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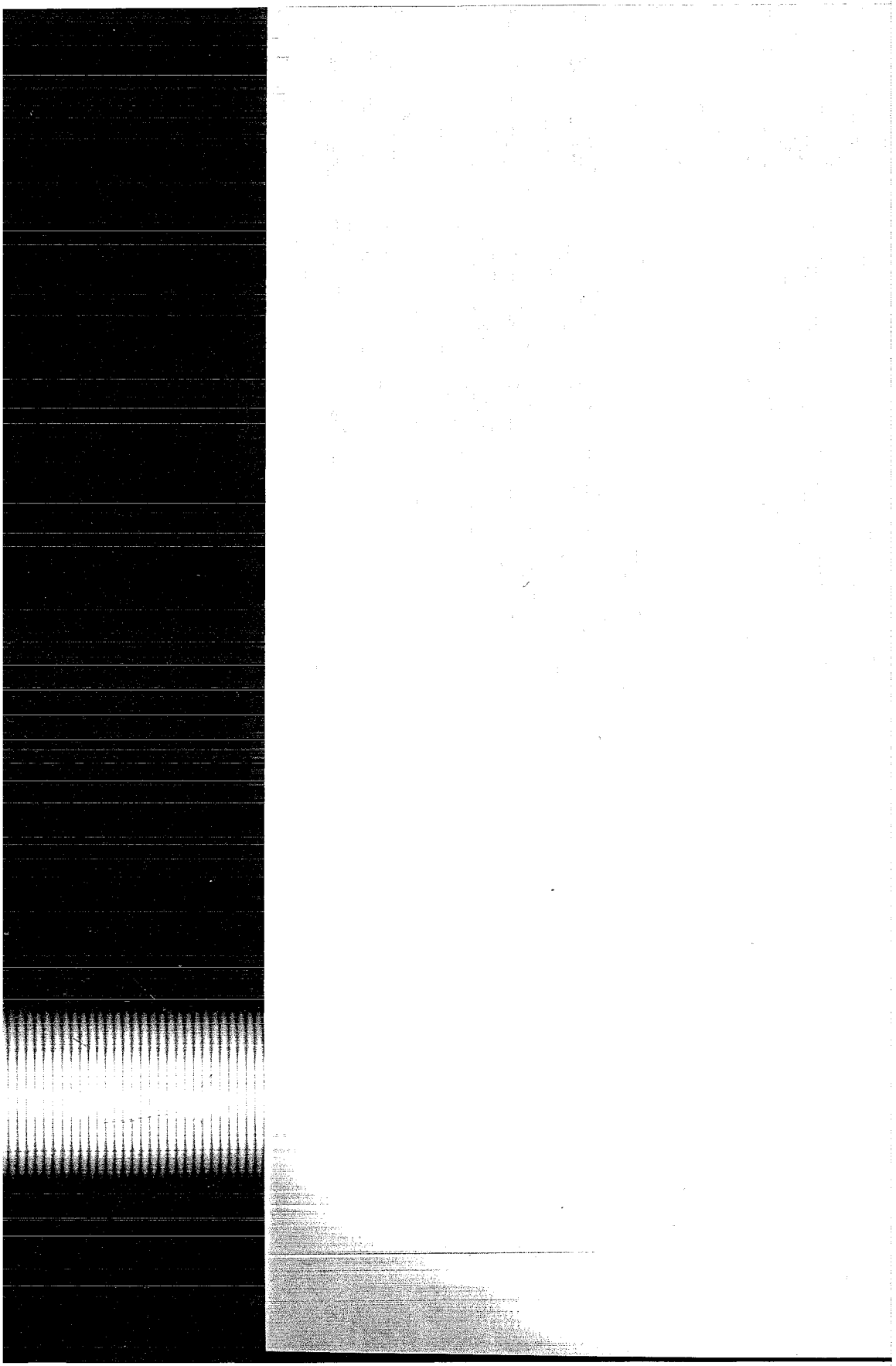

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A computer-controlled E.P.R. analysis of free radical formation in dry thymine and derivatives after electron irradiation

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Radical formation and transformation in electron irradiated dry thymine, thymidine, thymidine-5′-monophosphate, and DNA have been studied with 35 GHz electron paramagnetic resonance. Dry samples were irradiated at 77°K and the E.P.R. spectra taken at 77 and 300°K under anaerobic conditions. The spectra of the cation and the anion radical of thymine were identified. It was shown that the cation easily undergoes deprotonization. Conversely, the anion radical is transformed into the 5,6-dihydro-5-thymyl radical by proton capture. These processes were found to be significant for all the compounds investigated.

