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AND PYRIMIDINES**

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FREE RADICALS FROM BIOLOGICAL PURINES AND PYRIMIDINES*

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The purine and pyrimidine rings are the alphabet of the genetic language, the code letters with which the life processes and living forms are described. Since the establishment of the Watson-Crick model for DNA, few bioscientists doubt this. Now, it is beginning to be strongly suspected that what man learns—if he remembers it very long—is also coded in these same molecular groups. I hope that you are recording this discussion in the purine and pyrimidine letters of your brain. If you record it only in amino acid letters, it won't last.

Ionizing radiations are known to produce mutagenic, carcinogenic, aging, and other effects on living things. It is reasonable to postulate that such effects could result from chemical changes induced directly or indirectly by the radiation in the information groups—the purine and pyrimidine rings of the nucleic acids. Several years ago we set out to find what, if any, information could be gained about these chemical alterations from the electron spin resonance (ESR) of possible free radical intermediaries of these radiation-induced alterations. This method does not detect the normal molecules in which all electrons are paired; it detects only the free radicals. Because microwave radiation used for observation of electron spin resonance readily penetrates organic solids or liquids, the ESR method can be used to examine free radicals trapped in almost any biochemical in almost any form.

In this exploration, the first question to be asked is whether the free radicals produced by ionizing radiation in the purines and pyrimidines are sufficiently long-lived for detection with ESR. This question was soon answered in the affirmative.^{1, 2} All the purines and pyrimidines, including the nucleosides, nucleotides and the nucleic acid polymers, were found to give observable ESR signals after irradiation with suitable dosage of x-rays and γ -rays. What are the chemical forms and electronic structures of the observed free radicals? What are the specific mechanisms of their production? These questions are being answered today, but more slowly.

Scientists working in many laboratories other than ours have made ESR observations on irradiated purines and pyrimidines. These include: Muller and his collaborators working in Germany; the Ehrenbergs in Sweden; Shen, Blyumenfeld, Kalmanson, and Pasynskii in Russia; Van de Vorst, Duchesne and others in Belgium; Lacroix and Williams-Dorlet in France; Charlesby, Omerod and others in England; Pihl, Sanner and Henriksen in Norway; Schulman, Pershan and their collaborators at the Bell Telephone Laboratories; Heller and Cole at the Ford Laboratories; Myers and his collaborators at U.C.L.A. It is impossible to review here the many studies on the subject, but fortunately an excellent review by Müller³ is now available. Schoffa's monograph⁴ on the applications of spin resonance to the study of free radicals in biological systems, and Poole's book on experimental techniques⁵ for elec-

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tron spin resonance provide recent material on the subject. This paper will be concerned primarily with the work done in our laboratory at Duke University.

Early Work on Powdered Samples

In our original work^{1, 2} with Howard Shields, characteristically different ESR patterns were observed for various purine and pyrimidine derivatives irradiated in the powder form. FIGURE 1 shows the spectra we obtained for thymidine, cytidine, desoxyguanosine, and desoxyadenosine. The base thymine gave a pattern like that of thymidine, but a much weaker one for the same x-ray dosage. No signals could be detected for the pure bases adenine and guanine, although for adenylic acid and guanylic acid signals similar to those of adenosine and guanosine were detected.

A comparison of results indicated that the complex pattern of thymidine and the triplet pattern of the purines came from radicals formed in the basic ring structures. The sugar constituents irradiated separately produced entirely different patterns. We demonstrated that the observed thymidine pattern agreed with a theoretical pattern (represented by bars in FIGURE 1) consisting of a triplet from two equally coupling protons of the order of 40 gauss, a quartet substructure arising from the CH_3 group with equivalent proton couplings of 23 gauss. Likewise, it was evident that the triplet splitting observed for the purine derivatives indicated an equal coupling by two protons of the order of 40 gauss. It was not apparent to us, however, how radicals having two protons with equivalent coupling of 40 gauss could be produced directly by the radiation in these compounds. Now we know, from evidence which I shall describe, that these radicals are not produced directly by irradiation but are, in fact, produced by migrating H atoms which add to the purine or pyrimidine rings. The source of the H atoms is not definitely known, but it is probable that they are released by the irradiation from the sugar groups in the nucleosides and the nucleotides, as is indicated by the greater difficulty in production of observable concentrations of the radicals by irradiation of the unsubstituted bases. The H atoms may be released in pairs with the formation of a double bond, so that no primary free radicals are formed. However, in adenosine single crystals (see later discussion), we have observed both a radical formed by an H addition on the ring and a radical on the sugar group formed by loss of an H atom. The source of the H atoms which give rise to the weak signals observed in thymine may be the CH_3 group, or possibly an impurity.

The cytidine pattern (FIGURE 1) is a triplet having components of equal intensity and spacing of about 10 gauss. This hyperfine structure evidently arises from coupling of the N^{14} nucleus which has spin $I = 1$. This structure was not resolved in the ESR of the irradiated cytosine, for which the radical is evidently different. Although Shields and Gordy² recognized this N^{14} coupling, they could not ascertain the nature of the radical nor learn which of the N^{14} nuclei of the cytidine led to the triplet splitting. Later results in our laboratory on single crystals of cytidine revealed the probable structure of the radical, which will be described in the discussion of pyrimidine single crystals which follows.

From quantitative measurements of the intensities of the ESR lines, Müller and Köhnlein⁶ have derived values for the free radical yields for various

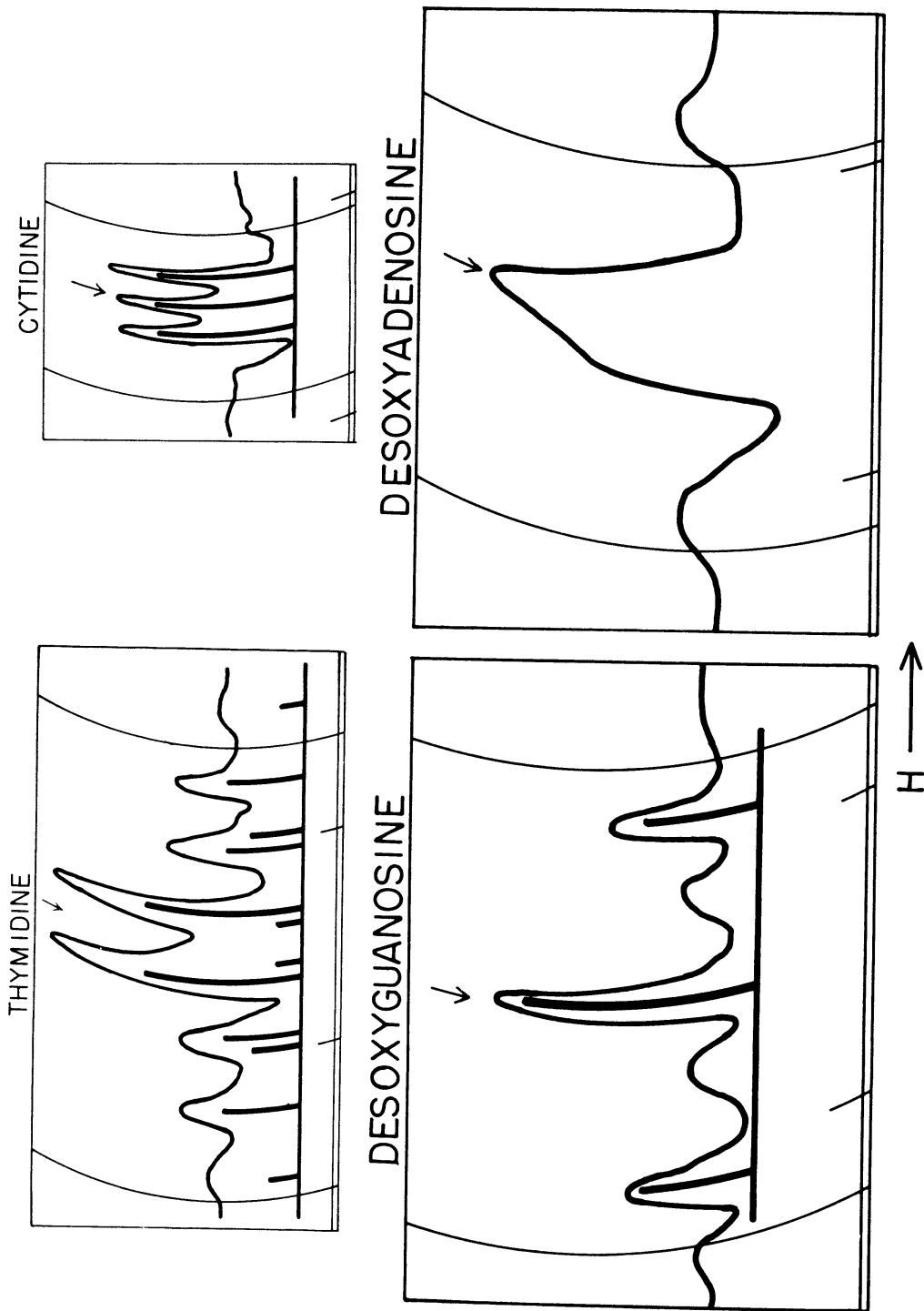


FIGURE 1. ESR patterns of γ -irradiated pyrimidine and purine powders at room temperature. (From Reference 2)

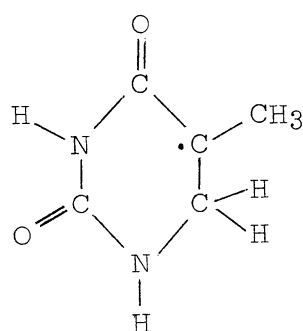
dosages of radiation in the purine and pyrimidine bases as well as in the nucleosides and nucleotides.

