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RADIATION-INDUCED RADICALS IN THYMINE: ESR STUDIES OF SINGLE CRYSTALS

Thormod Henriksen and Wallace Snipes

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RADIATION-INDUCED RADICALS IN THYMINE: ESR STUDIES OF SINGLE CRYSTALS[1]

Thormod Henriksen and Wallace Snipes

Donner Laboratory and Lawrence Radiation Laboratory
University of California
Berkeley, California 94720

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ABSTRACT

The radicals induced in single crystals of thymine monohydrate by ionizing radiation have been studied by ESR. Both at 77°K and at room temperature three different radicals are formed. The spectral changes observed at different temperatures are due to variations in the relative yields of these three radicals. In addition to the previously known 5-thymyl radical, two other radicals have been identified. The hyperfine splitting tensors and the directions of the principal axes show that for both radicals the unpaired spin density is mainly in a p_π orbital on C_6. One radical has hyperfine coupling to one α and one β proton; the other radical has coupling to one α proton only. The radicals are the 6-thymyl radical formed when a hydrogen atom is added to C_5, and a radical in which a hydrogen atom is added to the oxygen atom bonded to C_4. The latter radical is presumably formed by electron capture with subsequent proton tunneling in the hydrogen bond to the water molecule.

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KEY WORDS: Thymine, Single crystals, Electron spin resonance, Free radicals.

INTRODUCTION

The free radicals induced by radiation in the DNA component thymine have been studied in several different laboratories during recent years (1-4). These studies have so far been restricted to polycrystalline samples or frozen aqueous solutions (5-6), which has made the identification of the radicals very difficult. However, it has been found that the 5-thymyl radical, which is formed by adding a hydrogen atom to the 6-position in the thymine ring, is one of the radicals induced in this compound (7-10). This identification was made possible because the 5-thymyl radical exhibits a well-defined resonance spectrum extending beyond the resonances of the other radicals. The relative yield of this radical was studied under different experimental conditions, and it was found that the radical hardly accounts for more than 30% of the composite ESR spectrum of X-irradiated thymine (4). The other radicals induced in thymine, which seem to be precursors of the 5-thymyl radical, have not been identified so far.

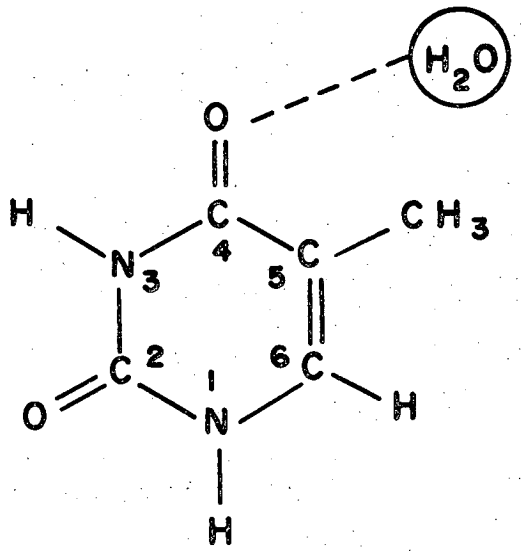
By extremely slow evaporation of aqueous solutions of thymine, single crystals can be grown that are large enough to allow the technique of "single crystal ESR studies." In this paper the details of the work are presented. Thymine crystals were irradiated both at 77°K and at room temperature. Three different radicals were formed and identified. The same three radicals are formed at both temperatures, but the two previously unknown radicals are identified at 77°K, at which the 5-thymyl radical makes a negligible contribution to the composite resonance spectrum.

EXPERIMENTAL PROCEDURE

Crystal preparation

The thymine crystals were obtained from dilute aqueous solutions that were allowed to stand at 4°C for 3 years. The crystals are parallelepipeds with a well-defined morphology. According to Gerdil (11) the crystals contain water of crystallization, are elongated in the c direction, and have {0 1 0} as well-developed form (see Fig. 1). The water is rapidly lost unless the crystals are kept with some of the mother liquor or covered with silicone grease. When the water of crystallization is lost the crystals turn white and the crystal structure collapses. Only transparent crystals were used in this work. They were approximately 3 mm in the c direction, 1.2 mm in the a' direction, and 0.5 mm in the b direction, and weighed between 2 and 3 mg.

Thymine monohydrate is monoclinic with space group $P2_1/c$. There are four molecules in the unit cell, which has the parameters a = 6.077 Å, b = 27.862 Å, c = 3.816 Å, and β = 94° 19'. The molecules are packed in stacks consisting of ribbons of two molecules in width, which are inclined about 30° to (0 0 1). The molecules within each ribbon are hydrogen bonded. Furthermore, the ribbons are bonded together by the water molecule, which is hydrogen bonded to the oxygen atom on C_4 (I).



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In this work the orthogonal a' bc coordinate system was employed (see Fig. 1).

Irradiation and Orientation of the Crystals

The crystals were irradiated with 6.5-MeV electrons from a linear accelerator. In order to obtain a good signal-to-noise ratio, doses of approximately 50 MR were used. The results were checked also for crystals irradiated with 5 MR. The dose rate was approximately 1 MR/min, which ensures that no heating of the crystals takes place. The irradiation temperature was either 77 °K or 295 °K.

Irradiation and observation at room temperature involved no problems with regard to crystal mounting. The crystals were mounted with silicone grease on Teflon rods, and they could be oriented very precisely. At 77 °K the crystals were irradiated in one end of a quartz tube. After irradiation the crystals were transferred to the other end, which was shielded during irradiation. A Teflon rod in the shielded end

was machined to fit the particular crystal in one fixed orientation. With some effort it was possible to mount the crystal in the desired orientation without any increase in the temperature. In this work three different crystals were used for measurements in the three mutually perpendicular planes a'b, bc, and ca'. The orientation was less accurate than that at room temperature, and the positions of the different axes in a plane may be off by 10° to 15°. Particularly, in the bc plane the crystal may also have been tilted slightly out of the plane.