1. Introduction

Since the discovery of the 5,6-dihydro-5-thymyl radical in irradiated DNA (Ehrenberg, Ehrenberg and Löfroth 1963, Salovey, Shulman and Walsh 1963, Pershan, Shulman, Wyluda and Eisinger 1964), an increasing number of investigations deal with the mechanism by which this species showing a characteristic octet E.P.R. spectrum is produced. Although at first sight this radical seems to be simply an adduct of atomic hydrogen to the C_6-position of the thymine molecule (Pruden, Snipes and Gordy 1965), there are indications of other reactions being involved in its formation. Pershan *et al.* (1964) observed the intensity of the octet spectrum in irradiated DNA to increase with increasing water content, while substitution of the normal water by D_2O yields the corresponding D-adduct. Deuteration effects have also been reported by Herak (1970) after irradiating single crystals of thymidine grown from D_2O. Similar results have been obtained in photolyzed frozen solutions of thymine and derivative compounds (Holroyd and Glass 1968). Recently it has been shown that bombardment of dry thymine with discharge-excited argon also yields the thymyl radical (Dertinger and Carpy 1971): i.e. under conditions where there was no hydrogen to react with the thymine as opposed to the experiments with a hydrogen discharge (Herak and Gordy 1965). Controversies also exist concerning the interpretation of other radicals found in irradiated single crystals of thymine monohydrate (Hüttermann 1970, Henriksen and Snipes 1970). This variety of results led us to re-investigate radical production in thymine and derivative compounds (including DNA) in order to get additional information on the radical transformation processes occurring in these compounds.

2. Experimental methods

2.1. *Irradiation*

Dry powders of the A Grade compounds thymine (Schuchardt), thymidine and thymidine-5′-monophosphate (Serva), calf thymus DNA (Worthington

Biochemical Corporation) and DNA of bacteriophage T1 (kindly prepared by Dr. G. Hotz at this institute) have been irradiated with a 50 nA beam of 2 MeV electrons from a Van de Graaff accelerator (High Voltage Engineering), the dose being approximately 3 Mrads. Irradiation was carried out at 77°K in a special cryostat supplied by AEG (Berlin). Cooling of the sample and the microwave cavity was achieved by a very intense stream of cold nitrogen gas obtained by electrical heating of liquid nitrogen in a large Dewar vessel. After irradiation at 77°K, the E.P.R. spectra were immediately recorded. Then the cryostat with the sample was warmed up, whereafter the 300°K spectra were taken. During the whole procedure the samples were not exposed to air. Weak quartz signals appeared in some of the E.P.R. spectra resulting from the irradiation of the Suprasil sample tubes, but were subtracted electronically.

2.2. *E.P.R. spectrometer*

E.P.R. measurements were performed with a Q-band spectrometer (AEG, Berlin) equipped with a tunable cylindrical H_{011} cavity and 125 kHz field modulation. The 35 GHz technique was selected since the g-factor resolution is increased approximately four times as compared to the commonly applied X-band. However, this advantage has to be paid for by strong power saturation and dispersion effects especially at low temperature. To overcome this difficulty, a special microwave bridge was constructed, allowing measurements at power levels below 1 μW. The resulting loss of sensitivity could be counteracted by using computer- and signal-averaging techniques.

2.3. *Computer technique*

With the aid of computer technique the following procedures could be performed: (i) signal-averaging and smoothing, (ii) subtraction or addition of measured or stored spectra with different coefficients, (iii) storage of measured or analysed spectra on magnetic tape. A computer of average transients ' CAT 1000 ' (TMC) was modified for this purpose allowing off-line addition and subtraction of spectra, as well as the use of a magnetic tape recorder. The CAT was triggered by a stimulus pulse given by a proton magnetic resonance Gaussmeter (AEG, Berlin). Thus the g-factors of the spectra were maintained throughout the storage and analysing procedures. A very precise handling of the spectra could be achieved by this device mainly for the following reasons: (i) field-linear sweep of the magnet controlled by induction coils, (ii) quartz-controlled stimulus pulse from the Gaussmeter for signal read-in, (iii) quartz-controlled tape code, (iv) tape controlled address advance for the CAT in the read-out phase with suppression of unwanted pulses.