H and D Bombardment

After single crystal⁷ measurements in our laboratory and work by Pershan and collaborators on deuterium substitution⁸ in the Bell Telephone Laboratories had shown that radicals observed in irradiated thymine or thymidine resulted from H addition on C₆ of the ring, Herak and the author⁹ bombarded various purines and pyrimidines with gaseous H atoms at thermal velocities, to determine whether H addition radicals could be produced in this direct manner. We used ESR to detect any radicals which might be formed. In order to distinguish radicals formed by H addition from those which might be formed by H abstraction or from thermal dissociation by the bombarding particles, we also observed samples similarly bombarded with D atoms. These experiments were successful in demonstrating beyond any doubt that H-addition radicals are formed not only on thymine but also on the basic rings of all the purines and of the other pyrimidines, and that the atoms added were from the gaseous streams. Although we were unable in our earlier experiments to obtain signals for H addition on cytosine at room temperature, we later found such radicals¹⁰ for cytosine bombarded at 77° K. Since these radicals disappeared as the cytosine was warmed to room temperature, the warming process evidently expelled the added H atoms. However, H-addition radicals were observed up to room temperature in desoxycytidylic acid.¹¹

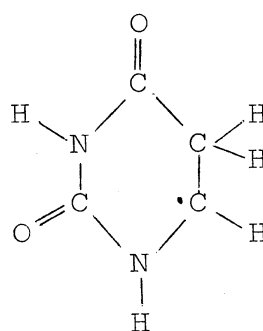
Independently, and almost simultaneously with us, Heller and Cole¹² reported radicals in thymine produced by H bombardment that were similar to the radicals produced previously by ionizing irradiation. They did not, at the same time, produce signals with D atoms. Holmes and coworkers¹³ have reported detection of addition radicals at room temperature when various purines and pyrimidines were bombarded with H and D atoms. They used a special technique of depositing the samples on quartz wool in order to increase the surface area. Their results agree with ours, except that they were able to obtain signals from cytosine bombarded at room temperature. Though the cause of these differing results is not entirely clear, it must be related to quartz wool.

The H-addition radicals observed in the purine and pyrimidine bases are:



thymine + H

(I)



uracil + H

(II)

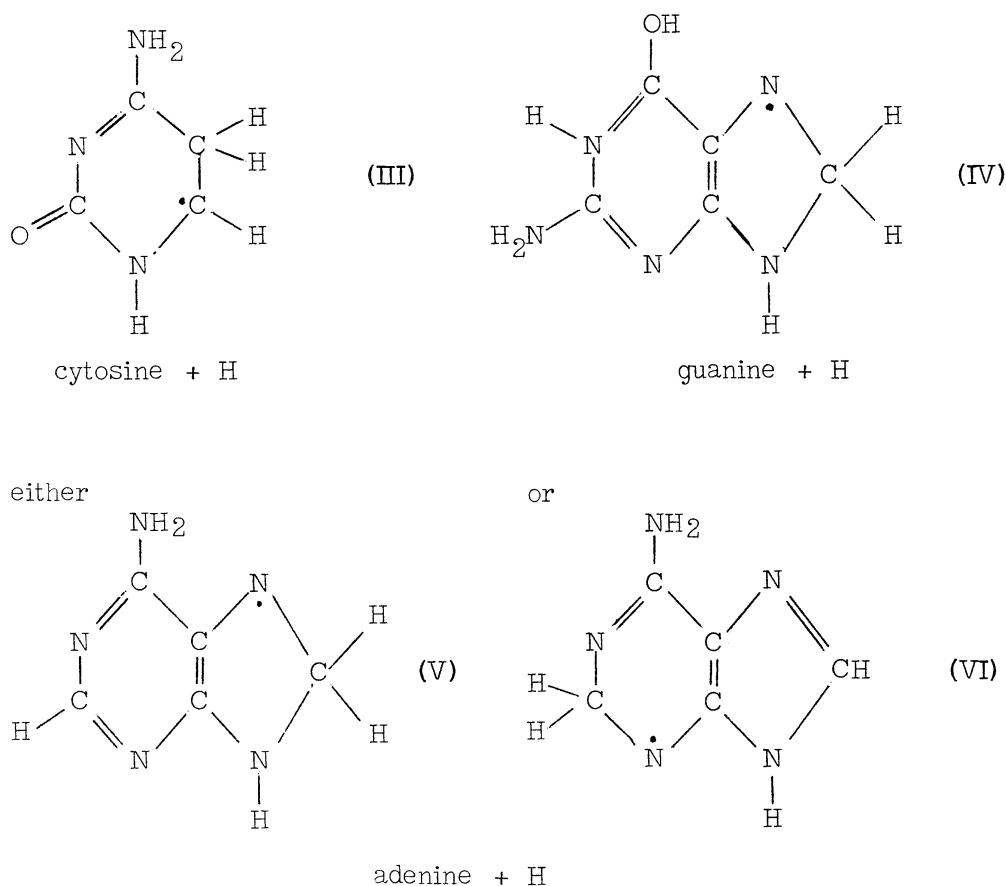


FIGURE 2 shows the characteristic ESR patterns produced by H bombardment of the powdered samples of thymine, uracil, guanine, and adenine at room temperature. FIGURE 3 shows a comparison of cytosine and uracil when H-bombarded and observed at 77° K. Note the similarity between these two spectra and the differences between the pattern for uracil at 77° K and that at 300° K. The latter difference is due to the change in orientation of the CH₂ bonds relative to the molecular plane. At 300° K, the two CH bonds have equivalent orientations relative to the molecular plane.

TABLE 1 gives the proton coupling for the nucleic acid bases at 77° K and at 300° K. TABLE 2 gives the proton couplings observed for the corresponding H-addition radicals¹¹ in the nucleosides and the nucleotides produced by bombardment of these various compounds with H atoms. The values listed in TABLE 2 are for 77° K unless otherwise indicated.

OH Bombardment

In a similar way, we bombarded the nucleic acid bases with gaseous OH radicals¹⁴ at thermal velocities. All the observations were carried out at room temperature. The OH radicals were made by the action of an electric discharge on H₂O₂ vapor. Direct OH-addition radicals were readily detected for uracil, but not for the other bases.

The observed pattern for uracil bombarded with OH atoms is shown in FIGURE 4. Note the differences between this pattern and that for H-bombarded uracil shown in FIGURE 2. The hyperfine pattern is what would be ex-

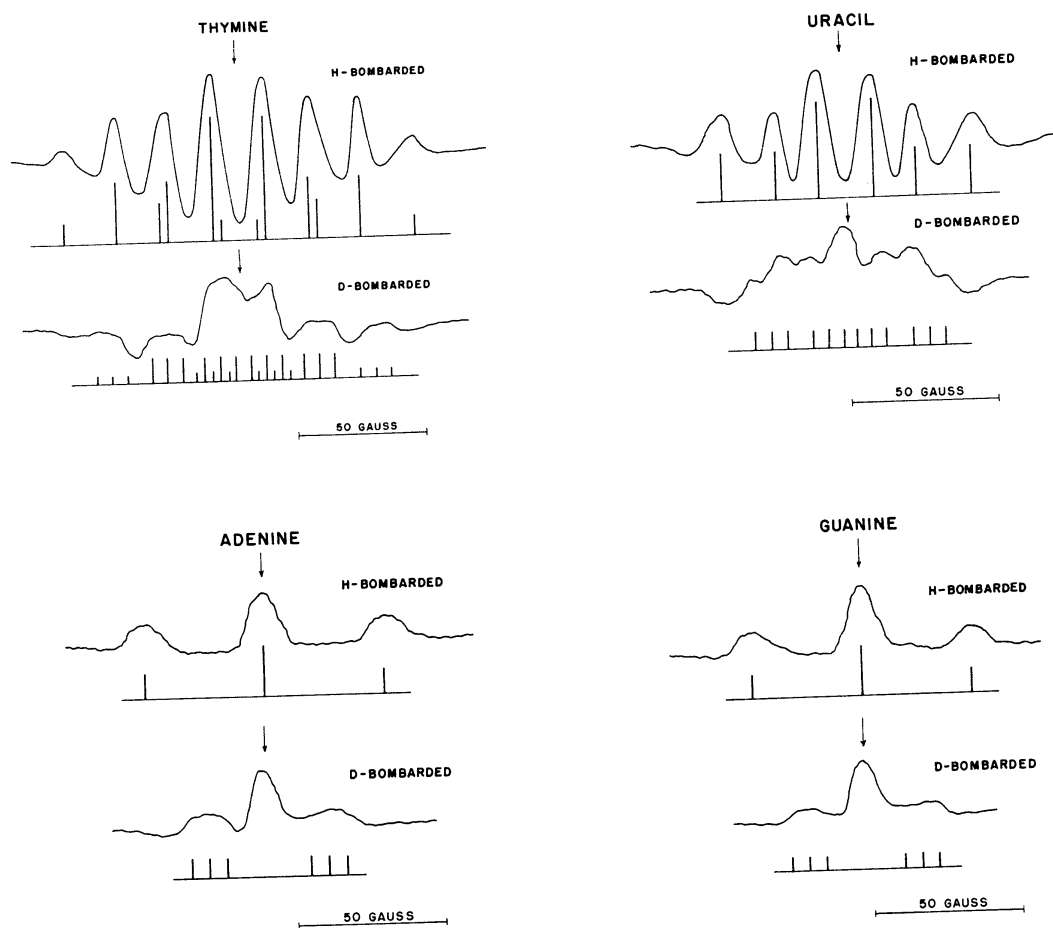


FIGURE 2. ESR patterns of pyrimidine and purine powders after bombardment with gaseous H or D atoms at room temperature. (From Reference 9.)