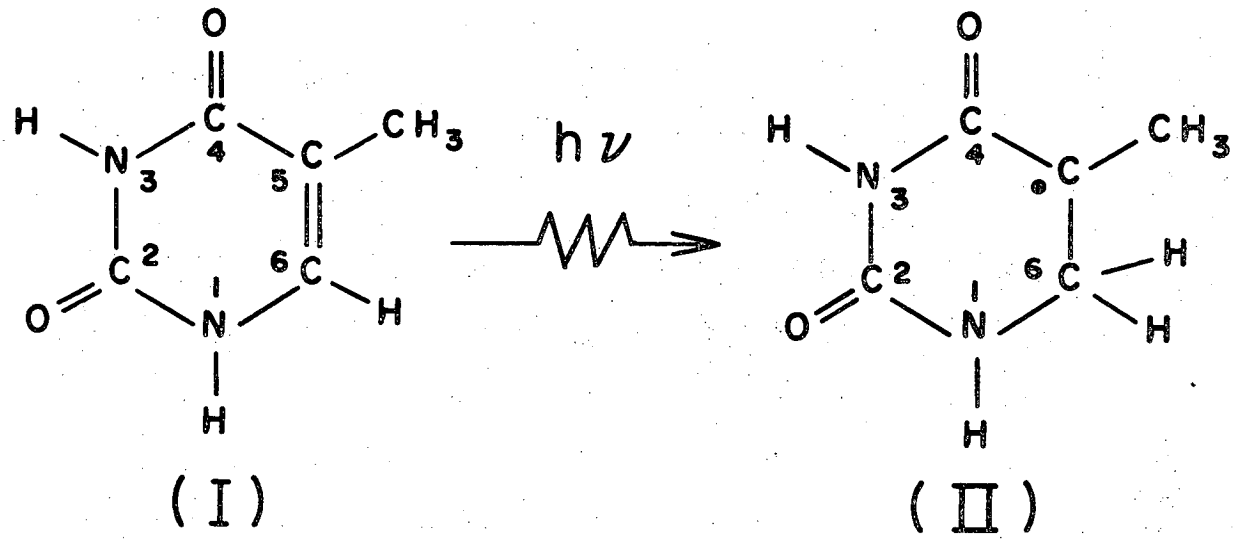
ESR Observations

A Varian 4502 spectrometer with the X-band rotating cavity was used. The results were checked at 16,200 MHz by use of a microwave bridge constructed in our laboratory. The observation temperature was mainly 77°K. Spectra were recorded at every 5° of rotation in three planes. The magnetic field was measured with a proton resonance field meter. The calculations necessary for diagonalizing the hyperfine splitting tensors were made with a CDC 6600 computer.

RESULTS

Observations at 77°K

It is well known from experiments on polycrystalline samples that the 5-thymyl radical (II) is formed in thymine both at room temperature and at 77°K (2-4). The ESR spectrum consists mainly of eight lines spread out over approximately 140 gauss. No attempts were made in this work to analyze in more detail the ESR parameters for this radical. It should be mentioned that the radical contributes significantly to the room-temperature spectra (see below). At 77°K the 5-thymyl radical spectrum is very weak and it is therefore possible to analyze



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the remaining part of the composite spectra. Throughout this work the 5-thymyl radical spectrum is indicated in the figures by arrows pointing towards the two outermost lines on each side.

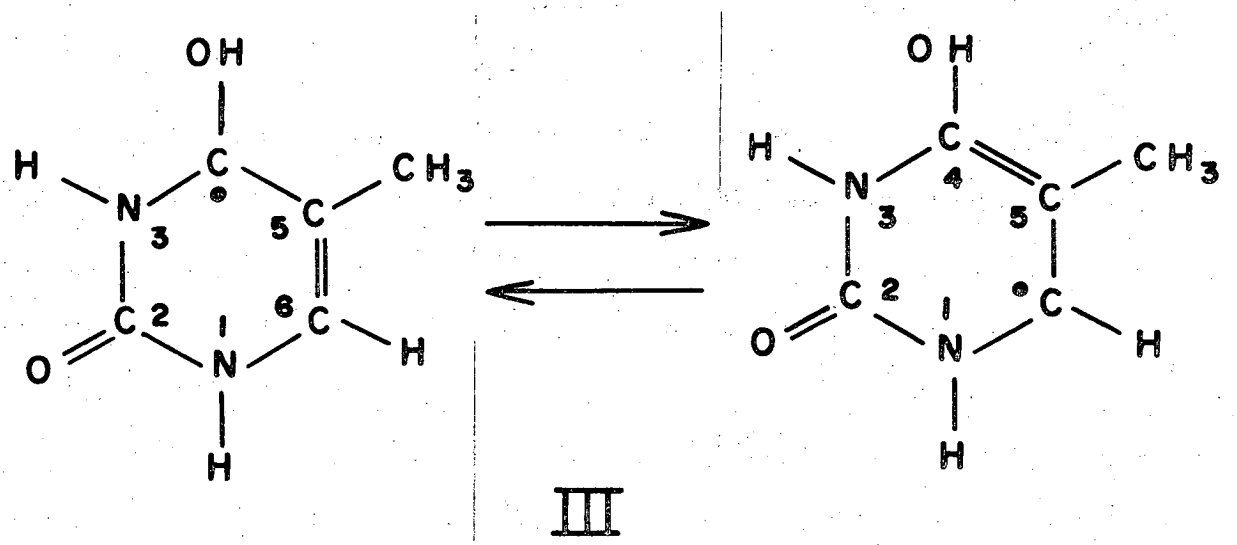
In Figs. 2 and 3 are presented some of the more typical ESR spectra obtained when a single crystal of thymine monohydrate is irradiated at 77°K. In addition to the 5-thymyl radical spectrum the resonance seems to consist of a doublet (see Fig. 2, middle spectrum) and a quartet. Thus, for a number of different orientations mainly six lines are observed (for example in the b and c directions). The possibility that this splitting is partly due to two magnetically different sites (space group $P\,2_1/c$) must be excluded, since the same spectrum is observed in the ca' plane perpendicular to the diad axis (see Fig. 3). The doublet and quartet have different relative intensities at different orientations, confirming that two radicals are formed.

The Doublet Resonance

At 77 °K the doublet may be observed for most orientations even though it partly overlaps the central quartet lines. The g value is nearly isotropic, and varies only from 2.0020 to 2.0030. The observed splitting in the three mutually perpendicular planes is given in Fig. 4. From these data it is possible to arrive at the maximum and minimum values in each plane. The method given by Schonland (12) was then used to determine the hyperfine splitting tensor. The principal values and the direction cosines for the principal axes in the a'bc system are given in Table I.

