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"ESR STUDY OF RADIATION DAMAGE IN PYRIMIDINES"

Technical Progress Report

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"ESR STUDY OF RADIATION DAMAGE IN PYRIMIDINES"

ABSTRACT

The primary objective of this project is to investigate radiation damage to biomolecules using substituted pyrimidines as a model system. Results this year include a general mechanism for iminoxy radical formation in nitropyrimidines, identification of free radical structures in 5-ethyl-5-isopropylbarbituric acid, 5-ethyl-5-isoamylbarbituric acid, and 5-allyl-5-isopropylbarbituric acid, demonstration that the uracilyl radical is formed by hydrogen addition to C(5) of the pyrimidine ring, construction of a k-band microwave cavity for 77°K single crystal studies, determination of several dose-response curves, and synthesis of new pyrimidine derivatives for study in the coming year.

The primary objective of this project is to investigate radiation damage to biomolecules using substituted pyrimidines as a model system. During the first nine months of the current contract year, major progress was made in the following areas:

1. The publication of the work on selective abstraction of groups from C(5) of the pyrimidine ring has now appeared in the literature. "Free Radicals in Pyrimidines: ESR Study of Selective Abstracts of C(5) Substituents by X-Irradiation". Radiat. Res. 58 141 (1974). (see accompanying reprints).

2. The nitropyrimidine work has now been completed and has resulted in two publications:

"Nitro-Centered Free Radicals in γ-Irradiated 5-Nitro-6-Methyluracil at 77°K." Radiat. Res. 60 405 (1974).

"ESR Single Crystal Study of the Mechanism of Free Radical Formation in Nitromethyl Pyrimidines". Radiat. Res. 61 36, (1975).

3. A single crystal ESR study of 5-ethyl-5-isoamyl barbituric acid (amobarbital) has been completed. The free radical structure at room temperature is formed by abstraction of the isoamyl group leaving the unpaired electron localized at C(5) of the pyrimidine ring. When irradiated at 77°K the precursor free radical is found to involve a hydrogen abstraction from the isoamyl group, suggesting a mechanism similar to that found for ethyl abstraction from other derivatives. (A preliminary draft of our paper is attached as an Appendix).

4. We have taken extensive ESR data at room temperature on a single crystal of 5-ethyl-5-isopropyl barbituric acid. Preliminary analysis of the spectra indicates that a hydrogen is abstracted from one of the terminal methyl groups. When the room temperature study is completed, we will heat this sample to determine whether there is a subsequent abstraction of a group from C(5). Conversion from the hydrogen abstraction to the group abstraction radicals has occurred at temperatures at or slightly below room temperature in other derivatives, so perhaps only a slight heating will cause the conversion in the case of amobarbital.

5. The free radical formed by irradiation of 1,3-dimethyl uracil has been shown by single crystal ESR analysis to be formed by hydrogen addition to either C(5) or C(6) of the pyrimidine ring. We have synthesized 1,3-dimethyluracil substituted with deuterium at C(5) (50% isotopic ratio). A careful analysis of the polycrystalline spectra shows that the hyperfine coupling is to a β-deuterium, leaving the unpaired electron of the free radical localized at C(6). Analysis of the polycrystalline spectra depended upon hyperfine coupling parameters determined in the single crystal study. This is the first direct determination of the site of hydrogen addition to any uracil derivative. In the renewal period, we will deuterate uracil itself and perform a similar determination. We will submit the work on 1,3 dimethyluracil for publication shortly.

6. Aprobarbital (5-allyl-5-isopropyl barbituric acid) has been investigated both as single crystals at room temperature and as a polycrystalline powder at 77°K. The room temperature data show that the predominant radical formed at low doses involves the abstraction of the isopropyl group, while at high doses the allyl abstraction becomes dominant. We have not yet been able to interpret the 77° polycrystalline spectra, but there is evidence that a single crystal analysis at 77°K should yield the radical structures. This experiment is awaiting success in growing more crystals.

7. Construction of a k-band microwave cavity for single crystal investigations at 77°K has been completed. The novel feature of this cavity design is that it can be irradiated in a cobalt source with the crystal mounted inside while being immersed in liquid nitrogen. This avoids having to manipulate the crystal while keeping it at 77° in moving it from the irradiation device to the proper orientation in the ESR cavity.