3. Results

3.1. *Thymine*

Figure 1 shows the spectra of thymine irradiated at 77°K and measured at 77 and 300°K, respectively. The low temperature spectrum (figure 1 (*a*)) consists mainly of a 1 : 3 : 3 : 1 quartet of approximately 19 G hyperfine coupling centred at $g = 2{\cdot}0061$. The lines in the right-hand section of this spectrum are somewhat distorted by dispersion effects originating from the

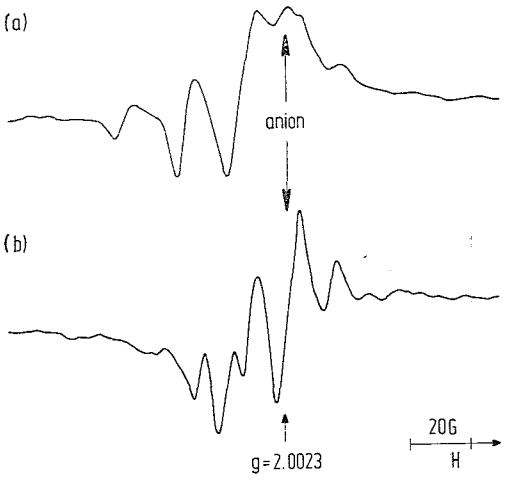
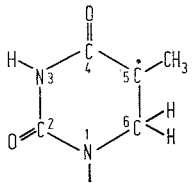
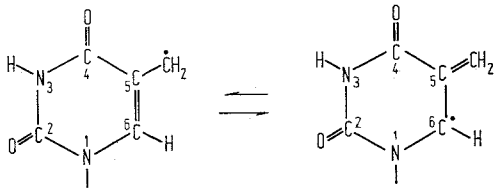
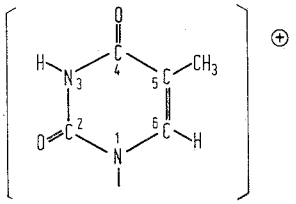
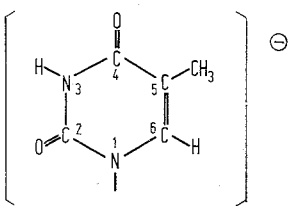


Figure 1. First derivative of the thymine E.P.R. spectrum after irradiation at 77°K, measured at (*a*) 77°K (gain 20), and (*b*) 300°K (gain 20). Microwave power approx. 1 μW.

superposition of an additional signal near $g=2{\cdot}0022$. The quartet, clearly representing methyl hyperfine structure, is attributed to the cation radical formed by ionization of the thymine. Recent spin-density calculations (Sevilla 1971) predict approximately 45 per cent of the unpaired spin to be concentrated at the C_5-position of this species. Adopting this value, the theoretical hyperfine splitting according to $A=\rho\,.\,Q_{CH_3}$ with $\rho=0{\cdot}45$ and $Q_{CH_3}=40$ G (cf. Sevilla 1971) amounts to 18 G which is in good agreement with the 19 G observed. Sevilla (1971), studying U.V.-photolyzed frozen solutions of thymine, also observed the cation, but additional nitrogen hyperfine structure was resolved in his spectrum. The absence of the nitrogen lines in our cation spectum is, however, not unusual since it has been found that these lines do not necessarily occur when the radicals are trapped in dry powders or even in single crystals (Dertinger 1967, Schmidt and Borg 1971).

As can be seen from figure 1 (*b*) the signal at $g=2{\cdot}0022$ persists after warming up to room temperature. The corresponding radical can be identified to be due to the thymine anion in § 3.2 when explaining figure 4.