The isotropic value of 21.1 gauss and the anisotropy suggest that the radical is of the type $> \dot{C}$-H, where the unpaired electron is in a p_π orbital on the carbon atom and interacts with one α proton. Unless drastic molecular rearrangements are assumed only a few possibilities exist. Proper identification of the radical is made possible by using the directions for the principal axes in Table I. Thus, the intermediate hyperfine value is along an axis only 1° from the perpendicular of the plane containing the thymine molecule. Furthermore, the smallest hyperfine value is in a direction only 13° from the C_6-H bond in the undamaged molecule. This orientation of the principal axes is hardly fortuitous and strongly suggests that the unpaired electron spin density is mainly in a p_π orbital on carbon atom 6 in the thymine ring.

As mentioned above, the water of crystallization is hydrogen-bonded to the oxygen on C_4. This suggests, therefore, that the absorption of radiation results in a proton transfer in the hydrogen bond, probably by a tunnel effect (13, 14), and that radical III, which is stabilized by resonance structures, is formed.



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It is expected that a radical of this type would exhibit hyperfine splitting characteristic for a $> \dot{C}$-H fragment, and additional splitting from the β hydrogen in the $> \dot{C}$-O-H fragment. In radical III the latter splitting is too small to be resolved. The main reasons for this are briefly given as follows:

1. The hyperfine splitting of the β proton in $> \dot{C}$-O-H depends upon the unpaired spin density localized on C_4. In radical III the unpaired spin density is shared between C_4 and C_6. When the formula given by McConnell and Chesnut (15) for the isotropic splitting in a $> \dot{C}$-H fragment is used, it appears that the spin density on C_6 is roughly 0.6 to 0.8. This would imply a relatively small unpaired spin density of the order 0.2 to 0.4 on C_4.

2. The isotropic splitting of the β proton in the $> \dot{C}$-O-H group also depends on $\cos^2\theta$, where θ is the dihedral angle between the direction of the orbital containing the unpaired electron (which in the case of

radical III is a p_π orbital on C_4) and a plane through C_4-O-H. Since the hydrogen bond in the undamaged molecule is in the plane of the thymine molecule, it is reasonable to assume a large dihedral angle and consequently a negligible coupling.

3. The dipolar coupling of the β proton can be given (16, 17) by

$$A_d = 28.2\ \rho/r^3, \qquad (1)$$

where ρ is the spin density on C_4 and r is the distance in angstroms from C_4 to the hydroxyl proton. If r is taken to be approximately 1.8 Å the dipolar splitting will at maximum be of the order 1 to 2 gauss.

In view of this it can safely be concluded that the contribution from the hydroxyl hydrogen would result in only a broadening of the doublet lines from the $> \dot{C}$-H fragment.

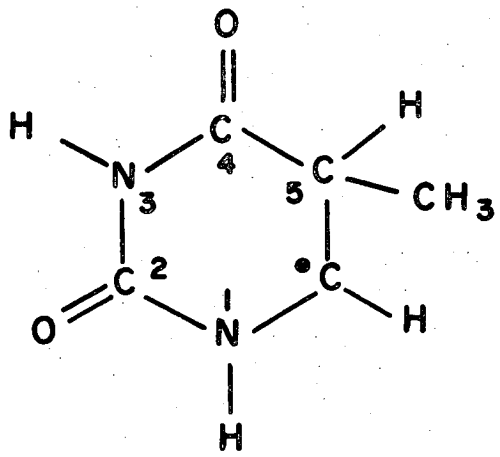
It can also be mentioned that another possibility for forming the $> \dot{C}$-H radical on C_6 would be by adding a hydroxyl group to C_5. In this case, there would be no resonance structures to reduce the unpaired spin density on C_6, and a larger isotropic coupling than that observed would be expected (see also below). We consider this possibility as being quite unlikely, and suggest that a hydrogen addition to the oxygen on C_4 gives rise to the doublet resonance.

The Quartet Resonance

The g value for the quartet shows only a small variation, ranging from 2.0020 to about 2.0033. Due to overlapping of the different spectra it is difficult to arrive at the intensity distribution of the lines. However, taking into account the somewhat more resolved spectra observed at 295°K in the ca' plane (an example is given in Fig. 5), the quartet lines appear to have almost equal intensities. The ESR spectra

can therefore be accounted for by a radical having unequal coupling to two protons. One coupling is quite anisotropic, whereas the other is only slightly anisotropic. This strongly suggests that the radical is of the type $-\dot{C}H_{\alpha}-CH_{\beta}<$, where the unpaired electron is mainly in a p_{π} orbital interacting with one α and one β proton. The α- and β-proton splitting data in the three crystal planes are given in Figs. 6 and 7.

By use of the maxima and minima and Schonland's method (12), the hyperfine splitting tensors and the direction cosines for the principal values in the a'bc system are obtained; they are given in Table I. It follows from these data that the direction of the intermediate α proton splitting constant is perpendicular to the plane of the thymine molecule. Furthermore, the direction of the principal axis for the smallest splitting constant is only 9° from the C_6-H bond in the undamaged thymine molecule. Consequently, these results are compatible with the 6-thymyl radical (IV), formed by adding a hydrogen atom to C_5 in the thymine ring.



(IV)

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As seen from Table I, the isotropic splitting of the α hydrogen is very large (28.3 gauss). For most $>\dot{C}$-H fragments the observed isotropic splitting can be fitted by the relation given by McConnell and Chesnut (15),

$$A = \rho Q, \qquad (2)$$

where ρ is the unpaired spin density on the carbon atom and Q is a constant. Even though Q is not well determined theoretically, most experimental data seem to indicate a value of the order 25 to 26 gauss (18). A possible explanation for the large isotropic value observed here may be that the hybridization of the carbon atom containing the unpaired electron in a p_π orbital has changed slightly from that dealt with in most $>\dot{C}$-H fragments. Thus, Bernal, Rieger, and Fraenkel (19) predict, from theoretical calculations, a strong dependence of Q on the hybridization angle (2 gauss/degree). In the undamaged molecule the angles for the $>\dot{C}$-H fragment are all different and the angle N_1-C_6-C_5 is 121.8°. When the 6-thymyl radical is formed the C_5-C_6 double bond is broken and some rearrangements presumably take place. It is reasonable to assume that the C_5-C_6 bond length increases, which would cause a decrease of the N_1-C_6-C_5 angle. If, for example, the C_5-C_6 bond became equal to the other carbon-carbon bond (the C_4-C_5 bond) the angle would decrease to about 115°, and the isotropic hyperfine splitting would increase significantly (19).