8. Dose response curves have been determined for secobarbital, amobarbital, and aprobarbital. These data, as well as dose response data reported in the last progress report on several methylated derivatives, will be published after the required accurate dosimetry

is completed. The determination of G-values from the initial slopes of dose response curves has been hindered by weak signals at low doses. The data analyzer requested in the renewal proposal should help to solve this problem when used as a signal analyzer. It can also be used to integrate the spectra automatically and hence reduce the time required for the long and tedious numerical integrations by hand.

9. We have recently synthesized dialuric acid, which has a double bonded oxygen at C(5), and benzalbarbituric acid, which has C(5) double bonded to a carbon atom of the benzal group. Radiation damage to these compounds will be investigated in the next contract period.

APPENDIX

FREE RADICALS IN PYRIMIDINES: ESR STUDIES OF IRRADIATED AMOBARBITAL (5-ethyl-5-isoamylbarbituric acid)*

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Introduction

An extensive investigation of the available barbituric acid derivatives may yield a more complete understanding of the effects of different substituents on free radical formation and on radical characteristics in pyrimidines. Previous studies of barbituric acid derivatives have had 5-position substituents of H in barbituric acid (Bernhard and Snipes, 1966), OH in alloxan (Benson and Snipes, 1967), C_2H_5 in barbital (Haak and Benson, 1971), H and C_2H_5 in 5-ethyl barbituric acid (Gutierrez and Benson, 1974b), CH_3 and C_2 in 5-ethyl-5-methyl barbituric acid (Gutierrez and Benson, 1974a), and C_2H_5 and C_6H_5 in phenobarbital (Gutierrez, Lorenz, and Benson, 1973). As part of this program ESR studies of amobarbital (I) irradiated at 77°K and 300°K were undertaken.

Experimental Procedures

Polycrystalline amobarbital was obtained from Sigma Chemical Company, St. Louis, Missouri. For the variable temperature studies, powder samples were irradiated in one end of a sealed ESR tube by a Gamma-Cell Co^{60} source. During irradiation the temperature was kept at 77°K by immersing the ESR tube containing the sample in a specially constructed thin-walled dewar containing liquid nitrogen. After an accumulated does of approximately 10 Mr, the ESR tube was invested into another reservoir of liquid nitrogen and the sample tapped into the lower end which had been carefully annealed to remove any free radicals generated in the quartz during the irradiation. For ESR measurements at 77°K the sample was then placed in a dewar fitted into a Varian X-band cavity and the spectra recorded with a Varian 4502 spectrometer. Intermediate temperature spectra were obtained with a Varian variable temperature apparatus.

Room temperature ESR studies of X-irradiated single crystals were also carried out. The single crystals of amobarbital were grown by slow evaporation at room temperature of a saturated solution of 95% ethanol and polycrystalline amobarbital. The crystal structure of the monoclinic plates obtained has been determined by Craven and Vizzini (1969) with the data indicating a C2/c space group, eight molecules in the unit cell and a melting point of 155°C. The single crystals were given a total dose of 30 Mr. Spectra were taken a 9.3 Ghz and ESR parameters were measured as previously reported (Haak and Benson, 1971).

Room Temperature Results

A typical second derivative ESR spectrum of X-irradiated amobarbital is shown in Figure 1. The major radical species exhibited a similar four line spectra at all orientations. Although there are eight crystallographic molecules in the unit cell, they are almost magnetically equivalent. Complete resolution of the eight molecules may be possible at very high microwave frequencies, but the only effect observed at 9.3 Ghz was a slight line broadening at some orientations. The major radical species was analyzed by taking data in three orthogonal planes at 5° intervals at 9.3 Ghz. The maximum variation in hyperfine coupling to the two inequivalent protons was three gauss, indicating that the protons are located two bond lengths away from the unpaired electron. The g-values are typical of those found in organic free radicals where the unpaired electron is in a $2p\pi$ orbital. The principal values of the hyperfine coupling tensors and the g-value tensor are shown in Table 1.

