Electron Spin Resonance of Irradiated DNA

Abstract. *When DNA is irradiated by ultraviolet light a paramagnetic damage center is formed in the thymine bases. The structure of this free radical has been determined by electron spin resonance and consists of a hydrogen addition at the carbon No. 6 position. The rate of formation of the radical is enhanced by water vapor, and it is quenched by paramagnetic ions bound to the DNA.*

Ultraviolet, x-ray, or particle irradiation of living systems is well known to be mutagenic (*1*). The identification of DNA as the molecular source of genetic information (*2*) suggests that radiation induces mutations by damaging the DNA molecule. The technique of electron spin resonance (ESR) can often provide detailed information about a particular site of radiation damage, and a number of workers have used it to study radiation damage in DNA (*3–5*). We have irradiated DNA fibers (calf thymus DNA) with ultraviolet light while the fibers were cold (77°K to 195°K) and then, without allowing them to warm up to 273°K (0°C), we observed the ESR spectrum at 77°K (Fig. 1). By comparison with the results (*6*) on radiation damage to the separate constituents of nucleic acids, the characteristic hyperfine structure of this resonance pattern can be attributed to damage located on the base thymine (*3, 4*). The model for this center of damage is shown in Fig. 2. Thus it may be that an incident ultraviolet photon provides the activation energy (possibly by way of a metastable triplet state) for a hydrogen atom to break the double bond between carbon No. 5 and carbon No. 6 by adding to carbon No. 6, leaving an unpaired electron on the No. 5 carbon.

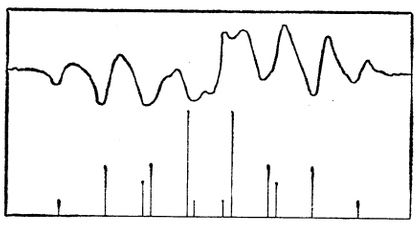

Fig. 1. Electron spin resonance spectra from moist fibers of calf thymus DNA after irradiation with ultraviolet light; 77°K. The bars represent the theoretical intensities. The outermost bars are separated by 20.8 gauss, while the thymine radical has a *g*-factor, measured at *x*-band of 2.0035.

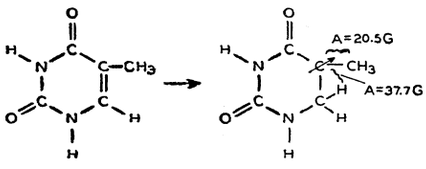

Fig. 2. Proposed model for the thymine radical responsible for the ESR pattern in Fig. 1.

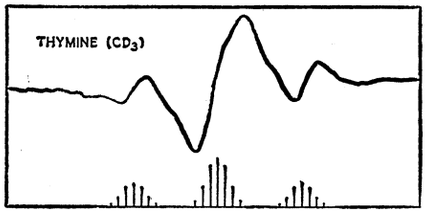

Fig. 3. ESR pattern for irradiated thymine in which the $-CH_3$ group is replaced by $-CD_3$.

The hyperfine pattern (Fig. 1) can thus be explained by assuming that the two protons on carbon No. 6 interact with the unpaired electron to produce three lines with intensities in the ratio 1:2:1 and a splitting of 37.7 gauss between adjacent lines. Each of these lines is further split into four lines with intensities in the ratio 1:3:3:1 and separated by 20.8 gauss by way of a hyperfine interaction with the $-CH_3$ group attached to the carbon No. 5. The net effect of these splittings is shown by the bars under the spectrum curve in Fig. 1.