Observations at Room Temperature

In experiments with polycrystalline samples of thymine considerable spectral changes were observed when a sample, initially irradiated at 77°K, was warmed to room temperature (4). The experiments on

single crystals presented here show (Figs. 5 and 8) that the same three radicals are formed at both temperatures and that the spectral changes with temperature must be ascribed to variations in the relative yields of these radicals. It is clearly seen (Fig. 8) that the 5-thymyl radical contributes far more to the spectra at room temperature. At room temperature the 5-thymyl radical spectrum interferes so strongly with the doublet and the quartet for so many orientations that it would be extremely difficult to solve the crystal data based only on these observations. On the other hand, the observations in the ca' plane (see Fig. 5) demonstrate that both the "doublet radical" and the 6-thymyl radical are formed at room temperature.

DISCUSSION

Three different radicals are formed and trapped when thymine is exposed to ionizing radiation. In addition to the well-known 5-thymyl radical (II), the 6-thymyl radical (IV) and a radical (III) giving rise to a doublet have been found in the work presented here. The relative yields of these three radicals depend strongly upon the experimental conditions. Thus, the 5-thymyl radical spectrum dominates the composite resonance when thymine is exposed to thermal hydrogen atoms (4, 8-10) or when irradiated in frozen acid and alkaline solutions. The other two radicals, which seem to be precursors of the 5-thymyl radical, dominate when thymine is irradiated in the solid state.

All three radicals are formed when a hydrogen atom is added to the proper position in the thymine ring. The "doublet type radical" may be formed by a proton transfer in the hydrogen bond between the oxygen on C_4 and the water of crystallization. It is likely that the

potential curve for the proton in this hydrogen bond changes considerably if the thymine molecule captures an electron released by the ionizations. Thus, if the depths of the two minima in the potential curve for the hydrogen bond are reversed a proton tunneling would result in a trapped neutralized radical (13, 14). The water of crystallization may therefore be very important for the formation of the radicals. In an experiment with polycrystalline samples we found almost the same resonance spectrum in crystals containing water of crystallization as that for a heat-treated sample from which a large amount of the water had disappeared. However, the yield was approximately three times as large in the thymine monohydrate crystals, demonstrating the importance of the bound water. This observation has certainly also some bearing on the large differences in radical yield reported from different laboratories (3, 4, 20, 21). Thus, polycrystalline thymine samples prepared in various ways presumably have different amounts of water bound to the molecules.

It is of interest to note that both the 5- and the 6-thymyl radicals are also formed in dihydrothymine (22). The 5-thymyl radical in the two compounds exhibits almost the same methyl-group splitting. The two β hydrogens seem to be equivalent in the case of dihydrothymine, whereas they give different splitting constants for thymine (the difference was found to be approximately 7 gauss at 77°K). The 6-thymyl radical, on the other hand, shows an interesting difference in structure for the two compounds. Thus the β-proton splitting, which for thymine has an isotropic value of 17.1 gauss (see Table I), is much larger when the same radical is formed in dihydrothymine (22). In that case the β-proton splitting was found to be isotropic, and had a value of 44.0 gauss at

room temperature and 46.8 gauss at 77°K. The isotropic part of the β-proton splitting seems to be adequately described by the empirical relation (23, 24)

$$A_\beta = (B_1 + B_2 \cos^2\theta)\,\rho, \qquad (3)$$

where ρ again is the unpaired spin density on the α-carbon atom, B_1 and B_2 are constants ($B_1 << B_2$), and θ is the dihedral angle between the plane formed by C_α-C_β-H_β and the direction of the p_π orbital of the unpaired electron. The large difference in the β-proton splitting observed here when the 6-thymyl radical is trapped in two different compounds must therefore be ascribed to a difference in the dihedral angle (ρ is probably quite similar in the two cases). Thus, whereas for dihydrothymine the C_5-H bond (the β-carbon atom is C_5) seems to be almost perpendicular to the molecular plane (θ is of the order 10° to 15°), the same bond is at least 50° away from this direction and more in the plane of the molecule when the radical is trapped in thymine.

ACKNOWLEDGMENTS

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FIGURE LEGENDS

Fig. 1. Crystal morphology and the coordinate axis system used for the thymine monohydrate crystals. The crystals are elongated in the c direction. ESR spectra are observed in the three mutually perpendicular planes a' b, bc, and ca' .

Fig. 2. Second-derivative spectra of a thymine crystal. The crystal was irradiated and observed at 77 °K in the a' b plane. For the orientation, 0 °, the magnetic field is in the b direction, whereas 90 ° corresponds to the a' direction. The solid bars associated with each spectrum give the predicted splittings based on the tensor parameters in Table I. The doublet is marked by the dashed lines. The arrows indicate the positions of the two outermost lines on each side of the 5-thymyl radical spectrum. Observation frequency, 9023 MHz.

Fig. 3. Second derivative spectra in the ca' - plane for a thymine crystal. The magnetic field is in the c direction for the orientation 0 ° . Otherwise as for Fig. 2. Observation frequency, 8958 MHz.

Fig. 4. The splitting (in gauss) between the two lines of the doublet. The filled circles represent observations in which the spectra are well resolved and the positions of the lines relatively well defined. The bars, on the other hand, refer to orientations in which the different resonances overlap. The solid lines are the calculated curves, based on the tensor data given in Table I.

Fig. 5. Second-derivative spectra for a thymine crystal irradiated and measured at 77 °K (top spectrum) and at 295 °K. The crystal

was oriented with the magnetic field in the ca' plane, 45° from the c axis in both experiments. The sweep rate is the same for the two spectra, but the frequencies are 8959 MHz (top spectrum) and 9536 MHz respectively.