Additional lines indicating a second radical species were observed which had characteristics indicating the radical was located in the ethyl or isoamyl group where coupling to many protons could occur. This radical decayed rapidly at room temperature and will be discussed fully in the low temperature section.

Room Temperature Free Radical Structure

The only free radical structures in amobarbital which agree with the above considerations are ones in which a five position substituent is removed, leaving the unpaired electron in a p_z orbital on C(5). This electron couples to the two β protons on the remaining substituent by hyperconjugation. The inequivalence of the hyperfine coupling indicates the two protons are relatively fixed, with one located near the plane of the ring and the other more in a direction perpendicular to the plane of the ring. Either structure II, formed by abstraction of the ethyl group, or structure III, formed by abstraction of the isoamyl group, can account for the above characteristics.

The amobarbital radical characteristics are very similar to the previously reported radical in X-irradiated barbital polymorph I (Haak and Benson, 1973) and in the rhomohedral prisim polymorph of phenobarbital (Gutierrez, Lorenz, and Benson, 1973). In both instances an ethyl substituent is the remaining group at the five position.

In the crystal structure reported by Craven and Vizzini (1969) a single ribbon is formed by hydrogen bonded barbiturate rings. In amobarbital this familiar crystal structure unit typical of the pyrimidine group of molecules is arranged in a "double ribbon", formed by two single ribbons interlocked with their ethyl group surfaces in close contact. The isoamyl groups project outward from the center of the double ribbon and the reported unusually large thermal motion indicates that they are not subject to large intermolecular forces. In contrast the terminal methyl groups of the ethyl substituents have a close contact with the oxygen

atom of a neighboring molecule, giving the ribbon a slightly puckered shape. Although the room temperature ESR data alone cannot distinguish between structures II and III, the crystal structure considerations indicate that the radical formed by an isoamyl abstraction, structure III, is more probable than structure II. The low temperature ESR results support this conclusion.

Low Temperature Results and Free Radical Structure

When amobarbital is irradiated and the ESR spectra recorded at 77°K, the radical structure which decayed rapidly at room temperature is observed as shown in Figure 2. The number of resonances and their 212 Gauss range indicate coupling to many protons. The narrow linewidths (~3 Gauss) of this powder sample, observed at 77°K and at subsequent higher temperatures, are typical of the small degree of anisotropy expected for β protons. The radical structure IV is proposed as the one responsible for the observed resonances. This structure has eight β protons which account for the numerous resonances, their range, and their linewidths. Also shown in Figure 2 are the resonances expected if hyperfine coupling constants of 25 Gauss for the six methyl protons, 17 Gauss for one methylene proton, and 46 Gauss for the other methylene proton are assumed. As the sample temperature is increased slight changes in the hyperfine coupling constants significantly alter the appearance of the spectra. Above 0°C the room temperature double doublet also appears as shown in Figure 3. The spectra predicted by assuming hyperfine coupling constants of 24.5 Gauss for the methyl protons, 15 Gauss for one methylene proton, and 40 Gauss for the

other proton is also shown. Neither the spectra observed at 77°K nor those taken at ten degree intervals from -160°C to +10°C showed resonances indicative of additional radical structures. The radical structure IV, which results from a simple hydrogen abstraction from C(11), is consistent with the observed data. It is interesting that this result is consistent with, although not necessarily related to, detoxification of the barbiturates in the liver in which the first step is the removal of the ω-1 proton. The ω-1 proton for amobarbital is the one attached to C(11).

Mechanism of Radical Formation

Although the primary ionization product of the interaction of the radiation with amobarbital is not observed at 77°K, it is reasonable to postulate that the radical structure IV is formed from a primary ionization cation by deprotonization. The conversion of IV to 11 which takes place rapidly at room temperature would involve proton transfer from C(10) to C(11) and a subsequent breaking of the C(5) - C(9) bond with a double bond formed between C(9) and C(10). Structure V is an intermediate in this conversion process and is undetectable in our data. This may be due either to a short lifetime of the intermediate or to the presence of anisotropic hyperfine coupling to the α proton with resultant increased linewidths and reduced intensity in our powder samples. This mechanism is very similar to and is supported by the process observed for the formation of the stable free radical in 5-ethyl-5-methylbarbituric acid (Gutierrez and Benson, 1974).