The most important result of figure 1 is that upon warming up the sample the cation spectrum (figure 1 (*a*)) is transformed into a 5 to 6 line spectrum of approximately 42 G total spacing centred at $g=2{\cdot}0032$. This signal has recently been obtained and analysed independently by Hüttermann (1970) and Henriksen and Snipes (1970) in irradiated single crystals of thymine monohydrate. However, the authors arrived at entirely different interpretations. According to Hüttermann the underlying radical results from the abstraction of a hydrogen atom from the methyl group (see the following table), whereas Henriksen and Snipes ascribe this spectrum to two different radicals, one showing an anisotropic doublet, the other producing a quartet with hyperfine interaction by one α- and one β-proton. After careful analysis of these results we favour Hüttermann's 'one-radical' interpretation, mainly on account of the following arguments: (i) as stated in both publications the hyperfine structure, clearly resulting from

Type of radical (authors)	(Overall) Splitting (G)	*g*-factor	Symbol†	Formula
Octet; 5,6-dihydro-5-thymyl (Pruden *et al.* 1965)	Thymidine (147) dTMP (139)	2·0038	$XH_2^{\cdot}$	
Quintet/ sextet (Hüttermann 1970)	(42)	2·0032	$X^{\cdot}$	
Quartet; thymine cation (Sevilla 1971 and this work)	19	2·0061	XH^+	
Doublet; thymine anion (Sevilla 1971 and this work)	Thymi(di)ne 7 dTMP 15 (?)	Thymi(di)ne: 2·0022 dTMP: 2·0041(?)	XH^-	

† Thymine moiety = XH.
Radicals identified in thymine, thymidine, and thymidine-5′-monophosphate.

three distinct protons, is anisotropic for each proton which seems to rule out β-proton interaction; (ii) the E.P.R. spectrum is independent of the applied microwave frequency as shown by Hüttermann by comparing the Q-band with the X-band. This does not seem to be compatible with the existence of two different radicals which, according to Henriksen and Snipes, should differ somewhat in their g-tensors; (iii) finally, the 'one-radical' interpretation is supported by Hüttermann's finding that U.V. irradiation of the crystal yielded exactly the same spectrum as ionizing irradiation.

3.2. *Thymidine*

The spectra obtained with thymidine at 77 and 300°K do not differ significantly (figure 2). The main signal structure is the thymyl octet at $g = 2{\cdot}0038$. As can be seen, this spectrum is seriously distorted in the central region, indicating the presence of other radicals. From visual inspection of the oscilloscope-displayed spectra we found that only the radical of figure 1 (*b*) is superimposed (hereafter denoted as X$^{\bullet}$; see the table). In fact, subtraction of its spectrum from the thymidine 300°K spectrum yields the pure thymyl octet with the known splittings and amplitude ratios (Pruden *et al.* 1965). This is shown in figure 3. The small distortion remaining thereby near $g = 2{\cdot}0022$ is due to the anion resonance. A careful analysis showed that this signal which is unfortunately always associated with the X$^{\bullet}$ radical taken from figure 1 (*b*) has been ' oversubtracted ' in figure 3. This does, however, mean that the anion is originally not present in irradiated thymidine at 300°K, nor at 77°K.

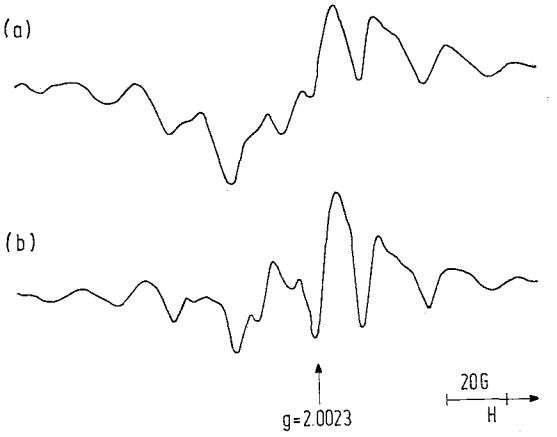


Figure 2. First derivative of the thymidine E.P.R. spectrum after irradiation at 77°K, measured at (*a*) 77°K (gain 16), and (*b*) 300°K (gain 16). Microwave power approx. 1 μW.