H - BOMBARDED

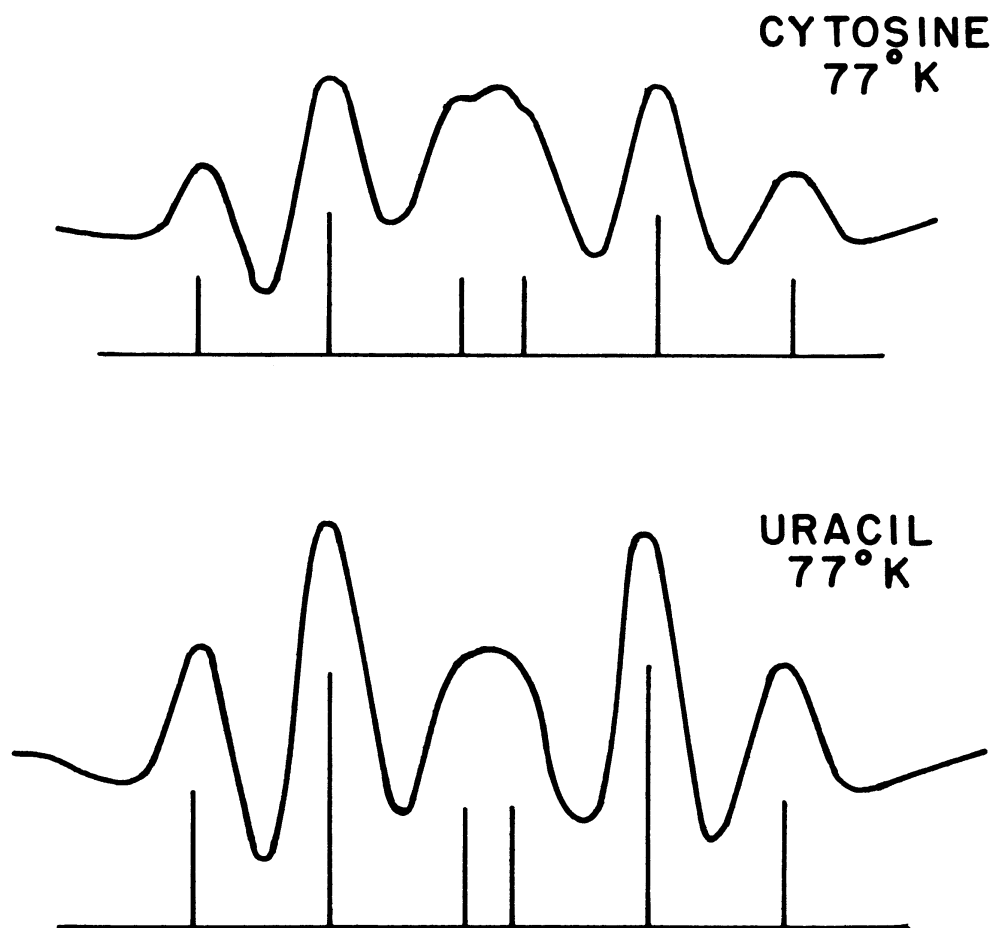


FIGURE 3. ESR patterns of cytosine and uracil powders after bombardment with H atoms at 77° K. (From Reference 10.)

TABLE 1
PROTON COUPLINGS AND SPIN DENSITIES OF H-ADDITION RADICALS IN
PURINE AND PYRIMIDINE BASES*

Radical Source	Coupling Group	Coupling in Gauss	
		77° K	300° K
Thymine	$>C_{(6)}H_2$	34	42
	$-CH_3$	20.5	20.5
Uracil	$>C_{(5)}H_2$	19.5	46
	$>C_{(6)}H$	19.5	18.5
Cytosine	$>C_{(5)}H_2$	19.5	48
	$>C_{(6)}H$	19.5	
Adenine	$>C_{(2)}H_2$		40
Guanine	$>C_{(8)}H_2$	38	38

* From Reference 10.

TABLE 2
 PROTON COUPLINGS AND SPIN DENSITIES OF H-ADDITION RADICALS IN
 NUCLEOSIDES, NUCLEOTIDES AND RNA*

Compound	Coupling Group	Coupling in Gauss	Spin densities on C_α
Cytidine	$C_{(6)}H$	$a_1 = 20$	0.76 ^b
	$C_{(5)}H_2$	$a_2 = 20; a_3 = 50$	
Cytidylic acid	$C_{(6)}H$	$a_1 = 19$	0.73 ^b
	$C_{(5)}H_2$	$a_2 = 19; a_3 = 46.5$	
Deoxycytidine	$C_{(6)}H$	$a_1 = 20$	0.76 ^b
	$C_{(5)}H_2$	$a_2 = 14; a_3 = 48.5$	
Deoxycytidylic acid	$C_{(6)}H$	$a_1 = 20; (a_1 = 21)^a$	0.76 ^b (0.80) ^a
	$C_{(5)}H_2$	$a_2 = 13; a_3 = 50$ ($a_2 = 12.5; a_3 = 50$) ^a	
Thymidine	$-CH_3$	$a_1 = a_2 = a_3 = 20.5$	0.70 ^c
	$C_{(6)}H_2$	$a_4 = a_5 = 39$	
Thymidine 5'- mono-phosphate	$-CH_3$	$a_1 = a_2 = a_3 = 20.5$	0.70 ^c
	$C_{(6)}H_2$	$a_4 = a_5 = 38$	
Uridine	$C_{(6)}H$	$a_1 = 20$	0.76 ^b
	$C_{(5)}H_2$	$a_2 = 20; a_3 = 46$	
Deoxyuridine	$C_{(6)}H$	$a_1 = 19.5$	0.74 ^b
	$C_{(5)}H_2$	$a_2 = 19.5; a_3 = 46$	
Uridylic acid	$C_{(6)}H$	$a_1 = 20$	0.76 ^b
	$C_{(5)}H_2$	$a_2 = 20; a_3 = 43$	
Guanosine	$C_{(8)}H_2$	$a_1 = a_2 = 37$	
Guanylic acid	$C_{(8)}H_2$	$a_1 = a_2 = 37$	
Deoxyguanosine	$C_{(8)}H_2$	$a_1 = a_2 = 37$	
Deoxyguanosine 5'- monophosphate	$C_{(8)}H_2$	$a_1 = a_2 = 37$	
RNA			
Pyrimidine	$C_{(6)}H$	$a_1 = 19.5$	0.75
	$C_{(5)}H_2$	$a_2 = 19.5; a_3 = 42$	
Purine	$C_{(8)}H_2$	$a_1 = a_2 = 38^a$	

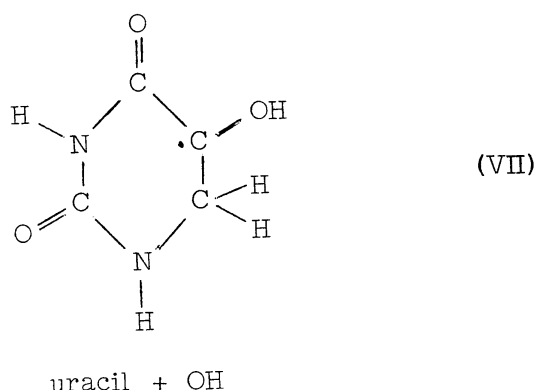
* From References 10, 11.

^a At 300° K.

^b Calculated with the equation $a_\alpha = Q_\alpha \rho_\alpha$ with $Q_\alpha = 26.2$ gauss.

^c Calculated from the equation $a_\beta = \rho_\alpha Q_\beta \cos^2\theta$ with $Q_\beta = 58.5$ gauss.

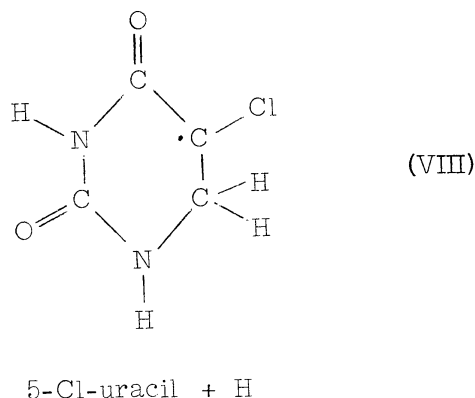
pected for two equally coupling protons of 28 gauss. The coupling is isotropic and arises from hyperconjugation. The radical giving this pattern is evidently



It could be formed by a direct OH addition on C₍₅₎ with a subsequent transfer of the H from C₍₅₎, to which it was originally bonded, to C₍₆₎. We also studied the interaction of OH radicals with 5-halogen uracils, as described below.

5-Halogen Uracils

When 5-I-uracil or 5-Br-uracil was bombarded with H atoms, the ESR signals obtained were like those for H-bombarded uracil itself. We concluded that the impinging H atoms replaced the bromine or the iodine to form uracil, which later captured an H atom to form the uracil radical. In contrast, 5-Cl-uracil formed the direct addition radical



The conditions for this addition are critical. At lowered temperature, Cl replacement occurred.

Mainly, the uracil + H radicals were observed when 5-F-uracil was subjected to gaseous H atoms. Since replacement of F by H is not energetically possible, the impinging H atoms must first interact with the 5-F-uracil to form HF and a primary radical, which is converted to uracil by H atoms arriving later.

Upon bombardment of 5-Br-uracil or 5-I-uracil with OH free radicals and H atoms, a signal is observed like that obtained when uracil itself is bombarded with OH free radicals (FIGURE 4). Either the OH replaces the Br or the I to form 5-OH-uracil, which then combines with H atoms to form radicals like (VII) above; or the H atoms first replace the halogens, and radicals like (VII) are then formed by interaction of the OH with uracil.

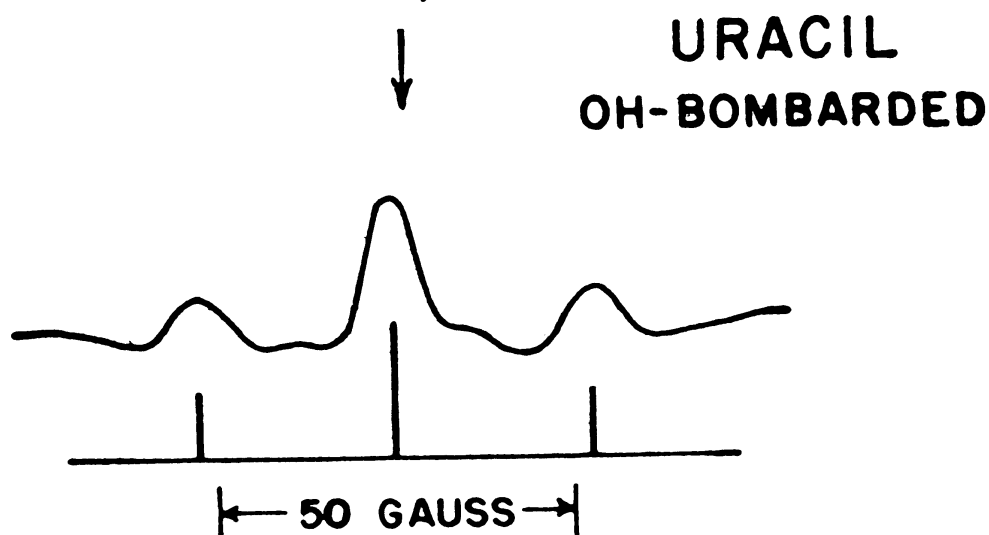


FIGURE 4. ESR pattern of uracil powder after bombardment with OH radicals at room temperature. (From Reference 14.)