Spin Density Calculations

In the case of inequivalent proton couplings with small anisotropic components the spin density may best be calculated using the empirical relationships suggested by Heller and McConnell (1960), $A_i = \rho(Bo \text{ t } B_2 \cos^2 \theta_i)$. A_i is the isotropic component of the hyperfine coupling and θ_i is the dihedral angle defined as the angle between the p_z orbital of unpaired electron and the plane formed by the C-H and C-C bonds. Assuming that the methylene protons are relatively fixed for the room temperature radical II, the isotropic components of 20.6 and 5.0 Gauss derived from Table I yield equations with a graphical solution of $\rho_C(5) = .75$ and $\theta = 46^o$ when $B_o = 3$ Gauss and $B_2 = 50$ Gauss. This result compares favorably with the value found for the identical radical structure in phenobarbital (Gutierrez, Lorenz and Benson, 1973). The remainder of the spin density is delocalized onto O(4) and O(6).

The coupling constants to the two inequivalent methylene protons in the low and intermediate temperature spectra may also be checked for consistency through the use of the Heller & McConnell relationship. Although the accuracy of the low temperature A_i cannot be expected to be as accurate as those for the room temperature radical the agreement is good, especially if one allows $\rho c(5)$ to decrease from 0.9 at 77^oK to 0.8 at 0^oC. The values obtained for the dihedral angle indicate that a small amount of reorientation occurs as the temperature increases. Fessenden and Schuler indicate that a value of 0.8 is expected for a radical structure similar to III with the two methyl groups causing the loss of spin density at C(11).

Discussion

The suggestion has been made (Gutierrez, Lorenz, and Benson, 1973) that one factor important to the mechanism of radical formation is the difficulty encountered in reorienting a substituent group into the plane of the pyrimidine ring when the electronic configuration of C(5) goes from sp^3 in the undamaged molecule to sp^2 in the free radical. The present study supports this hypothesis in that the large isoamyl group, although it has a greater degree of motional freedom, is abstracted rather than the ethyl group. Previous examples of the generality of this principal are found in the studies of phenobarbitol (Gutierrez, Lorenz, and Benson, 1973) and alloxantin (Benson and Snipes, 1969).

The importance of the extent of delocalization of the unpaired electron as a factor in the mechanism of free radical formation can not be directly assessed from the room temperature amobarbital data. Structures II and III should both allow the same extent of delocalization onto O(4) and O(6). It may be significant, however, that the low temperature free radical is not stable at room temperatures. Although there is delocalization in structure IV into the methyl groups (Fessenden and Schuler, 1963), the extent of delocalization is less than that observed in structure III. This may account for the observation that when the isoamyl group acquires sufficient thermal motion to allow reorientation to occur, structure V converts to structure III.

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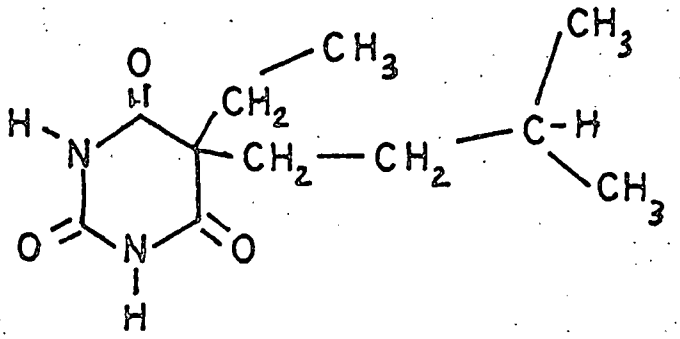
I



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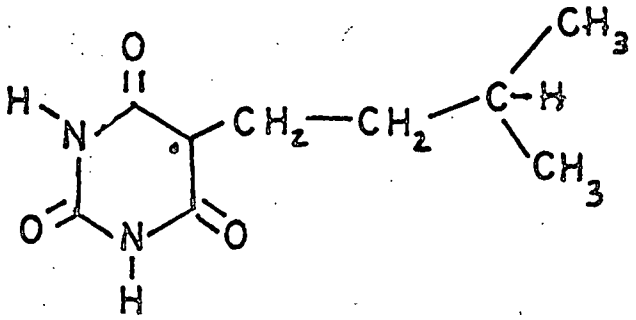