Although theoretical arguments present reasonably convincing evidence to support this model, there are more convincing experimental proofs (*5*). For example the $-CH_3$ group attached to carbon No. 5 was replaced by a $-CD_3$ group which for our purposes has a negligible hyperfine interaction. The experimental trace for deuterated thymine (not DNA) is shown in Fig. 3. The simple three-line, 1:2:1 pattern produced by the two protons on carbon No. 6 proves that at least in thymine the unpaired electron interacts with the $-CH_3$ group as suggested. In order to establish a connection between the model for damage to thymine and the observations in DNA we grew *Escherichia coli* 15T$^-$ on deuterated thymine. This bacterium is a mutant which cannot synthesize thymine and thus will survive only if it is fed thymine. Comparison between irradiated DNA's extracted from *E. coli* grown on deuterated and on normal thymine shows differences similar to the differences in Figs. 1 and 3 (*5*); these differences prove that in DNA, too, the major radical formed by irradiation is the one shown in Fig. 2.

Early in our studies we observed slightly different ESR spectra depending on the amount of moisture the DNA fibers had absorbed before irradiation. Actually the first observation was that if the fibers were perfectly dry the damage center was stable at room temperature and higher, but slight amounts of moisture caused the radical to vanish completely in a few seconds at temperatures above 0°C. Figure 4 shows the resonance pattern observed from samples of calf thymus DNA that had been held at different

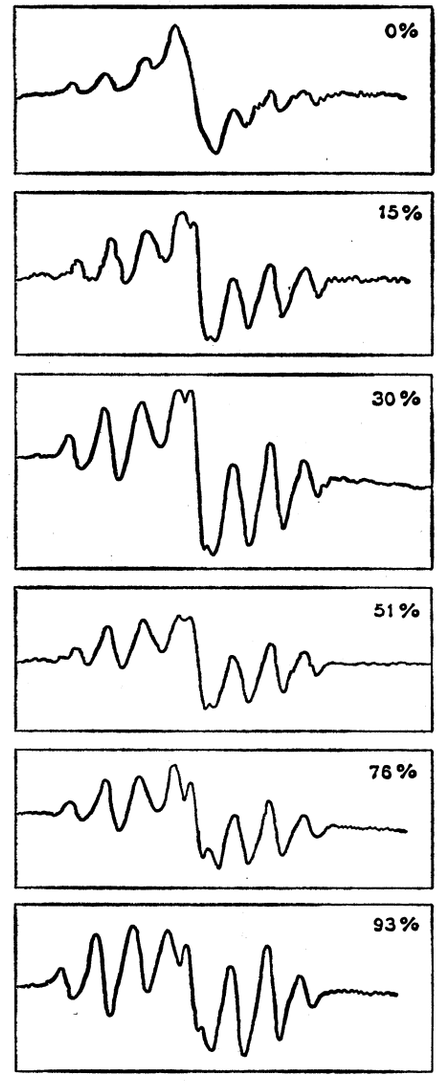

Fig. 4. Effects of equilibrating calf thymus DNA with different relative humidities of water vapor in percent at 298°K before irradiating and observing the resonances at 77°K.

relative humidities for 48 hours at 25°C. Irradiation and ESR measurements were made at 77°K. Removal of water from DNA destroys some of the regular structure observed by x-ray diffraction (*7*), and in the limit of extreme drying the hydrogen bonds linking the bases on opposite chains are broken, causing effects similar to denaturation (*8*). In a separate experiment we showed that native and denatured DNA samples which contain equal amounts of water give identical ESR signals; thus the differences between dry and wet samples are not explained by different secondary structures. The similarities between the native and denatured samples provide no support for the hypothesis that the aforementioned damage arises from excitation traveling along the molecule from base to base.

If the DNA is thoroughly dried and then exposed to D_2O vapor instead of to H_2O vapor, the different ESR spectrum observed (*5*) can be interpreted as deuteron addition to carbon No. 6 rather than as the hydrogen addition shown in Fig. 2. Therefore, in moist DNA the extra hydrogen comes either from an exchangeable hydrogen or directly from the water. However, the signal from dry DNA is exactly the same whether the DNA is dissolved in H_2O or D_2O before being dried at reduced pressure. There must be two sources of hydrogen, one of which predominates in moist DNA and one in dry.