Pyrimidine Single Crystals

Although it is tedious and difficult, work on single crystals can give specific and useful information about the free radicals when suitable crystals can be grown and a detailed analysis is possible. Pruden, Snipes and I succeeded first in obtaining the ESR spectra of irradiated single crystals of pyrimidines.⁷ From the analysis of the results on thymidine, they concluded that the hydrogen-addition free radical (I) already mentioned gave rise to the observed spectra. Briefly, this is the evidence from the single crystal experiment: the hyperfine structure was found to be isotropic such as that expected from the CH_2 and CH_3 in radical (I), whereas the g factor had a dependence upon the orientation of the crystal in the magnetic field expected for an unpaired π electron on the rings. No nuclear coupling from the nitrogens was observed. The only resolvable hyperfine structure was that from the CH_2 and CH_3 protons, which have isotropic values essentially the same as those listed in TABLE 1.

In 1964, Snipes, Pruden and I began a study of the ESR spectra of irradiated single crystals of cytidine. Because of some doubt about the exact identity of the principal radical giving the observed spectra, we have withheld publication of our findings in the hope of clearing up the uncertainty. However, some of the results are reported in the Ph.D. thesis of Pruden (1965), together with a description of the radical which agrees best with these data. Snipes and Pruden are no longer at Duke, but this investigation is being continued with the assistance of Chester Alexander. I shall describe briefly the essential results and conclusions.

The principal radical is not an H-addition radical such as that in thymidine, although weak signals appearing in some crystals may arise from H-addition radicals. The surprising feature is that the predominant spectrum has a hyperfine structure, which seems definitely to arise from an almost isotropic coupling to only one N^{14} nucleus, giving a triplet with spacing of approximately 9 gauss for most orientations of the crystal. For orientations of the magnetic field along the a , b , or c axis, all molecules in the unit cell have equivalent orientations, and only one triplet appears, as in FIGURE 5. For

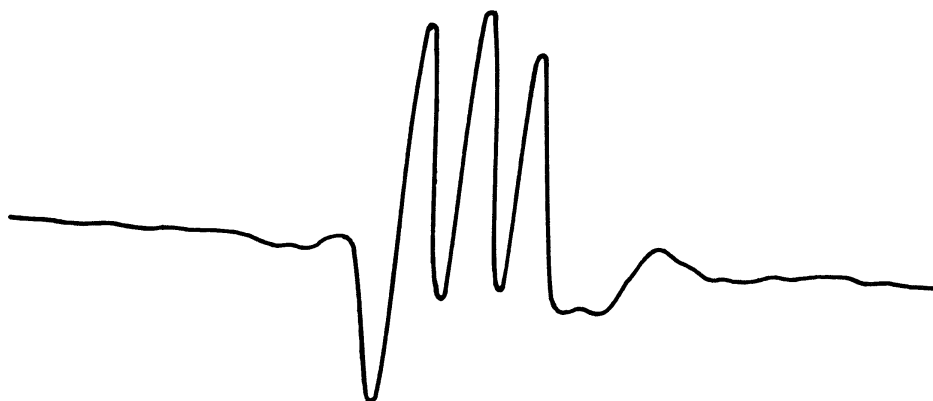
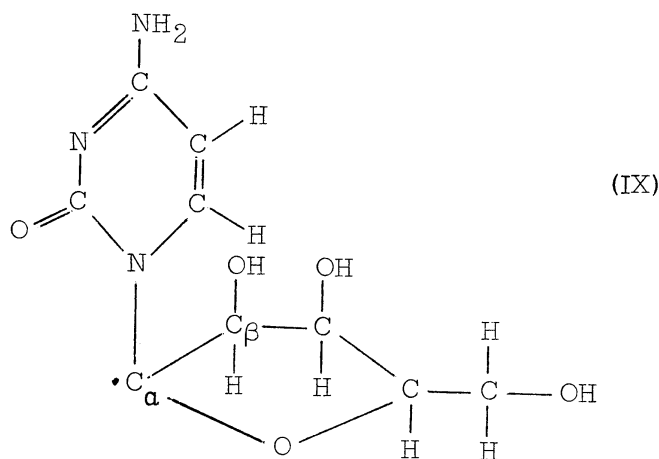


FIGURE 5. ESR pattern of a γ -irradiated single crystal of cytidine with the magnetic field parallel to the a axis of the crystal. (From Snipes, Pruden and Gordy, 1965.)

other orientations, two sets of triplets are observed because of variation in the g values for two inequivalent orientations of molecules of the unit cell.

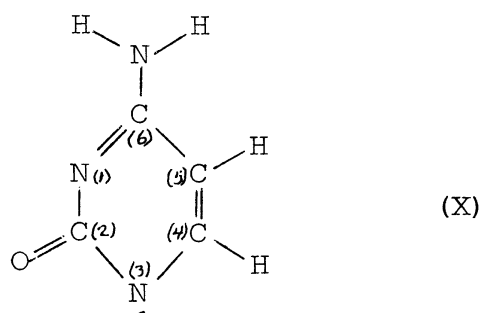
The principal ESR spectrum of the irradiated cytidine crystal can be ascribed to the radical



formed by the loss of a hydrogen from the carbon of the ribose ring which links the ring to the base. The plane of the ribose ring is approximately at right angles to that of the x base ring, with C_{α} approximately in both planes. The electron spin density is mainly in an sp_3 hybrid orbital directed almost in the plane of the base ring, and normal to that of the sugar. No noticeable coupling arises from the hydrogen on C_{β} because the C_{β} -H bond is approximately normal to the orbital of the unpaired electron. The triplet splitting arises primarily from exchange interaction of the unpaired electron with that of the C_{α} -N σ bond. There is little π -bonding of the unpaired electron with the N because of unfavorable orientation of the orbitals. The N-bonding orbital of the CN has approximately one-third 2s character, and it is this component which is responsible for most of the N^{14} coupling. The coupling of a 2s electron in N^{14} is 549 gauss. The observed isotropic coupling of 9 gauss indicates a 2s spin density of 0.016. With only one-third 2s character in the bonding

orbital, this indicates 3 (0.016) or 4.8% spin density in the sp^2 -bonding orbital of the N. This spin density would not cause significant anisotropy in the splitting because the coupling of a 2p electron of N^{14} is only 17 gauss and, with this small spin density, would give an anisotropic coupling constant B of only 0.18 gauss.

As this is being written, an interesting paper by Cook and collaborators¹⁶ reports successful investigation of irradiated single crystals of cytosine. These researchers found the principal radical to be



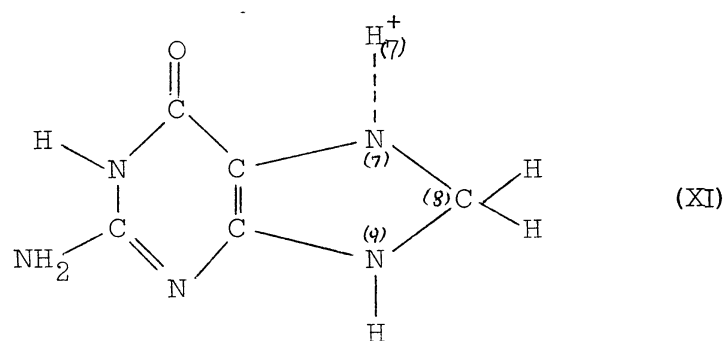
which is formed by loss of a hydrogen from $N_{(3)}$. Both $N_{(1)}$ and $N_{(3)}$ gave measurable hyperfine structure with anisotropic coupling constants of 2.5 gauss and 5 gauss (7 and 14 Mc). The corresponding spin densities are 0.15 and 0.29, respectively. In addition to the principal radical, Cook and coworkers observed lesser signals from H-addition radicals of the type earlier observed in the H-bombarded powder. These signals were about one-tenth the strength of the principal signals.

Although in our work on the cytidine single crystal we observed weak satellite signals, which may come from radicals such as those found by Cook and coworkers¹⁵ in the cytosine crystal, the principal radicals found in the two crystals are basically different. This conclusion is in agreement with that reached from the original observations on powdered samples.

Bernhard and Snipes¹⁷ have reported preliminary ESR studies of an irradiated single crystal of 3'-cytidylic acid, and have attributed the observed signals to a free radical having the form $R-CH_\beta R-CH_\alpha-R$, in which R represents groups which have no appreciable hyperfine interactions.

Purine Single Crystals

The first ESR study of an irradiated purine single crystal to be reported is that on guanine hydrochloride, undertaken with my student, Chester Alexander.¹⁸ The radical observed is one formed by hydrogen addition on $C_{(8)}$ and already identified by H-atom bombardment of the powdered sample. From the powdered samples, however, only the isotropic CH_2 coupling constants could be ascertained, whereas from the single crystal both the N^{14} nuclear coupling and the g tensor could be evaluated. In addition, an isotropic coupling was measured for an associated proton, apparently from the HCl and attached to $N_{(7)}$ of the guanine, mainly through electrostatic bonding. The observed radical is



Principal elements of the g tensor and the various nuclear couplings are given in TABLE 3. The direction cosines of the principal elements of g and the nuclear coupling tensor are in good agreement with those expected from the structure of the crystal, as determined by Iball and Wilson¹⁹ from x-ray analysis. The coupling for the $H_{(7)}$ proton is also in agreement with this structural determination. From the N^{14} coupling, the spin densities of the p_{π} orbitals of $N_{(7)}$ and $N_{(9)}$ are found to be 0.38 and ≤ 0.08 , in agreement with the theoretical value of 0.39 on $N_{(7)}$ and 0.08 on $N_{(9)}$, previously calculated by Pullman and Mantione²⁰ with the Hückel approximation.

FIGURE 6 shows ESR patterns obtained for three different orientations of the crystal in the magnetic field. For the crystal orientation corresponding to the top curve, only the isotropic CH_2 proton coupling is resolved. For the middle curve, the $H_{(7)}$ proton doublet is also resolved, but no N coupling is resolved. In the lower curve the $N_{(7)}$ nitrogen triplet is resolved, but not the $H_{(7)}$ doublet.