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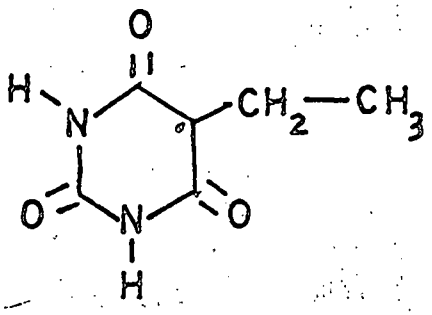

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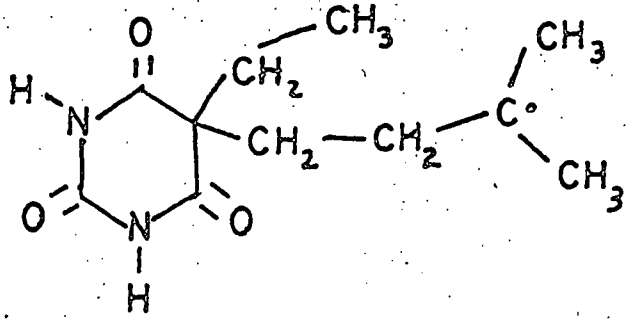

IV V III

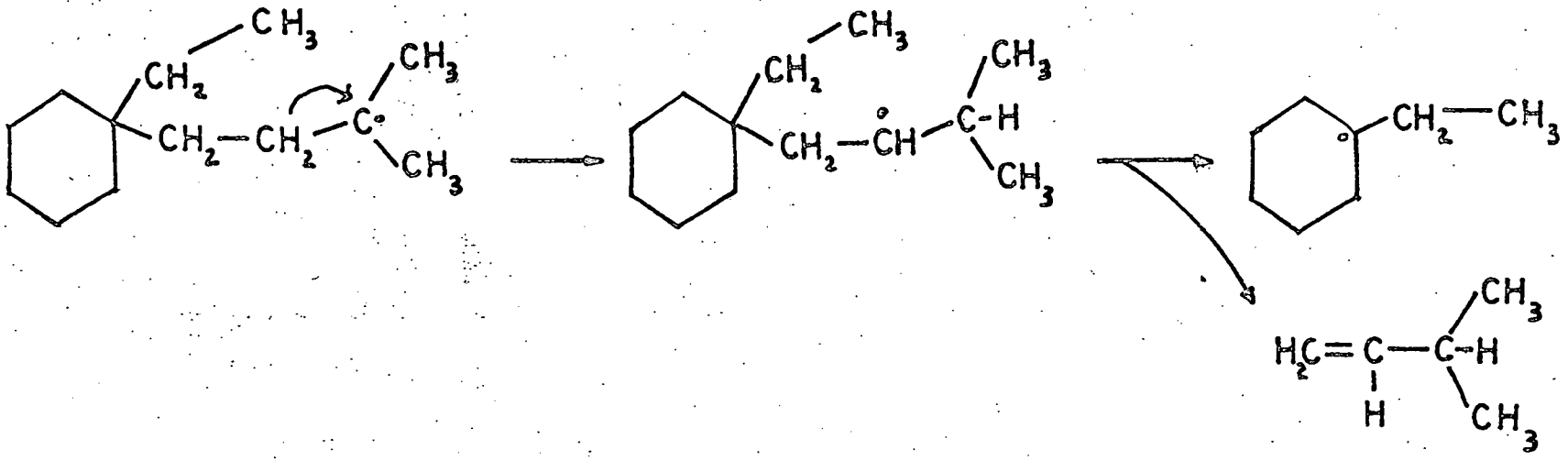

Table I. Hyperfine coupling tensors (in Gauss) and g-value tensor for the stable room temperature free radical formed in amobarbital. The tensors are shown in diagonal form to give the principal values.