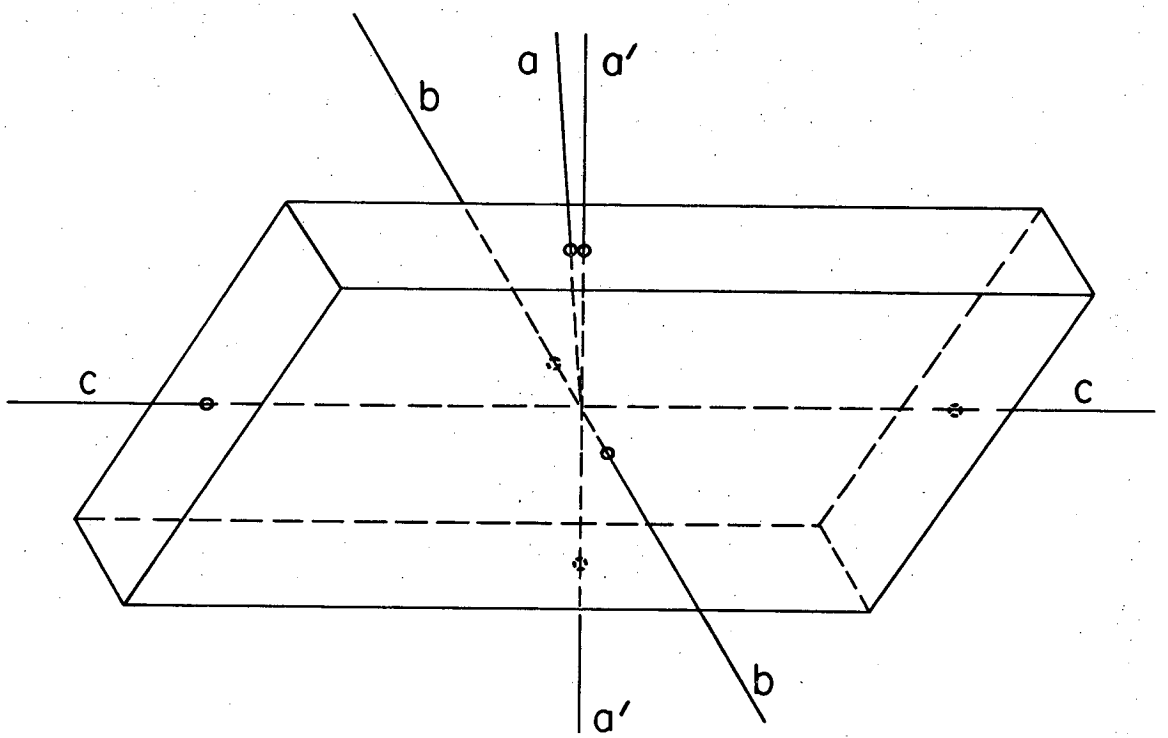
Fig. 6. The splitting (in gauss) for the α hydrogen of the quartet spectrum. Otherwise as for Fig. 4.

Fig. 7. The splitting (in gauss) for the β hydrogen of the quartet spectrum. Otherwise as for Fig. 4.

Fig. 8. Second-derivative spectra of an irradiated thymine crystal. The crystal was irradiated at 77°K and measured (the two top spectra). Subsequently, the crystal was warmed to room temperature and measured again. The orientations in the two experiments are the same. Thus, for the two spectra to the left the magnetic field is in the b direction, whereas for the two other spectra the magnetic field is 45° from b in the a'b plane.

Table I. The principal splitting constants and the orientation of the principal axes for the radicals induced in thymine.

Resonance	Principal splitting values (gauss)	Direction cosines for the principal axes in the a' bc system		
		a'	b	c
Quartet	A_1 = 26.1	-0.40	0.24	0.88
α splitting	A_2 = 36.7	0.29	0.95	-0.12
	A_3 = 22.1	-0.87	0.21	-0.45
Quartet	A_1 = 16.0	-0.72	0.20	0.67
β splitting	A_2 = 20.5	0.25	0.97	-0.03
	A_3 = 14.7	-0.65	0.15	-0.74
Doublet	A_1 = 21.0	-0.42	0.17	0.89
splitting	A_2 = 27.2	0.21	0.97	-0.09
	A_3 = 15.2	-0.88	0.14	-0.45



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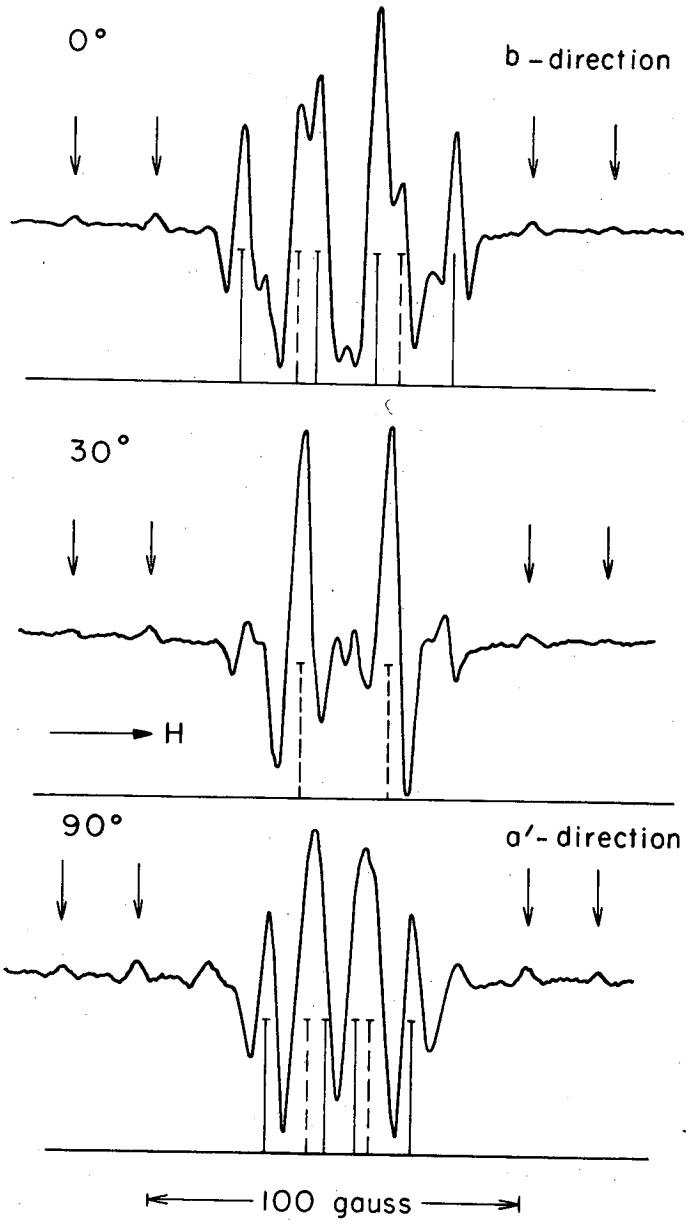