TABLE 3
ESR CONSTANTS OF H-ADDITION RADICALS IN SINGLE CRYSTALS OF
GUANINE HYDROCHLORIDE DIHYDRATE¹⁸

Coupling Atom	Nuclear Coupling	
	Principal Values (gauss)	Direction in Guanine Molecule
Nitrogen $N_{(7)}$	$\begin{cases} 21.0 \pm 0.5 \\ 1.5 \\ 1.5 \end{cases}$	\perp to plane of molecule $\parallel N_{(7)}-H_{(7)}$ bond \perp to above directions
Hydrogen $H_{(7)}$	$\begin{cases} -9.0 \pm 0.5 \\ 1.0 \\ -15.0 \end{cases}$	\perp to plane of molecule $\parallel N_{(7)}-H_{(7)}$ bond \perp to above directions
Hydrogen $H_{(4)}$	$\begin{cases} -2.9 \\ -0.9 \\ -5.0 \end{cases}$	\perp to plane of molecule $\parallel N_{(9)}-H_{(4)}$ bond \perp to above directions
Hydrogen CH_2	36.0 ± 0.5	Isotropic
g-Tensor		
	Principal Values	Direction in Guanine Molecule
	2.0023 ± 0.0002	\perp to plane of molecule
	2.0048	\parallel to $N_{(7)}-H_{(7)}$ bond
	2.0048	\perp to above directions

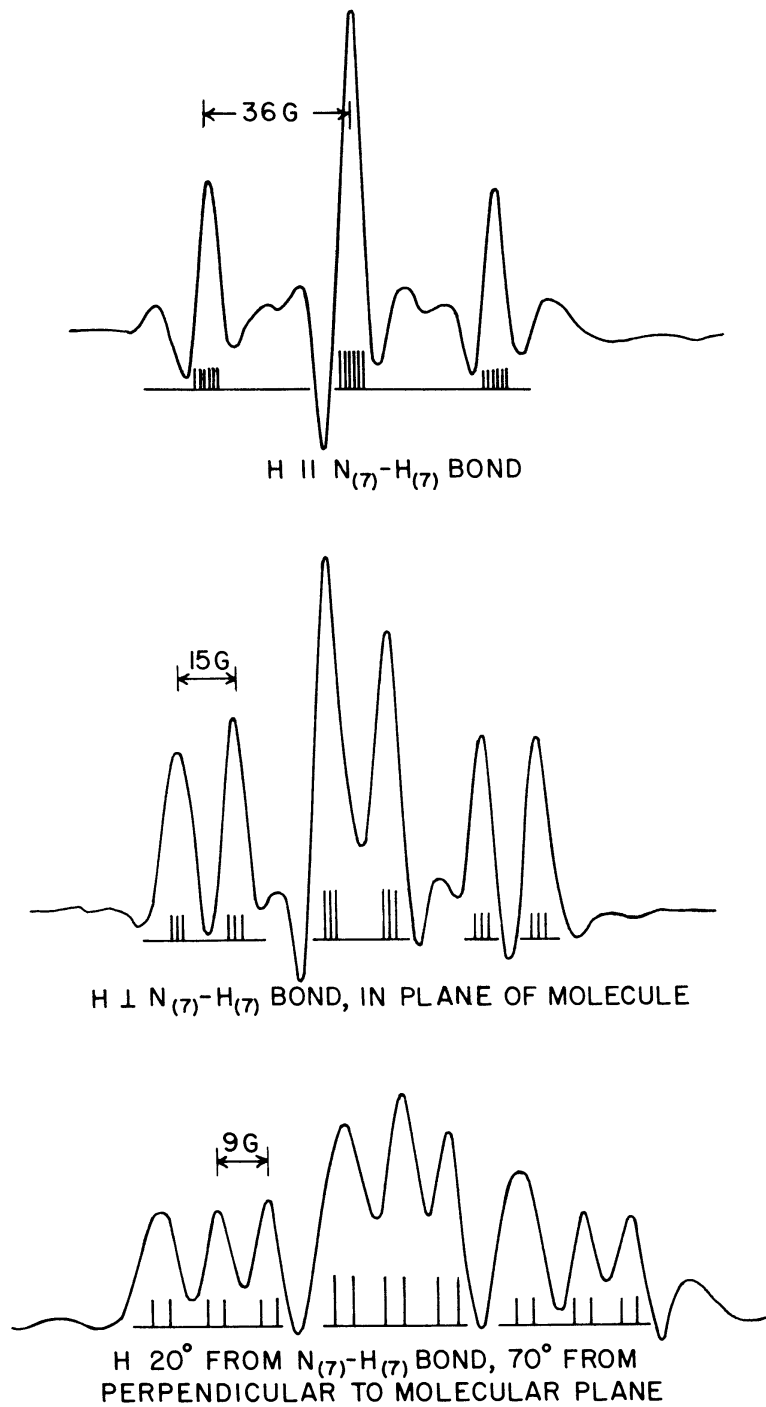


FIGURE 6. ESR patterns of a γ -irradiated single crystal of guanine hydrochloride dihydrate for different orientations of the crystal in the magnetic field. (From Reference 18.)

James Lichter and I are studying single crystals of deoxyadenosine. FIGURE 7 shows the ESR of a γ -irradiated single crystal of deoxyadenosine monohydrate for two orientations of the crystal in the magnetic field. There are evidently strong signals from two chemically different species of radicals. One

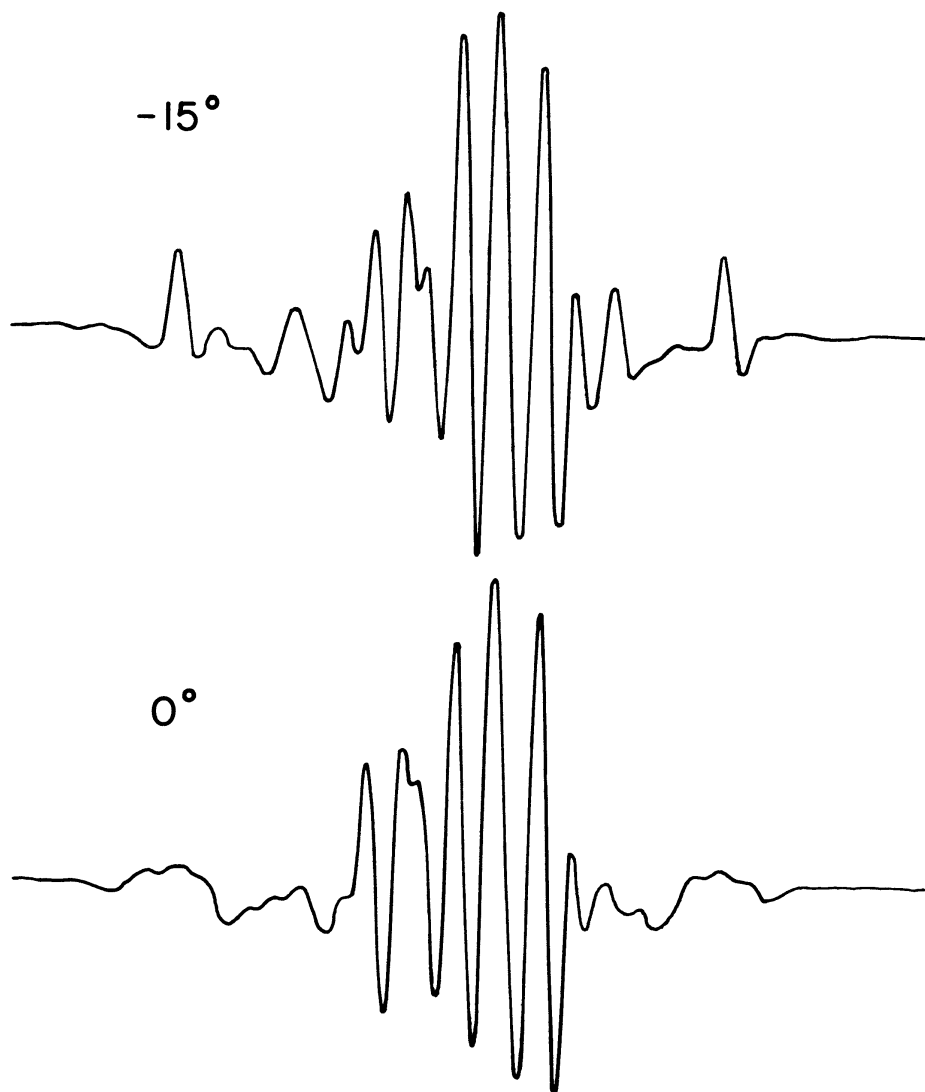


FIGURE 7. ESR patterns of a γ -irradiated single crystal of deoxyadenosine monohydrate for different orientations of the crystal. There are two chemically different radicals giving the patterns, and molecules having two different orientations in the unit cell. (From J. J. Lichter and W. Gordy, 1968.) *Proc. Nat. Acad. Sci.* **60**, 450-455.

of these is the H-addition radical of the basic ring structure, which was revealed by the earlier H-atom bombardment of the powder. The other is a radical formed in the ribose group by the loss of an H atom.

Fortunately, the isotropic triplet-splitting by the CH_2 protons is sufficiently great that the outer two components of the triplet are outside the range of the spectrum of the ribose radical. This allows resolution and analysis of the N^{14} hyperfine structure of these outer components from which the N^{14} coupling constants can be obtained. The analysis shows that there are two nitrogens in the radical that produce resolvable splittings. The coupling of each nitrogen is axially symmetric about the normal to the plane of the ring, and has maximum values when the field is normal to the rings. X-ray diffraction measure-

ments by Watson and collaborators²¹ show that there are two molecules in unit cell with the planes of their rings approximately 33° apart. These usually lead to radicals with two different orientations in the crystal, but for certain directions the orientations are magnetically equivalent. FIGURE 7 (lower curve) shows one recording for which the orientations are equivalent. At certain other orientations, the lines for one orientation are sufficiently sharper and stronger to be distinguishable from those of the other one.