The ESR spectra of many substances that had been damaged by radiation shows on careful analysis that although the major paramagnetic resonance pattern is due to the thymine radical (Fig. 2) there are five other radicals which have been observed in irradiated DNA. Although these have been characterized (*5*) by their line widths, *g*-factors (magnetic field for resonance), and in some cases by their hyperfine splitting, still they have not been identified with particular molecular sites. In typical moist calf thymus DNA all other radicals together contribute about one half of the total ESR absorption intensity, the remaining half coming from the thymine radical.

Because the thymine radical disappears at room temperature in moist or wet DNA it cannot cause biological damage. Nevertheless it could be the precursor of a stable photoproduct and thus represent an intermediate step in the photochemistry of damage. The

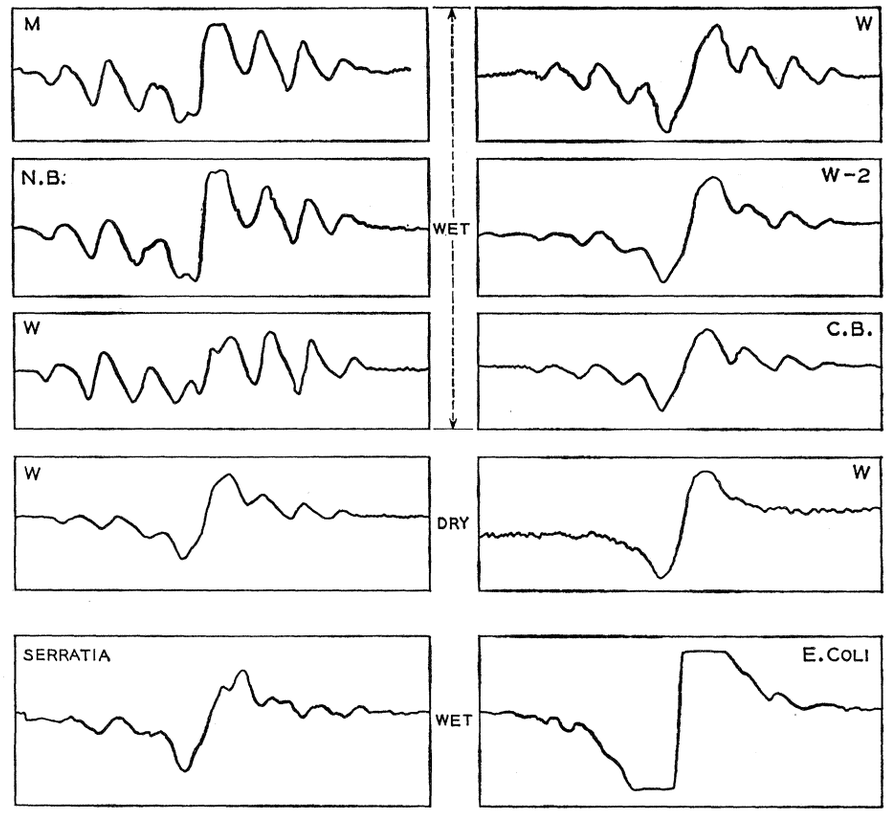

Fig. 5. Reproducible differences in the relative amounts of thymine radical and center line in DNA from different animals (left, calf thymus; right, salmon sperm) and different sources (*M*, Mann; *N.B.*, Nutritional Biochemical; *W*, Worthington; *C.B.*, Cal Biochemical). This figure also shows the difference between dry and moist DNA from calf thymus and salmon sperm. The bottom two traces are from *Serratia cetals* and *Escherichia coli*. The magnetic field increases from left to right.

following two measurements show that the thymine radical is formed almost as abundantly and efficiently as other stable photoproducts of DNA. In thin films of DNA irradiated until the ESR signal stopped growing approximately 5 percent of all the thymine in the sample was converted to the radical shown in Fig. 2. This compares favorably with values of 5 to 20 percent of all thymine in DNA converted to the thymine dimer (*9*) under different experimental conditions. Since the dimer is an important source of damage (*10*) the comparison shows that the thymine radical is present to a measurable extent. Our preliminary evidence (*5*) indicates that the radical is not a precursor to the dimer, but Wacker (*11*) has shown there are other stable thymine photoproducts besides the dimer which are approximately equally abundant. The thymine radical could be a precursor to one of these.