Fig. 2

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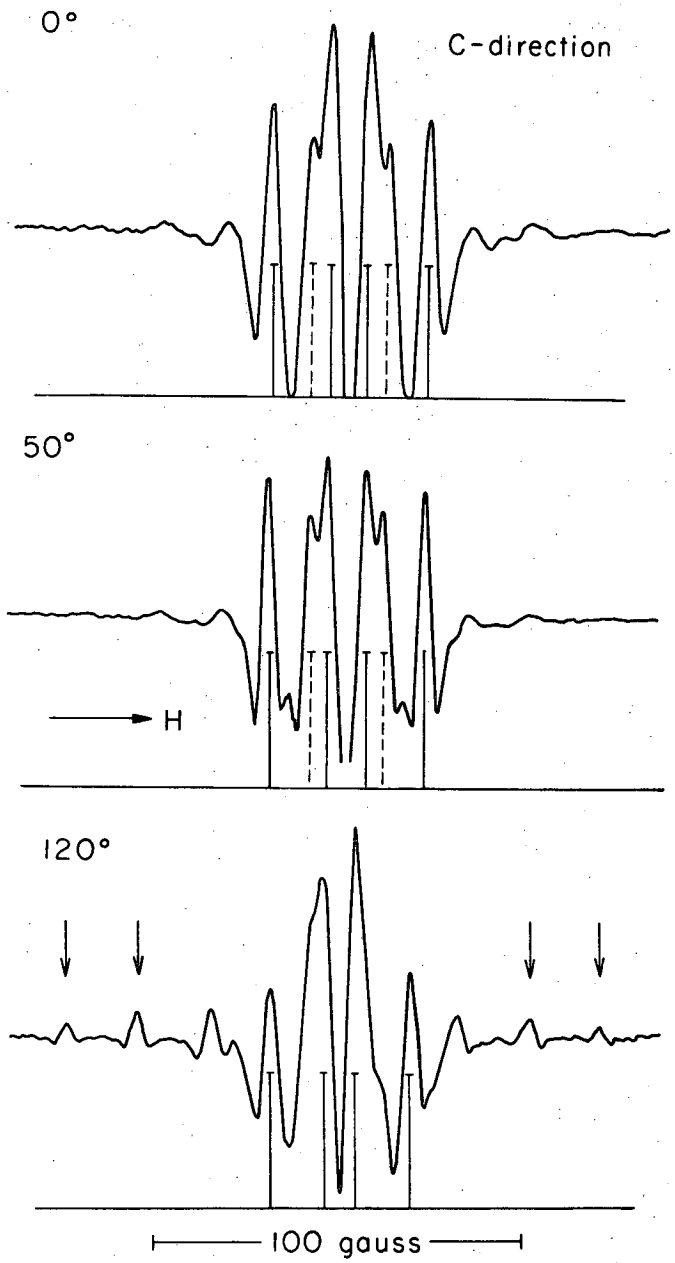