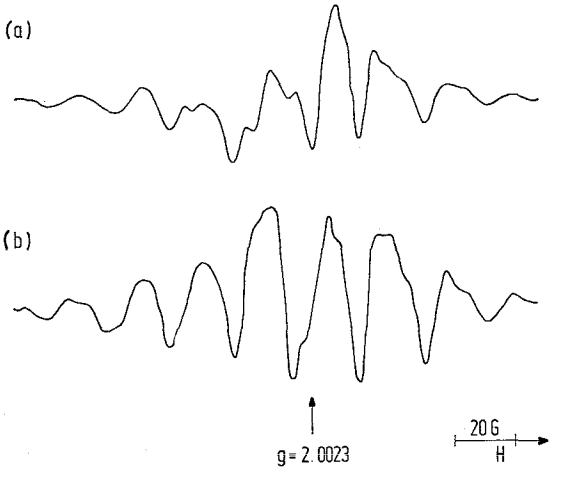


Figure 3. (*a*) Thymidine measured at 300°K (cf. figure 2 (*b*). (*b*) 300°K thymidine spectrum after subtraction of the X$^{\bullet}$ spectrum (cf. figure 1 (*a*); see also table).

From the heuristic point of view it is quite unsatisfactory that the thymidine spectra at 77 and 300°K are almost identical. Therefore, we carried out an irradiation experiment at 4°K during which the cryostat was filled with liquid helium and the irradiation procedure performed as before. Due to strong E.P.R. relaxation effects the thymidine Q-band spectrum could not be taken at 4°K. Therefore we had to warm up to approximately 25°K. The spectrum obtained at this temperature is shown in figure 4. It is characterized by the dominating anion resonance at $g = 2{\cdot}0022$. Furthermore, the X$^{\bullet}$ lines and the octet are present, however, the latter being weak.

From figure 4 it can be seen that the anion resonance at $g = 2{\cdot}0022$ is a doublet of approximately 7 G splitting. The association of this signal with the thymine anion is based on spin-density calculations from which a doublet structure resulting from the hyperfine interaction of the hydrogen bound at C_6 can be inferred. Sevilla (1971) calculated that approximately 45 per cent of the unpaired spin resides at the C_6-position of the thymine anion. Adopting a Q_{CH} value of -24 G (cf. Sevilla 1971) the predicted proton splitting should be -11 G. Apparently this spacing is 4 G larger than the 7 G observed by us. On the other hand, Sevilla arrived at the conclusion that the doublet resonance of 16 G observed in his frozen solutions should be associated with the thymine anion. However, this value is larger than the calculated value. An explanation of these discrepancies may derive from the fact that the anion is trapped in different matrices in each experiment.

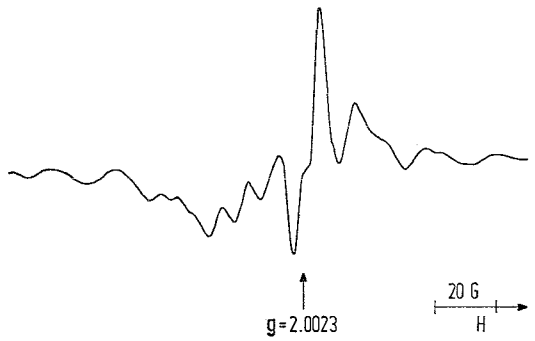


Figure 4. First derivative of the thymidine E.P.R. spectrum after irradiation at 4°K, measured at 25°K (Microwave power approx. 0·1 μW).

3.3. *Thymidine-5′-monophosphate (dTMP)*

The E.P.R. spectra of dTMP obtained at 77 and 300°K, respectively are presented in figures 5 (*a*) and (*b*). An essential component of the 77°K spectrum is a distorted doublet while after warming up a complex spectrum appears consisting of the octet and the radical X$^{\bullet}$ (see the table). From figure 5 (*a*) it can be seen that the satellite peak on the right-hand side of this spectrum indicates a contribution of the X$^{\bullet}$ radical. Therefore subtraction of its spectrum can be performed yielding the spectrum shown in figure 6 (*b*). This is a well-shaped doublet with 15 G hyperfine splitting centred at $g = 2{\cdot}0041$. Since this g-factor is typical for uncharged carbon-centred radicals and, furthermore, doublets of this kind have also been analysed in other DNA nucleosides and nucleotides after ionizing irradiation (Dertinger and Hartig, manuscript in preparation) the