A second experiment measured (*5*) the quantum yield for thymine radical production, that is, the number of thymine radicals produced per photon absorbed by the DNA, as 0.0005. This can be compared with a value of 0.001 for the thymine dimer in calf thymus DNA obtained by extrapolation of Deering and Setlow's (*12*) results on dithymidylic acid to DNA. A more direct measure of the number of dimers formed per photon absorbed by DNA can be obtained from Wolff's (*9*) study of *E. coli* DNA. Extrapolating his results to calf thymus DNA, we find an efficiency of about 0.004. Hence the yield of radical is one-half to one-eighth as great as the dimer yield, and the radical is not a negligible photoproduct.

A more definite experiment that could be done to identify the stable photoproduct, if any, would be to moisten DNA with tritiated water vapor before ultraviolet irradiation. The DNA could then be hydrolyzed, and the stable photoproducts could be measured for radioactivity. Since we have proved that the radical is formed by the addition of hydrogen at carbon No. 6, where the hydrogen has ultimately come from water, it is likely that the

radical anneals by the addition of something at carbon No. 5 and by quenching the unpaired spin. The ESR signal vanishes, but the final photoproduct would be tagged. Alternatively, when the radical vanishes, it could lose either of the protons on carbon No. 6. In this case half the photoproducts would lose the tritium and half the hydrogen, so the final product would again be tagged.

Beukers and Berends (*13*) have reported that paramagnetic metal ions reduce the photodamage of the pyrimidine derivative, orotic acid. Experiments were performed when Co++ and Cu++ ions were bound to the DNA before irradiation in the ratio of one metal ion to ten phosphates. Samples containing the paramagnetic metal ions showed that the concentrations of free radicals were reduced by a factor of about 6 as compared with similar samples irradiated under the same conditions, but containing no metal.

Finally we present the ESR signals from different DNA samples (Fig. 5). The four traces on the upper left are calf thymus DNA; three are moist and one is dry. The three wet samples are from different sources, but the values attained are in excellent agreement. There are significant differences between these and the salmon sperm DNA on the upper right where in comparison the thymine hyperfine lines are less intense with respect to the unidentified central lines. At the bottom are traces of samples of moist DNA from *E. coli* and *Serratia cetals*. The thymine hyperfine structure can be identified in all cases, but in different species its intensity relative to the center line varies. These variations from species to species could originate in the chemical techniques by which the samples are extracted from their parent cells.

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References and Notes

1. H. J. Muller, *Amer. Nat.* **60** (1926).
2. O. T. Avery, C. M. MacLeod, M. McCarty, *J. Exp. Med.* **79**, 137 (1944).
3. R. Salovey, R. G. Shulman, W. M. Walsh, Jr., *J. Chem. Phys.* **39**, 839 (1963); J. Eisinger and R. G. Shulman, *Proc. Nat. Acad. Sci. U.S.* **50**, 694 (1963).
4. A. Ehrenberg, L. Ehrenberg, G. Lofroth, *Nature* **200**, 376 (1963).
5. P. S. Pershan, R. G. Shulman, B. J. Wyluda, J. Eisinger, *Physics* **1**, 163 (1964).
6. H. Shields and W. Gordy, *Proc. Nat. Acad. Sci. U.S.* **45**, 269 (1959).
7. R. Langride, H. R. Wilson, C. W. Hooper, M. H. F. Wilkins, L. D. Hamilton, *J. Mol. Biol.* **2**, 19 (1960).
8. M. Falk, K. A. Hartman, Jr., R. C. Lord, *J. Amer. Chem. Soc.* **85**, 391 (1963).
9. D. L. Wulff, *Biophysical J.* **3**, 355 (1963).
10. R. B. Setlow, and J. K. Setlow, *Proc. Nat. Acad. Sci. U.S.* **48**, 1250 (1962).
11. A. Wacker, *Progr. Nucleic Acid Res.* **1** (1963).
12. R. A. Deering and R. B. Setlow, *Biochem. Biophys. Acta* **68**, 526 (1963).
13. R. Beukers and W. Berends, *ibid.* **49**, 181 (1961).
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Peptides Attached to Thrombin: Their Influence on Proteolysis