Fig. 3 XBL698-3520

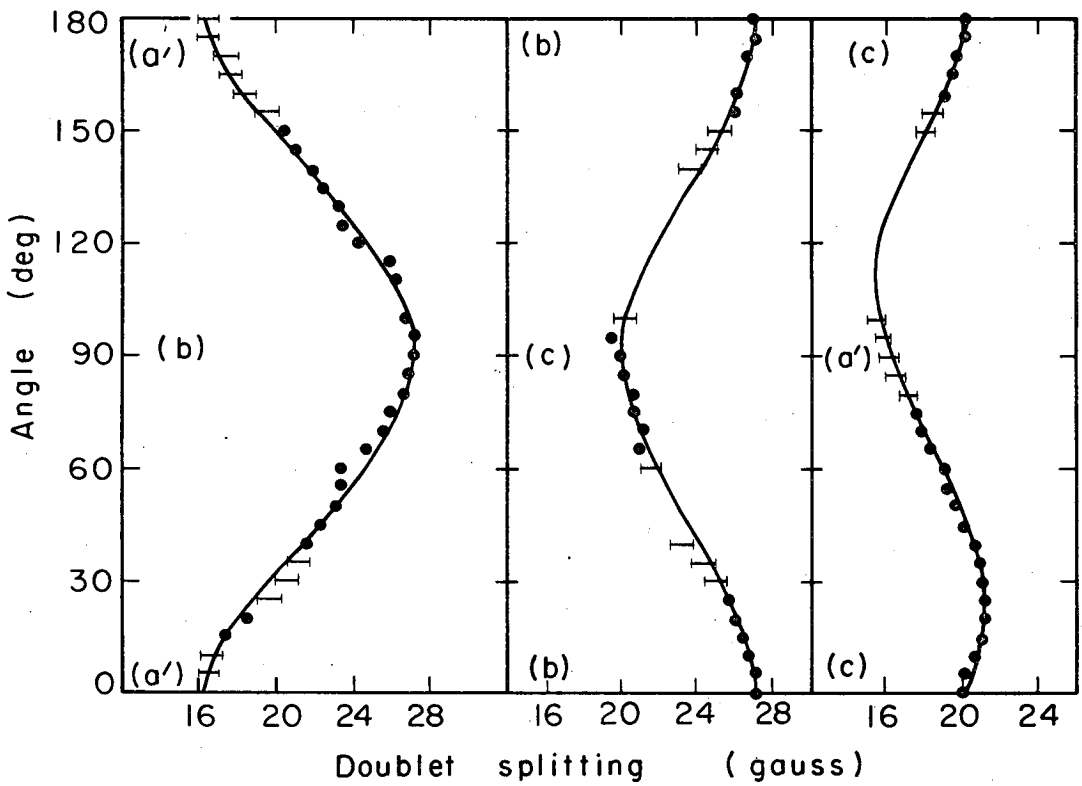

Fig. 4

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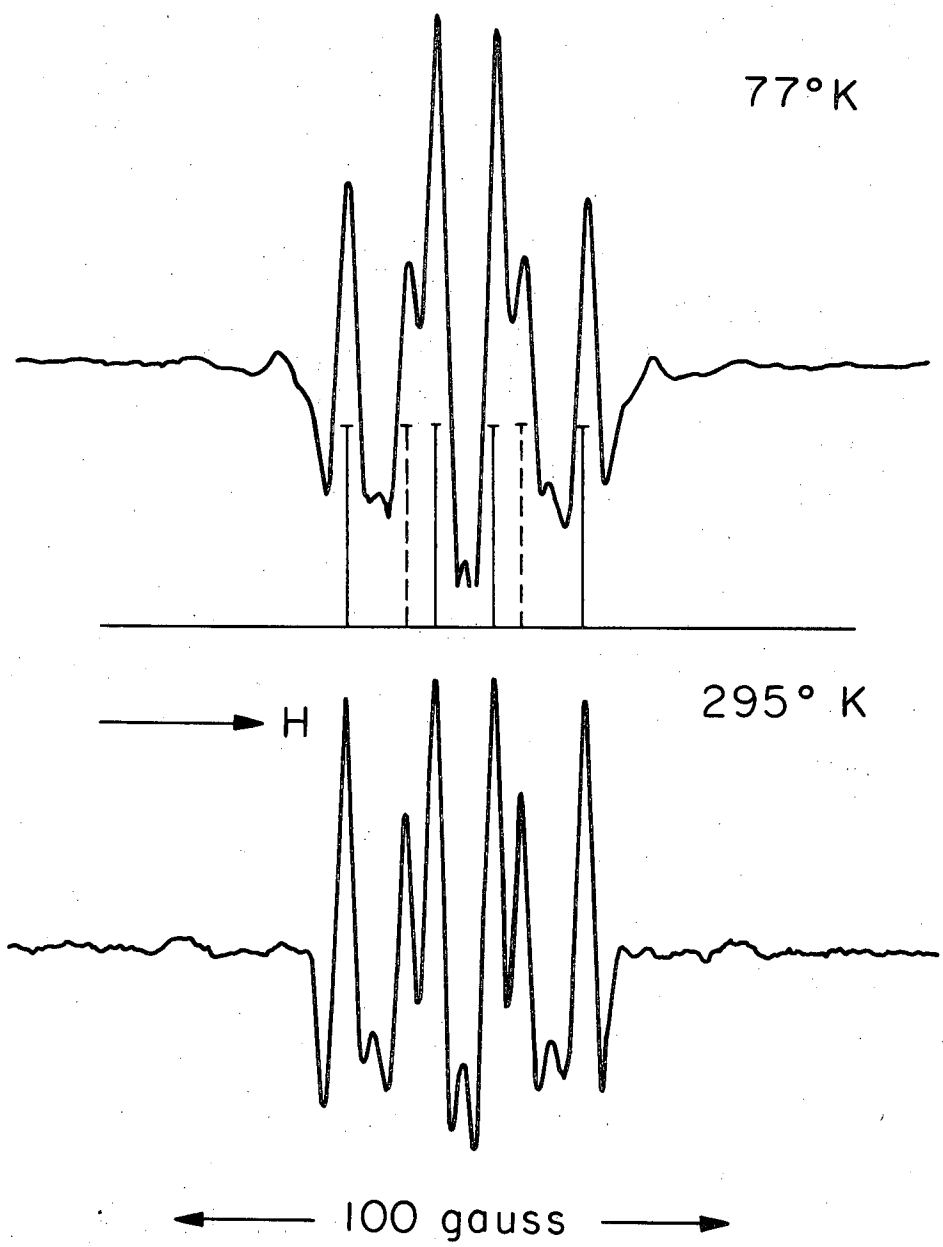