Hyperfine Coupling Tensor (Proton 1)

22.5	0.0	0.0
0.0	20.0	0.0
0.0	0.0	19.3

Hyperfine Coupling Tensor (Proton 2)

6.2	0.0	0.0
0.0	4.7	0.0
0.0	0.0	4.1

g-value Tensor

2.0054	0.0000	0.0000
0.0000	2.0043	0.0000
0.0000	0.0000	2.0024

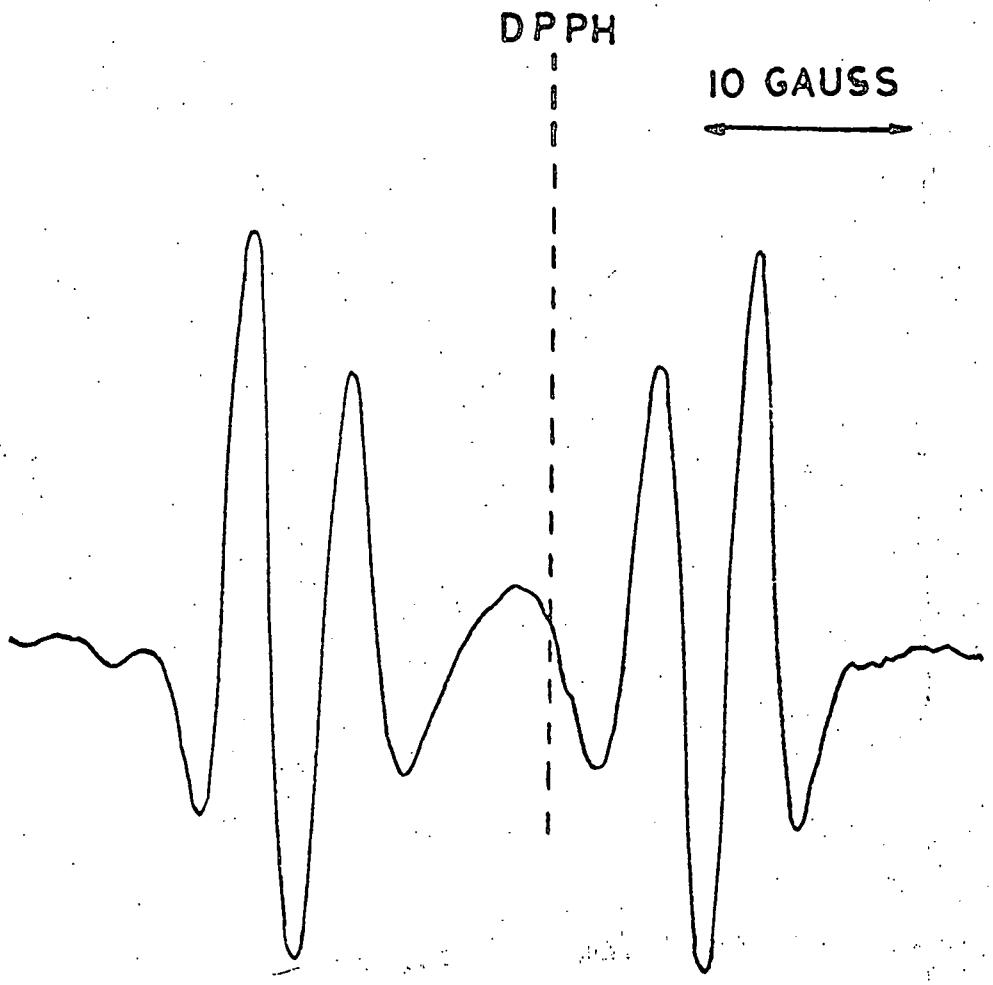

figure 1.