TABLE 4 gives preliminary values for the coupling constants for the nitrogens and the hydrogens of the H-addition radical. The couplings of the two

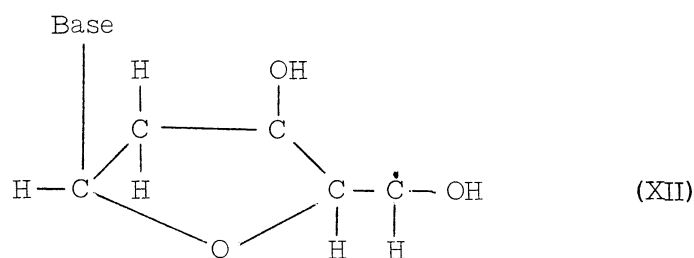
TABLE 4
NUCLEAR COUPLING CONSTANTS FOR RADICALS IN SINGLE CRYSTALS OF
DEOXYADENOSINE MONOHYDRATE*

Coupling Constant for	Principal Values (gauss)	Direction in Adenine Radical
Nitrogen N ₍₃₎	19 ± 1 ≤ 1	\perp to radical plane In plane of radical
Nitrogen N ₍₁₎	8 ± 1 < 0.5	\perp to radical plane In plane of radical
Hydrogen CH ₂	44 ± 1	Isotropic

* From Lichter, J. J. & W. Gordy. 1968. Proc. Nat. Acad. Sci. 60: 450-455.

nitrogens are significantly different from those of the two nitrogens of the guanine single crystal. Likewise, the coupling of the CH₂ hydrogens is larger than in the guanine. These comparisons, together with a comparison between the observed spin densities and those predicted theoretically by Pullman and Mantione,²⁰ lead to the conclusion that the radical observed in the adenosine is formed by H addition to C₍₂₎ rather than to C₍₅₎ as it is in the guanine, i.e., that the radical has a structure similar to (VI) rather than (V).

The measurement and analysis of the radical found on the deoxyribose group is still incomplete. It is complicated by the superposition of the stronger central component of the H-addition radicals as well as by the two different orientations of molecules in the crystals. Nevertheless, observations for certain orientations of the crystal for which these complicating factors are minimized indicate that the hyperfine structure comes from an anisotropic coupling of a C _{α} H fragment combined with the isotropic coupling of about 6.5 gauss of a C _{β} H proton. Tentative assignment of the structure of the radical is



Polynucleotides

Several irradiated polynucleotides in powder form²² have been studied in our laboratory. These include poly U, poly C, and poly A and also the co-

polymers poly CU and poly AU. We were unable to obtain samples of poly T and poly G. Only γ -irradiation was used in these experiments.

Noticeable effects of moisture content on the formation of H-addition radicals on the basic rings of poly U and poly A were observed. These effects are illustrated in FIGURES 8 and 9. The patterns expected for H-addition radicals

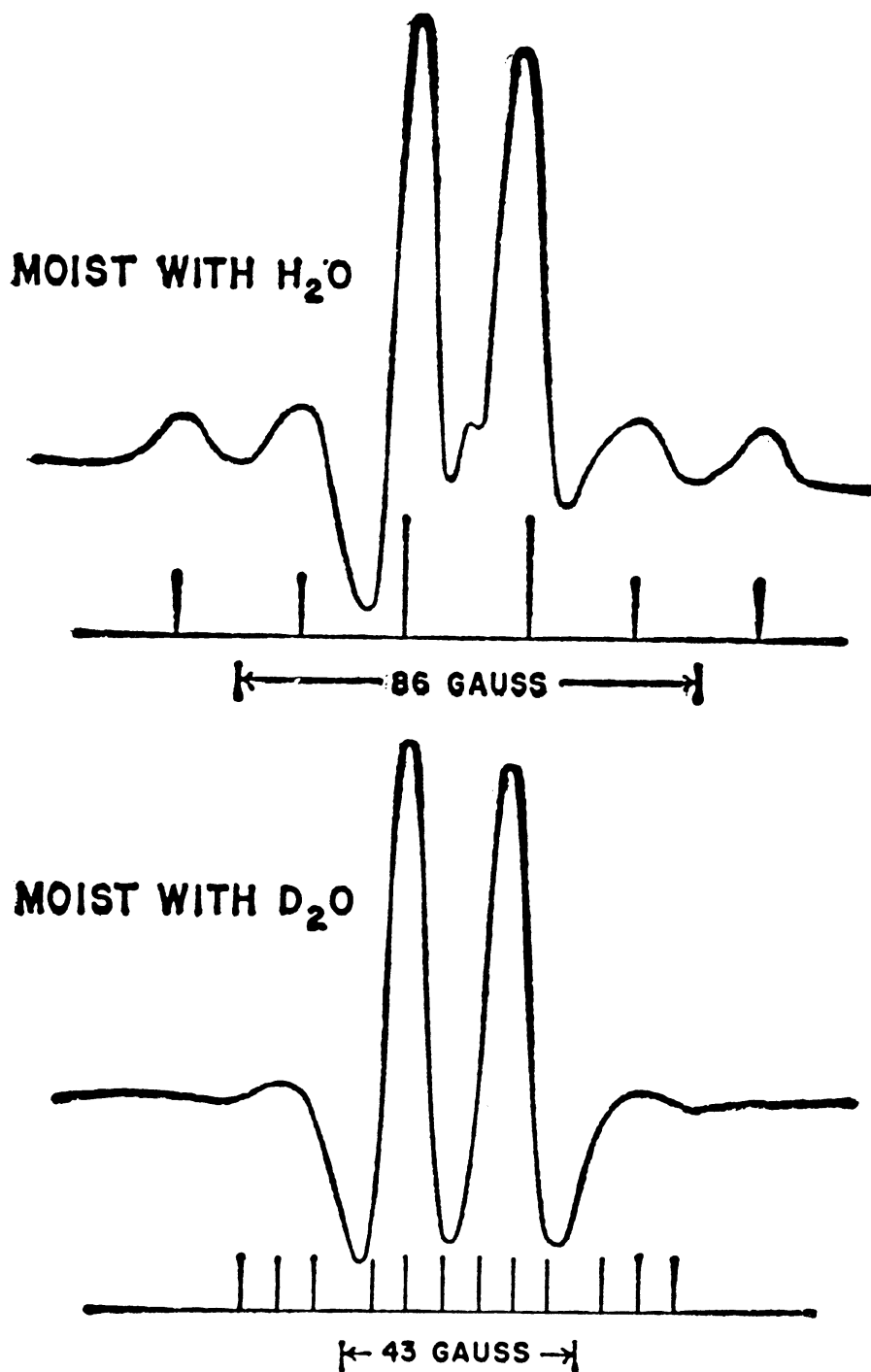
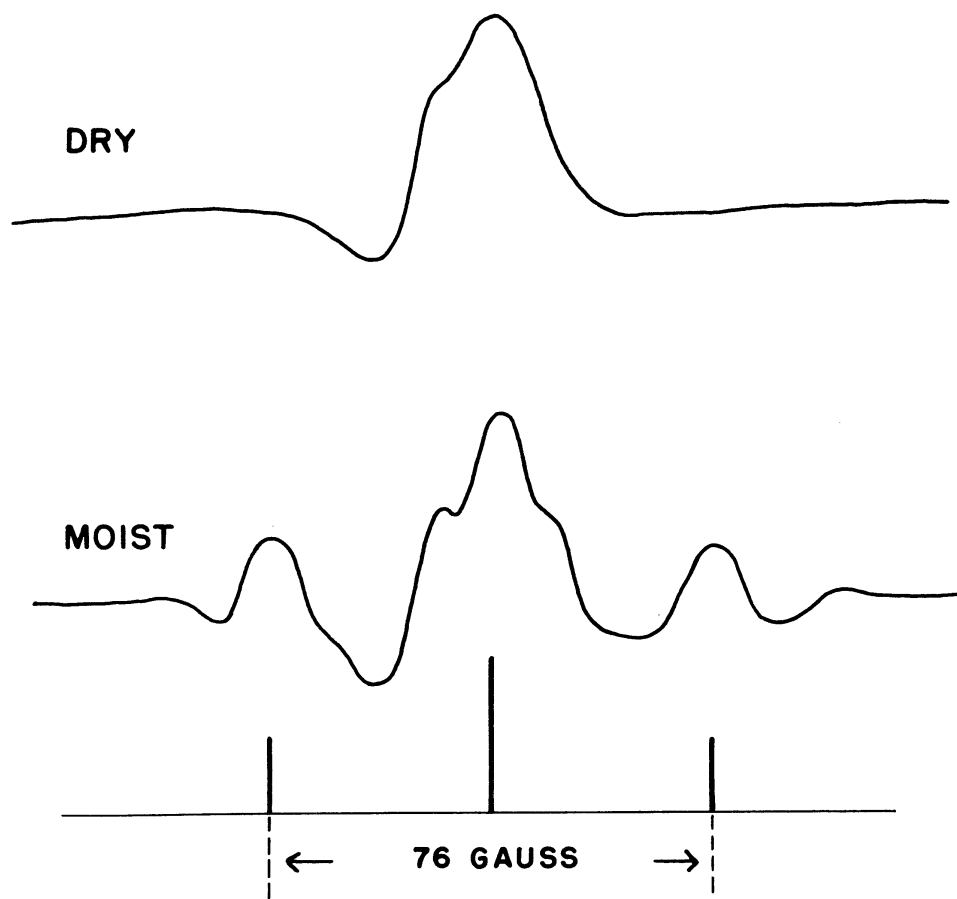


FIGURE 8. ESR pattern of γ -irradiated, moist samples of polyuridylic acid. (From Reference 22.)



ESR OF POLYADENYLIC ACID
 γ -IRRADIATED AT 200°K
OBSERVED AT 240°K

FIGURE 9. ESR patterns of γ -irradiated samples (dry and moist) of polyadenylic acid. (From Reference 22.)

are indicated by the bars in the lower graphs. In each polymer, considerable enhancement of the H-addition radicals was caused by the absorbed H_2O . That the H atoms which are added to the rings are produced from the H_2O is shown by the marked change in the pattern when D_2O is substituted for H_2O . These effects are similar to the enhancement of the thymidinelike resonance in DNA first observed by Pershan and coworkers.⁸

The strong doublet in the center of the poly U pattern, which is the same for samples moist with D_2O or H_2O , is believed to result from a radical formed by H abstraction from the ribose group. In agreement with the observations on cytidine powder, no evidence for significant formation of H-addition radicals was obtained for poly C even when the samples were moist when irradiated. The ESR pattern for γ -irradiated poly C resembles closely that of cytidine.