Abstract. *During the activation of prothrombin at least two peptides are split off the molecule. These peptides become attached to thrombin, changing its proteolytic activity. Depending on the peptide adsorbed the endopeptidase activity of thrombin increases or diminishes. Acidification of thrombin by acetylation of amino groups uncovers the influence of peptides on the properties of thrombin.*

The activation of prothrombin is presumed to be an orderly degradation of the protein molecule (*1*). Enzymes with endopeptidase activity, which use prothrombin as substrate, are derived from prothrombin itself. Thrombin (*1*) and autoprothrombin C (*2*) can hydrolyze the prothrombin molecule. Thrombin will be the end product of prothrombin activation whenever this process goes to completion. Thrombin, acting on fibrinogen (as substrate), releases two peptides, thus allowing fibrin to form by polymerization. This is the primary role of thrombin in the clotting mechanism. Thrombin can also utilize synthetic substrates such as tosyl arginine methyl ester (TAMe), showing an activity which is differentiated from the clotting activity; this is called the esterase activity (*3*). Determinations of thrombin activities, performed with equivalent calibrations, show that whenever prothrombin is activated, the first activity that develops is the esterase activity. This is shortly followed by the development of the clotting activity. When activation is completed the esterase activity equals clotting activity (*4*).

These properties of thrombin have been associated with free amino groups, since the acetylation of 50 percent of the free amino groups results in a product without clotting activity but with the same or increased esterase activity (*5*). The affinity for both substrates, namely fibrinogen and TAMe, changes with the acetylation. The acidification of thrombin by acetylation does not allow the interaction of thrombin with fibrinogen to occur, although fibrin is still acted upon by acetylated thrombin (*5*).

The thrombin molecule is known to be associated with two peptides, one containing *N*-terminal glutamic acid (peptide A), and the other containing threonine (peptide B) (*6*). Thrombin as it is formed during activation or after chromatography on amberlite IRC-50 shows glutamic acid as *N*-terminal and after chromatography on phosphate cellulose, shows threonine. This suggests that the peptides may become redistributed on the surface of the molecule as a result of the purification procedures. Only by treating thrombin with a 30-percent solution of urea will the molecule be freed of adsorbed peptides and show the *N*-terminal isoleucine (*6, 7*).

The only form of thrombin that is free of peptides is the one obtained by activation of prothrombin with trypsin (*8*). Consequently, there may be four types of thrombin product: (i) thrombin chromatographed with amberlite, having adsorbed on its surface the peptide with glutamic acid as *N*-terminal, (ii) thrombin chromatographed on phosphate cellulose, having adsorbed on its surface the peptide with threonine as *N*-terminal, (iii) thrombin treated with concentrated urea solutions and free of peptides, and (iv) thrombin obtained by trypsin activation.

To gain some insight into the significance of the peptide structures ad-

Table 1. Changes in esterase activity and the endopeptidase activity induced by acetylation of thrombin.

Esterase (units/ml)	After acetylation		
	Esterase (units/ml)	Endopeptidase activity (expressed as milligrams of tyrosine)	
		500 units thrombin	1000 units thrombin
Amberlite-purified thrombin			
10,000	21,000	0.12	0.19
Cellulose-purified thrombin			
12,000	6,000	0.10	0.18
Trypsin-purified thrombin			
10,000	10,000	0.11	0.20
Urea-treated thrombin			
10,000	11,000	0.10	0.19