Fig. 5

XBL698-3521

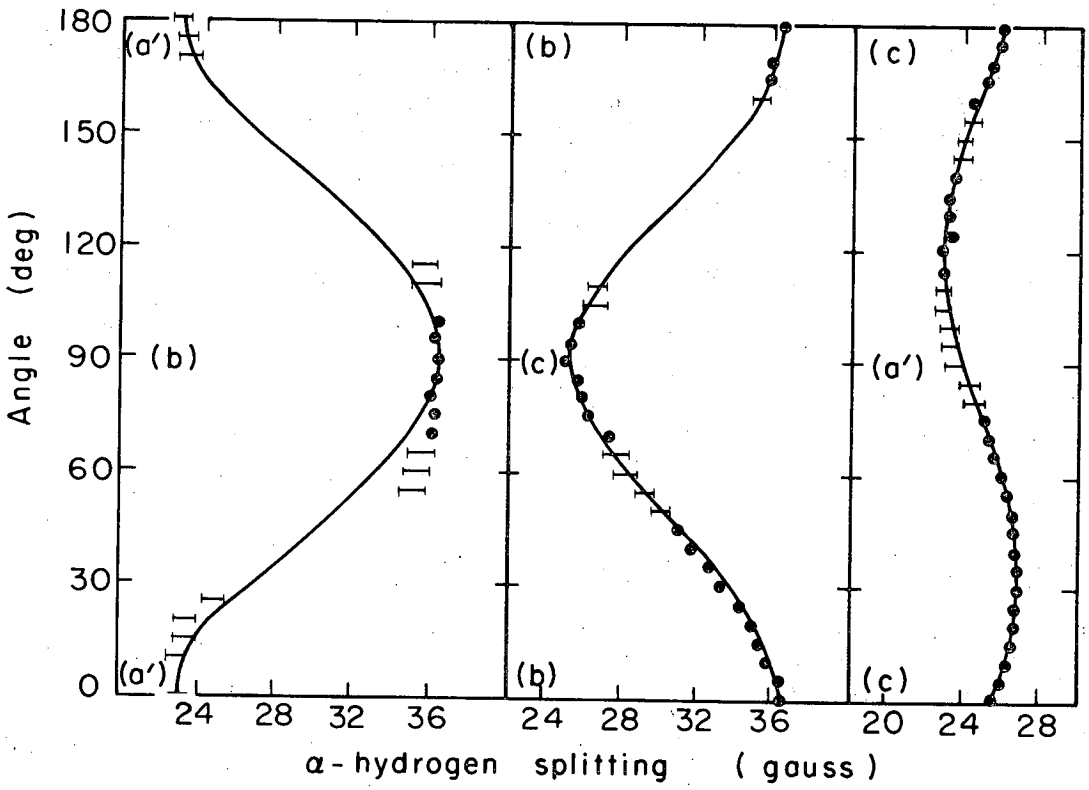

Fig. 6

XBL698-3513

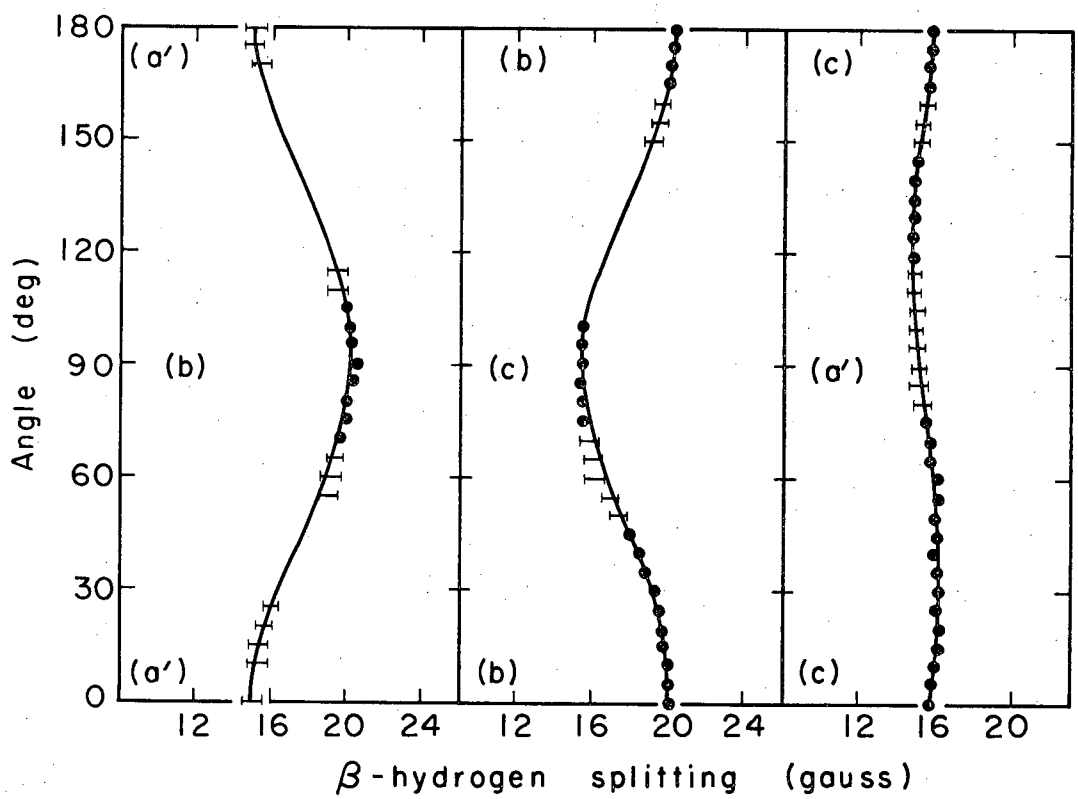

Fig. 7

XBL698-3514

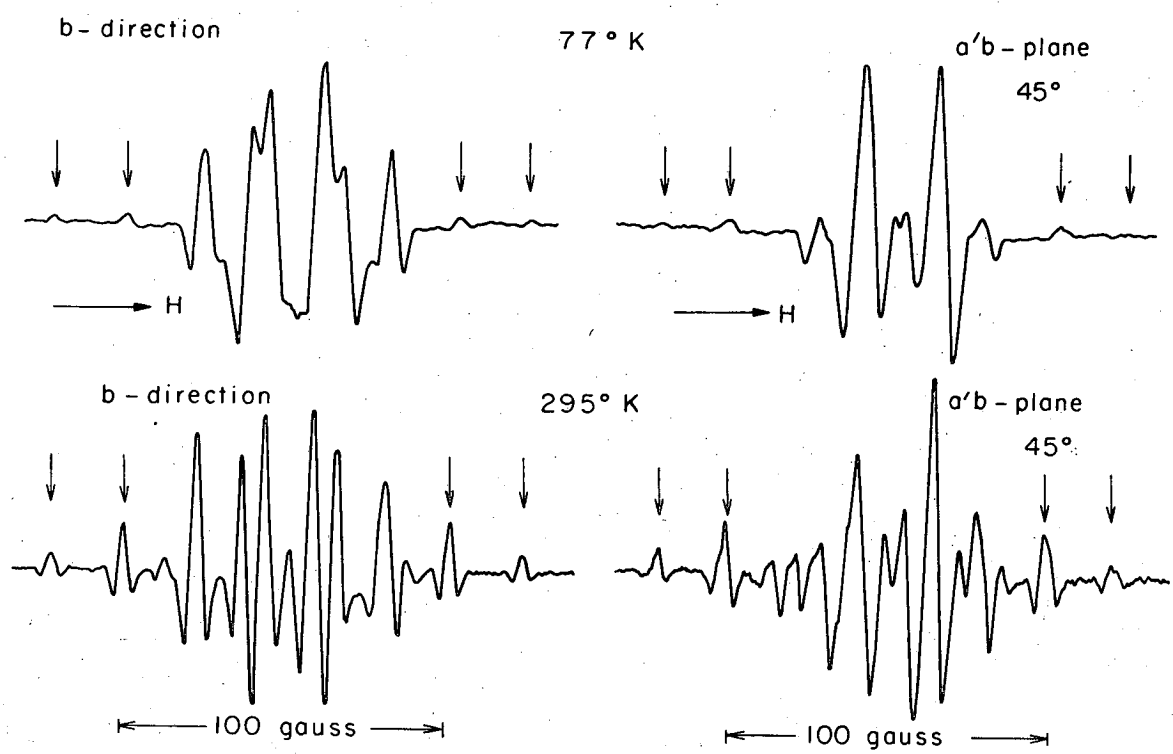

Fig. 8

XBL698-3523

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TECHNICAL INFORMATION DIVISION
LAWRENCE RADIATION LABORATORY
UNIVERSITY OF CALIFORNIA
BERKELEY, CALIFORNIA 94720