Müller²³ also observed the ESR of polynucleotides but did not report any study of moisture effects.

Purine and Pyrimidine Resonance in DNA and RNA

An important question to be considered is whether the various free radicals formed in the purines and pyrimidines described above are also formed by irradiation in DNA or RNA. Shields and I were surprised that the signals we observed for irradiated DNA and RNA were unlike any of the various signals we observed for their constituent purine and pyrimidine components. For both DNA and RNA irradiated with x-rays or γ -rays, we obtained a singlet resonance of 30 to 40 gauss in width but evidently having some unresolved structure. Although some people questioned the purity of our samples, it now appears that our samples may have been too pure or too dry to allow observation of H-addition radicals on the basic rings. We observed a number of different samples of DNA, but all of them were dry and evacuated. Several laboratories obtained results in agreement with ours. However, Salovey and collaborators²⁴ observed a thymidinelike component in DNA by large irradiation dosage at lowered temperature combined with postirradiation annealing of the sample. Ehrenberg and coworkers²⁵ obtained the thymidinelike resonance by special chemical treatment of the DNA samples before irradiation. Pershan and collaborators⁸ obtained the thymidinelike resonance by irradiation of moist samples of DNA with U.V.

After our single crystal studies of thymidine revealed that the thymidine signal comes from H-addition radicals, it occurred to us that the difficulty in observation of thymidinelike signals in evacuated DNA may be due to the unavailability of H atoms at the proper sites. Consequently, we irradiated samples of DNA under pressure of hydrogen gas, and thereupon were able to produce relatively strong thymidinelike resonance²⁶ in dry DNA.

To my knowledge, no one has detected H-addition radicals either on the cytosine rings in DNA or, with certainty, on the purine rings in DNA. Gordy and coworkers,²⁶ however, observed a cytidinelike triplet, possibly from a radical such as (IX), in a sample of highly polymerized calf thymus DNA γ -irradiated and observed under vacuum at room temperature. Dorlet and collaborators²⁷ obtained a purinelike triplet in a sample of trout-sperm DNA containing 20% H₂O and 1/10% protein, which was irradiated and observed at room temperature.

When they subjected DNA to H atoms, Heller and Cole¹² obtained an unresolved resonance similar to that usually obtained for dry γ -irradiated DNA. Although we have been unable to produce definite signals from H-addition radicals such as those in the purine or pyrimidine bases by H bombardment of DNA, we did obtain a singlet resonance¹⁰ similar to that shown by Heller and Cole.

On the other hand, we were able to produce both purine and pyrimidine signals when we subjected RNA to H atoms at lowered temperature (see FIGURE 10). Because of the similarity in patterns, it is not possible to learn whether the pyrimidine signals are from the cytosine group or the uracil group, or from both; nor can one tell whether the purine signals are from the adenosine group or the guanine group, or from both. However, the pyrimidine signal, like that of cytosine, is unstable at room temperature, whereas the purine signal remains after the sample is brought to room temperature. (See the lower curve of FIGURE 10).

An interesting recent paper by Ehrenberg and coworkers²⁸ reports the observation of definite orientation-dependence of the ESR resonance of strands of DNA irradiated at 77°, both when containing water and when dry. The ef-

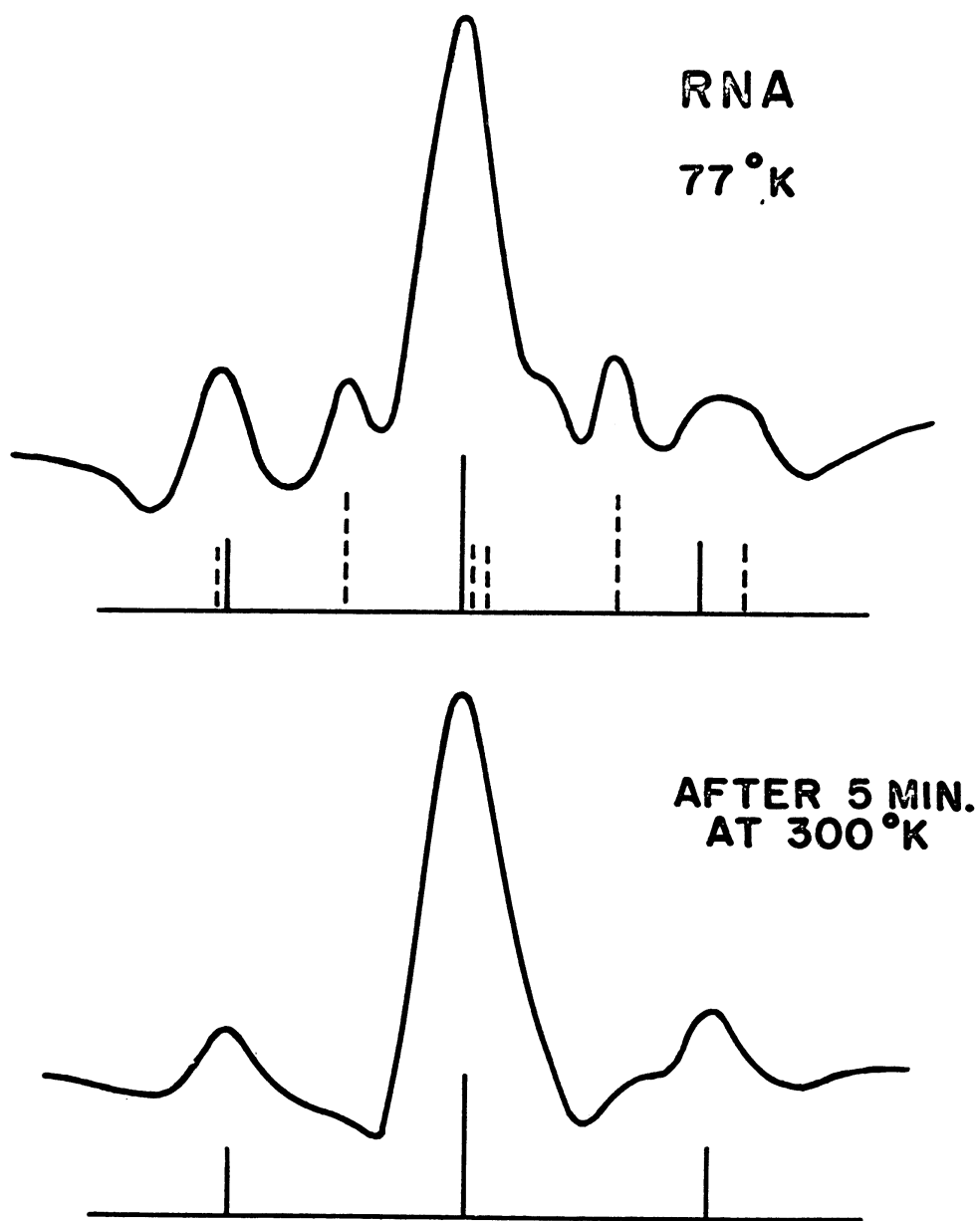
H - BOMBARDED

FIGURE 10. ESR patterns of RNA after exposure to gaseous H atoms at 77° K and then after warming. The dotted lines represent the pattern for the uracil H-addition radical at 77° K (see FIGURE 3), and the solid bars represent that for guanine or adenine (see FIGURE 2). (From Reference 10.)

facts are more pronounced in the moist strands than in the dry strands (see FIGURE 11). Note that the signals in the wet samples after annealing are predominantly the thymidinelike resonance, whereas the signals for the dry sample show no evidence of H-addition radicals even after annealing. Note also that the signals obtained for the moist samples show no structure such as that

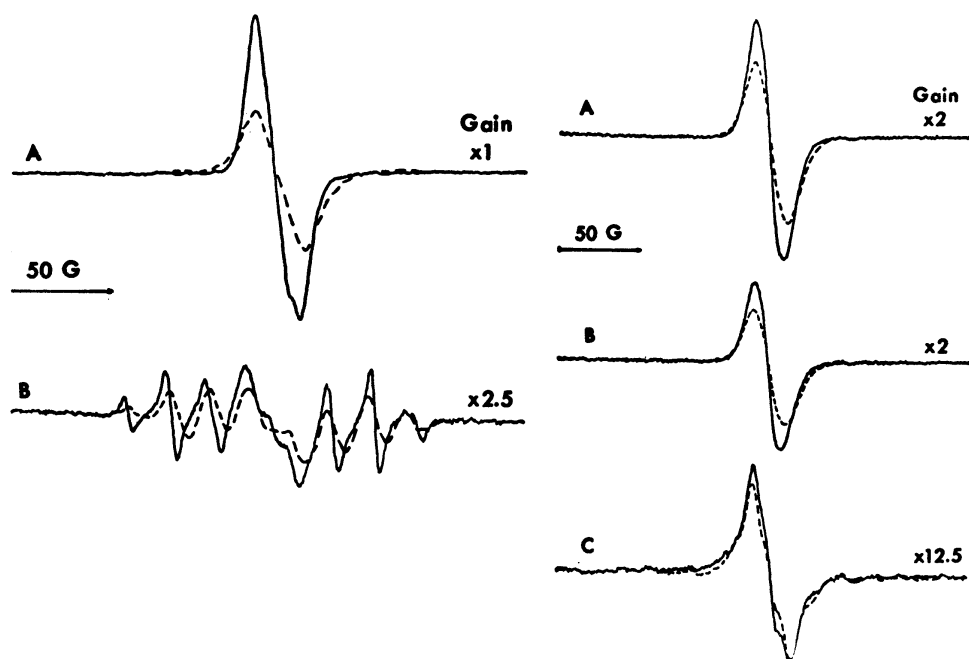


FIGURE 11. (Top left) The ESR spectra from a moist sample of oriented calf-thymus lithium DNA after γ -irradiation at 77° K. (A) Signals obtained at 77° K with the DNA helices parallel with (----) and perpendicular to (—) the field; (B) signals obtained at 77° K after annealing of the sample at 199° K for 5 minutes. (The amplification, relative to that in (A) is 2.5.) (From Reference 28.)

from the H-addition radicals until the samples are annealed. This is consistent with the earlier work²⁹ on DNA + H₂O irradiated at 4.2° K, in which a singlet resonance for the DNA was obtained and separate signals from H atoms. The H atoms are produced by the irradiation at low temperature, but they evidently cannot move to attack the DNA rings until the sample is warmed.