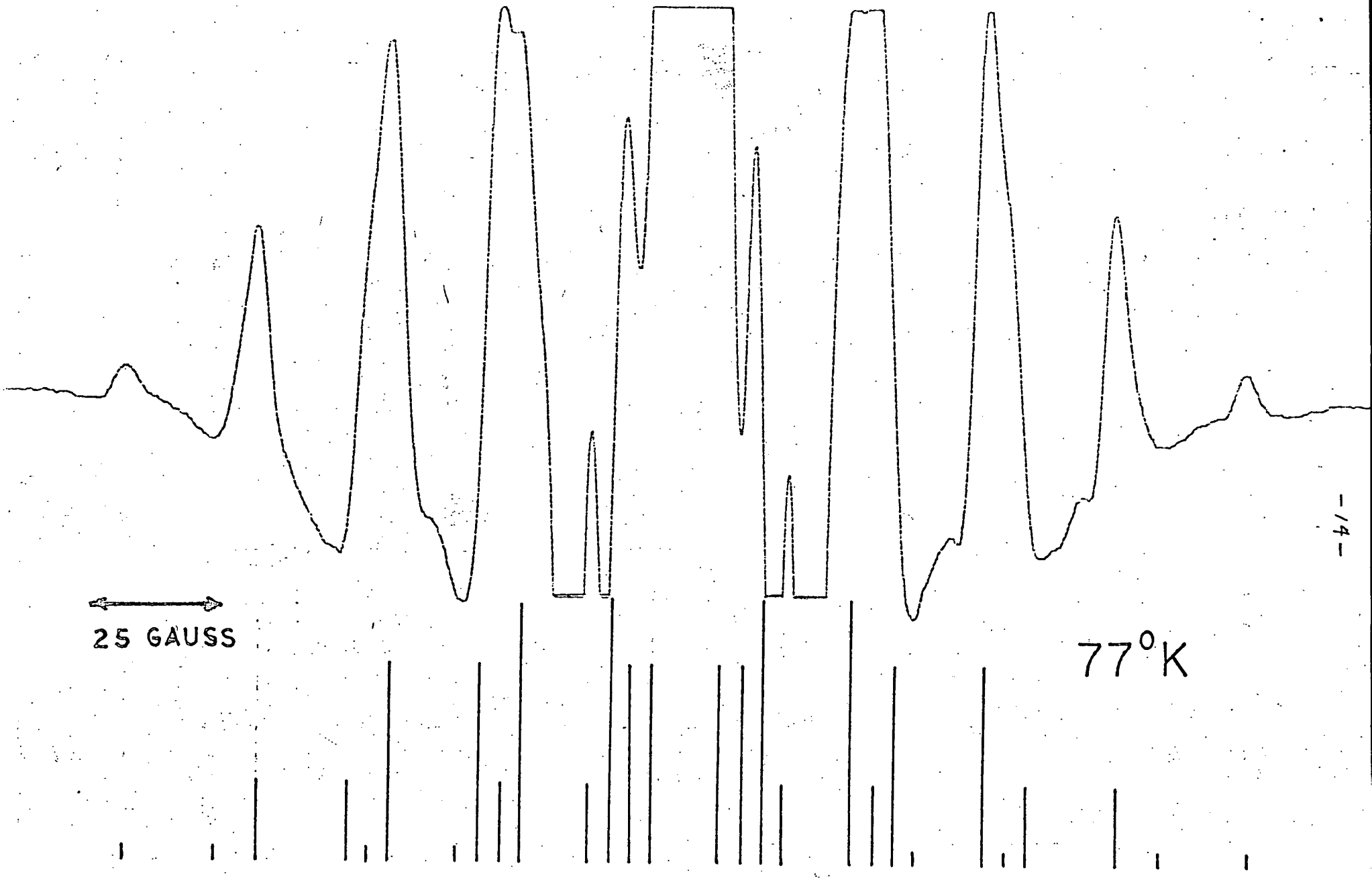

figure 2

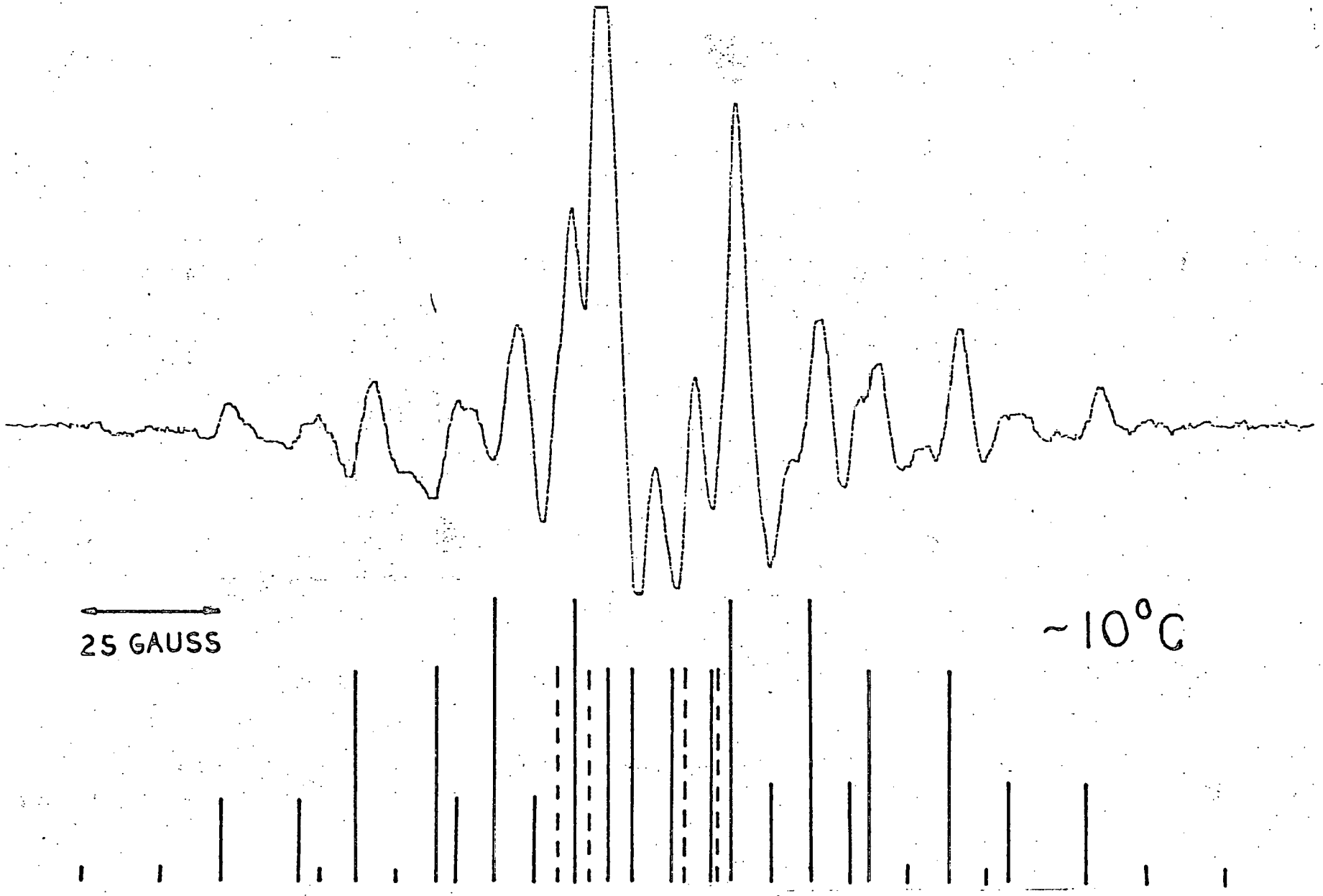

Figure 3

Figure Captions

Figure 1. Typical second derivative spectra of X-irradiated single crystals of amobarbital I taken at 9.3 GHz.

Figure 2. X-band second derivative ESR spectrum of irradiated polycrystalline amobarbital taken at 77°K. The stick diagram indicates the resonances expected if hyperfine coupling constants of A_{CH_3} = 25G., A_{CH_2} = 17G and 46G and a spin density of 0.9 are assumed for radical structure IV.

Figure 3. X-band second derivative ESR spectrum of polycrystalline amobarbital irradiated at 77°K and warmed above 0°C. The stick diagram indicates the resonances expected for radical structure IV if a spin density of 0.8 and hyperfine coupling constants of A_{CH_3} = 24.5G, A_{CH_2} = 15 G and 40G. The dotted lines indicate the room temperature radical, structure III.