bombarded guanine with conditions and explanations as given for Fig. 1.

experimental error, the same as the π spin density on C₍₅₎ of the thymine radical. Evaluation of the spin density on C₍₅₎ can also be found from the C₍₆₎H₂ coupling, but the calculations are more difficult and the results more uncertain.

Figure 3 shows the observed ESR for guanine induced by H and D bombardment. The bars indicate the expected proton and deuteron hyperfine structure for the free radicals:



The corresponding comparisons for adenine appear in Figure 4. In adenine there are two ringed carbons to which the H might be added to give radicals of structures (VII) or (VIII).





FIG. 4.—ESR spectra of H- and D-bombarded adenine with conditions and explanations as given for Fig. 1.

the free radical formed by H addition on $C_{(8)}$. The $C_{(8)}$ coupling constants observed for this radical are also listed in Table 1.

The experimentally observed CH₂ proton coupling in the adenine and guanine radicals indicates that the spin densities predicted by Pullman and Mantione¹⁰ are at least approximately correct, although an accurate test of their predictions is not possible. The coupling of these H's would arise from π orbital spin density of the entire molecule, but principally from hyperconjugative coupling with spin densities on the N₍₇₎ and N₍₉₎, located on either side of the coupling CH₂ group.



Either of these structures would give a triplet similar to that which is observed. Without an accurate knowledge of the relative magnitude of the couplings of the CH₂ protons of the radicals, it is impossible to decide which of these two radicals is the one observed. The molecular orbital calculations of Pullman and Mantione¹⁰ indicate, however, that (VII) would be the more probable, as do the relative couplings for adenine and guanine.

In Table 1 are given the various proton coupling constants measured for the different purine and pyrimidine radicals. We have obtained a triplet spectral pattern for xanthine (not shown) which is similar to that shown for H-bombarded guanine and which must also arise from

Bombarded compound	Coupling group	Proton coupling in gauss
Thymine	$\int >C_{(6)}H_2$	38
Uracil	$-CH_3$ $>C_{(5)}H$	$\begin{array}{c} 20.5 \\ 18.5 \end{array}$
Guanine	$\begin{cases} > C_{(6)} H_2 \\ > C_{(6)} H_2 \end{cases}$	33
Adenine	$>C_{(8)}H_2$	40
Xanthine	$>C_{(8)}H_{2}$	39

TABLE 1 PROTON COUPLING FOR FREE RADICALS PRODUCED BY ATOMIC HYDROGEN BOMBARDMENT OF PYRIMIDINE AND PURINE BASES

For the guanine radical (V) Pullman and Mantione predict $\rho_{(7)} = 0.39$ and $\rho_{(9)} = 0.08$; for the adenine radical (VII), $\rho_{(7)} = 0.43$ and $\rho_{(9)} = 0.09$. The observed CH₂ couplings are in agreement with the slightly higher spin densities predicted for (VII) over (V). Unfortunately, the knowledge about the amount of hyperconjugative coupling to be expected from an N which is coupled to a $C_{\beta}H_2$ group is not as good as that about a carbon coupled to this group. From electronegativity considerations, however, the spin density on the α N would be expected to give rise to a larger $C_{\beta}H_2$ coupling than would the same amount of π spin density on an α carbon. This is in accord with the results of comparison between the CH_2 coupling in the

purine and pyrimidine radicals and the spin densities predicted by Pullman and Mantione.

Our failure to resolve N¹⁴ hyperfine structure in the present experiment is not inconsistent with the relatively large spin density, ~ 0.40 , on one of the N's. This is p_{π}^{N} orbital density, but there is a small positive *s* orbital spin density, ρ_{2s} , induced by configuration interaction, which gives rise to an isotropic component in the N¹⁴ coupling given by

$$a_f{}^{\mathrm{N}} = \rho_{\pi}{}^{\mathrm{N}}Q,$$

where $Q \approx 25$ gauss.¹¹ The anisotropic component caused by dipole-dipole interaction with the nucleus is:

$$a_{\mu}{}^{\mathrm{N}} = \rho_{\pi}{}^{\mathrm{N}}A_{\mu}(3\cos^2\theta = 1),$$

where θ is the angle between the magnetic field and the axis of the p orbital and where $A_{\mu} \approx 15$ gauss.¹² The total coupling for a given radical with orientation θ is:

$$A^{N} = \rho_{\pi}^{N} (Q + A_{\mu} (3 \cos^{2} \theta - 1)).$$

The displacement of a given line by this N¹⁴ coupling would be $\nu = M_I A^N$, where $M_I = 1, 0, \text{ and } -1$. To obtain the line shape caused by the N¹⁴ coupling, one must find the number of molecules with orientations giving frequencies within various increments $\Delta \nu$ over all values of θ . For $M_I = 0$ there will, of course, be no N¹⁴ displacement, and the N¹⁴ coupling will have no effect on the line shape, which in the absence of N¹⁴ coupling is assumed to be gaussian. For $M_I = +1$ or -1, the axially symmetric coupling will give rise to an asymmetric curve with a peak of maximum intensity corresponding to the perpendicular orientation $\theta = 90$ and to a much weaker shoulder corresponding to the end-on, or parallel, orientation for which $\theta = 0$. For $M_I = +1$, the maximum peak will have an N¹⁴ displacement of

 $\rho_p(Q - A_\mu) \approx 0.40 \ (25 - 15) \approx 4$ gauss and a shoulder displaced by $\rho_p(Q + 2A_\mu) \approx 0.40 \ (25 + 30) \approx 22$ gauss. Thus the peak is separated from the shoulder by about 18 gauss. The corresponding values for $M_I = -1$ are -4 and -22 gauss. The composite line shape for all values of θ and all values of $M_I(1, 0, -1)$ as are present in the powdered samples used here has three central peaks with separations of about 4 gauss and two weak outer shoulders separated by about 44 gauss. The three central peaks are too close for resolution, and the outer shoulders are too weak for observation in the present experiment. However, observed line widths are consistent with the 8-gauss spread for the central components. In γ -irradiated polyadenylic acid we have observed the expected outer shoulders and found that they are in good agreement with the spin density $\rho_{(7)} = 0.43$ on N₍₇₎ as predicted by Pullman and Mantione.¹⁰ This work on the polynucleotides will be described in a later communication.

ESR patterns similar to those observed here have been reported for X-irradiated purine and pyrimidine nucleosides and nucleotides, except those derived from uracil, by Shields and Gordy³ and by Müller and Köhnlein,^{13, 14} although they did not identify the free radicals as those produced by H-addition reaction. After the thymidine radical was found by single crystal analysis to arise from an Haddition reaction, it was pointed out by Gordy, Pruden, and Snipes¹ that Haddition reactions could also account for the triplets in the patterns of irradiated adenosine and guanosine and for the triplet observed by Dorlet *et al.*¹⁵ in DNA. This prediction was supported by the theoretical work of Pullman and Mantione.¹⁰ The present work provides experimental confirmation.

We are continuing this investigation through H and D bombardment of the nucleosides, the nucleotides, and DNA and RNA themselves. Already we have obtained a triplet like that for guanine through atomic H bombardment of guanosine. However, special conditions may be required to cause H atoms from the outside to penetrate sufficiently to the sites on the rings of the nucleic acids as to give detectable ESR signals. In the solid compounds, the sites are evidently well shielded by the sugar and the phosphate groups.

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