Free Valencies and Spin Densities

It is reasonable to expect that the attack by H atoms or OH free radicals on the purine or pyrimidine rings would occur on the carbon having the highest free valence. In uracil and in the 5-halogen uracils (except 5-F-uracil) it has been possible to show that this is true. TABLE 5 gives the calculated

TABLE 5
CORRELATION OF FREE VALENCE WITH ATTACK BY H OR OH RADICAL¹⁵

Compound	Theoretical Free Valence		Atom Attacked (experimental)
	On C ₍₅₎	On C ₍₆₎	
Uracil	0.526	0.434	C ₍₅₎
5-I-uracil	0.459	0.423	C ₍₅₎
5-Br-uracil	0.446	0.438	C ₍₅₎
5-Cl-uracil	0.435	0.443	C ₍₆₎
5-F-uracil	0.400	0.434	F

free valences on $C_{(5)}$ and $C_{(6)}$, which have the highest values of the atoms of the ring. Also indicated is the carbon, which is found by ESR to be the point of attack by the H atom or OH radical. In 5-F-uracil, the attack occurs on F rather than on a carbon of the ring. For the other 5-halogen-uracils, the initial attack occurs on the carbon having the highest free valence. This is also true for 5-methyl-uracil (thymine).

In guanine, the H addition occurs on $C_{(8)}$, where calculations by Pullman and Pullman³⁰ predict the free valence to be highest, but spin densities on the nitrogens as derived from the single crystal measurements on adenosine (see comparisons in TABLE 6) indicate that the H addition occurs on $C_{(2)}$, whereas the predicted free valence is highest on $C_{(8)}$ for the free base adenosine.

TABLE 6 provides a comparison of observed and theoretical spin densities on certain atoms of H-addition free radicals derived from the measured nuclear coupling constants. The theoretical values are calculated with molecular orbital theory and with the Hückel assumption. The overall agreement is good in view of the approximations involved in the derivations.

Possible Significance for Radiation Damage and Mutations

The observed attack of H atoms at thermal velocities on all the information groups of the nucleic acids—the purine and pyrimidine rings—combined with the prevalent release of H atoms by radiation from H₂O and many other constituents of the biological cells already demonstrated by ESR studies, strongly indicates that attack by H atoms on the nucleic acids is one of the

TABLE 6
COMPARISON OF OBSERVED AND CALCULATED SPIN DENSITIES OF H-ADDITION
RADICALS IN PURINES AND PYRIMIDINES

Radical Source	H-Addition on	Spin Density on	Spin Density			
			Observed at 300° K	Ref.	Calculated	Ref.
Thymine	$C_{(6)}$	$C_{(5)}$	0.70	7	0.72	^d
Uracil	$C_{(5)}$	$C_{(6)}$	0.71	9	0.72	^d
5-OH-uracil (radical)	$C_{(6)}$	$C_{(5)}$	0.64	14	0.67	14
5-Cl-uracil (radical)	$C_{(6)}$	$C_{(5)}$	0.80	15	0.77	14
Cytosine	$C_{(5)}$	$C_{(6)}$	0.71 ^a	10	0.71	20
Deoxyadenosine (monohydrate)	$C_{(2)}$	$N_{(1)}$	0.15	^b	0.12 ^c	20
		$N_{(3)}$	0.37	^b	0.38 ^c	20
Guanine (hydrochloride dihydrate)	$C_{(8)}$	$N_{(7)}$	0.38	18	0.39	20
		$N_{(9)}$	0.08	18	0.08	20

^a Derived from observed value of 0.75 at 300° K.

^b From Lichter, J. J. & W. Gordy. 1968. Proc. Nat. Acad. Sci. 60: 450-455.

^c Theoretical value for radical formed with adenine base by H addition on $C_{(2)}$. For H addition on $C_{(8)}$, predicted spin densities on $N_{(7)}$ and $N_{(9)}$ are 0.43 and 0.09, respectively.

^d From Mantione, M. J. & B. Pullman. 1964. Biochim. Biophys. Acta 91: 387.

most important sources of radiation damage in biology. Many of the biochemicals which release H atoms when irradiated—H₂O, nucleoproteins, enzymes—are so closely associated with the DNA of the cell that H atoms released from them do not have to migrate far before they reach one of the sites where they readily add to the purine or pyrimidine rings. Of course, the H₂O or protein or other chemical which loses the H is also damaged, but such chemicals are more expendable and replaceable than is the DNA of the cell. The addition of an H atom to the base rings of DNA does not necessarily kill the cell nor constitute a mutation per se, but it could well be the initial step in a chain reaction which leads to either result.

We have demonstrated that the OH radical, also a radiation product of H₂O, will readily add to at least one of the pyrimidine rings, uracil. Thus far, our attempts to prove by the ESR method that gaseous OH likewise adds to other pyrimidine and purine groups have been negative, but this may be due to steric factors which prevent the OH from getting to the favorable sites of attack in the solid samples employed. Spectroscopic evidence has been obtained for OH addition to thymidine in solution. Although OH radicals are produced from H₂O, the ESR evidence indicates that they are not released as readily from other chemicals in the cell as are H atoms.

The evidence obtained for radical (IX) in irradiated cytidine suggests a possible cause of mutation. The existence of a radical with its unpaired electron mainly on the carbon that connects the base ring to the sugar group indicates that the formation of this radical might be the first step in the process of breaking off the cytosine base ring and replacing it with another group, perhaps a thymine ring.

References

1. SHIELDS, H. & W. GORDY. 1956. Bull. Amer. Phys. Soc. **1**: 267; GORDY, W. 1958. Electron spin resonance of free radicals in irradiated biochemicals. In Proc. Internat. Cong. Rad. Res. Burlington, Vt. (Suppl. 1, 1959. *Radiation Research*): 491–510. Academic Press. New York, N. Y.
2. SHIELDS, H. & W. GORDY. 1959. Proc. Nat. Acad. Sci. **45**: 269–281.
3. MÜLLER, A. 1967. The formation of radicals in nucleic acids, nucleoproteins, and their constituents by ionizing radiations. In *Progress in Biophysics and Molecular Biology*. **17**: 99–147. Pergamon Press. New York, N. Y.
4. SCHOFFA, G. 1964. Elektronenspinresonanz in der Biologie. G. Braun. Karlsruhe, Germany.
5. POOLE, C. P., JR. 1967. *Electron Spin Resonance: A Comprehensive Treatise on Experimental Techniques*. John Wiley & Sons. New York, N. Y.
6. MÜLLER, A. & W. KÖHNLEIN. 1964. Int. J. Rad. Biol. **8**: 121–130.
7. PRUDEN, B., W. SNIPES & W. GORDY. 1965. Proc. Nat. Acad. Sci. **53**: 917–924.
8. PERSHAN, P. S., R. G. SHULMAN, B. J. WYLUDA & J. EISINGER. 1965. *Science* **148**: 378–380.
9. HERAK, J. N. & W. GORDY. 1965. Proc. Nat. Acad. Sci. **54**: 1287–1292.
10. HERAK, J. N. & W. GORDY. 1966. Proc. Nat. Acad. Sci. **55**: 1373–1378.
11. HERAK, J. N. & W. GORDY. 1966. Proc. Nat. Acad. Sci. **56**: 7–11.
12. HELLER, H. C. & T. COLE. 1965. Proc. Nat. Acad. Sci. **54**: 1486–1490.
13. HOLMES, D. E., L. S. MYERS, JR. & R. B. INGALLS. 1966. *Nature* **209**: 1017–1018.
14. HERAK, J. N. & W. GORDY. 1966. *Science* **153**: 1649–1650.
15. HERAK, J. N. & W. GORDY. 1966. Proc. Nat. Acad. Sci. **56**: 1354–1360.
16. COOK, J. B., J. P. ELLIOTT & S. J. WYARD. 1967. *Molec. Phys.* **13**: 49–64.
17. BERNHARD, W. & W. SNIPES. 1967. Abstr. 15th Ann. Meet. Rad. Res. Soc. **15**. Academic Press, New York, N. Y.
18. ALEXANDER, C. JR. & W. GORDY. 1967. Proc. Nat. Acad. Sci. **58**: 1279–1285.
19. IBALL, J. & H. R. WILSON. 1965. Proc. Roy. Soc. (London) **A288**: 418–429.
20. PULLMAN, B. & M. J. MANTIONE. 1965. *Compt. Rend.* **260**: 5643–5645.

21. WATSON, D. G., D. J. SUTOR & P. TOLLIN. 1965. *Acta Crystallogr.* **19**: 111-124.
22. HERAK, J. N. & W. GORDY. 1966. *Proc. Nat. Acad. Sci.* **55**: 698-704.
23. MÜLLER, A. 1964. *Akad. Wiiss. Lit. (Mainz) Abhandt. Math.-Nat. Kl.* **5**: 143-261.
24. SALOVEY, R., R. G. SHULMAN & W. M. WALSH, JR. 1963. *J. Chem. Phys.* **39**: 839-840.
25. EHRENBERG, A., L. EHRENBERG & G. LÖFROTH. 1963. *Nature* **200**: 376-377.
26. GORDY, W., B. PRUDEN, & W. SNIPES. 1965. *Proc. Nat. Acad. Sci.* **53**: 751-756.
27. DORLET, C., A. VAN DE VORST & A. J. BERTINCHAMPS. 1962. *Nature* **194**: 767.
28. EHRENBERG, A., A. RUPPRECHT & G. STRÖM. 1967. *Science* **157**: 1317-1319.
29. PATTEN, R. A. & W. GORDY. 1964. *Nature* **201**: 361-363.
30. PULLMAN, B. & A. PULLMAN. 1963. *Quantum Biochemistry*: 683. John Wiley & Sons, New York, N. Y.