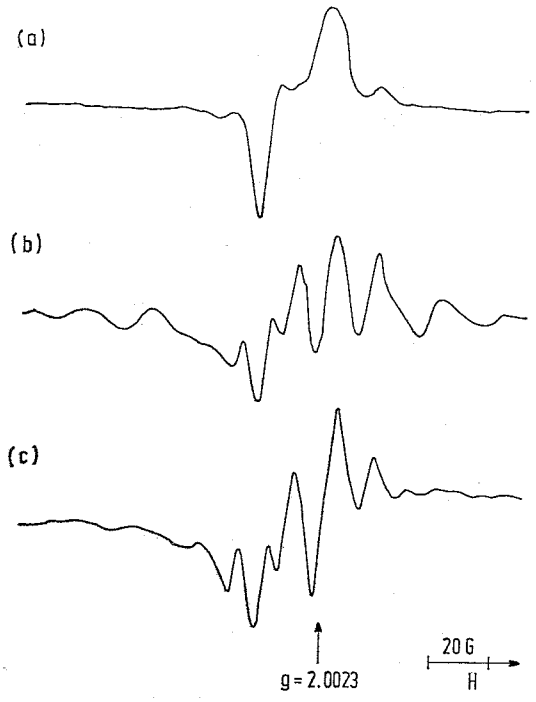


Figure 5. First derivative of the dTMP E.P.R. spectrum after irradiation at 77°K, measured at (*a*) 77°K (gain 12), (*b*) 300°K (gain 16), and (*c*) spectrum of the $X^{\bullet}$ radical depicted for comparison (cf. figure 1 (*a*); see also table). Microwave power approx. 1 μW.

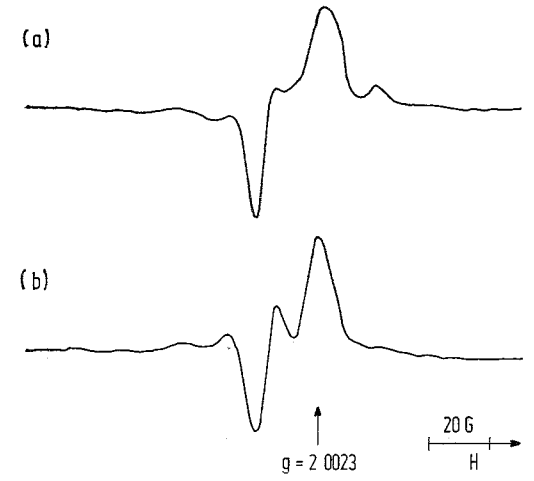


Figure 6. (*a*) dTMP measured at 77°K (cf. figure 5 (*a*)). (*b*) 77°K dTMP spectrum after subtraction of the $X^{\bullet}$ spectrum (cf. figure 1 (*a*), see also table).

corresponding radical probably resides on the deoxyribose moiety. This assignment is supported by irradiation experiments of β 2-deoxy-D-ribose at low temperature, where a doublet was shown to be the most prominent signal structure (Müller 1964). But in view of the 16 G doublet found by Sevilla (1971) in photolyzed frozen thymine solutions and attributed to the thymine anion, we can, of course, not rule out such an interpretation to be also applicable to the dTMP doublet. Structural differences between the dTMP and the thymidine matrix could possibly account for different anion doublet splittings in either compound (cf. figure 4 and 6 (*b*)). It is interesting to stress here that structural differences between the two compounds are responsible for the different overall splitting and shape of the thymyl octet (147 G in thymidine, 139 G in dTMP).

3.4. *DNA*

Figures 7 and 8 show the EPR spectra of calf thymus DNA and DNA of bacteriophage T1 (the latter containing lines of Mn^{++} ions for reasons that are not quite clear). While the 77°K spectra are quite unspecific (figures 7 (*a*) and 8 (*a*)) the room temperature spectra exhibit pronounced hyperfine structure (figures 7 (*b*) and 8 (*b*)). In figures 7 (*c*) and 8 (*c*) the signals resulting from the subtraction of the 77°K spectra from the 300°K spectra are plotted which show a striking similarity (apart from the Mn^{++} lines in the T1 DNA). However, it should be stressed that this subtraction is only an *ad hoc* procedure lacking a rigorous foundation, as it is not clear which of the low temperature radicals persist at room temperature. A comparison of the spectra in figures 7 (*c*) and 8 (*c*) with those obtained in this laboratory from all DNA bases, nucleosides

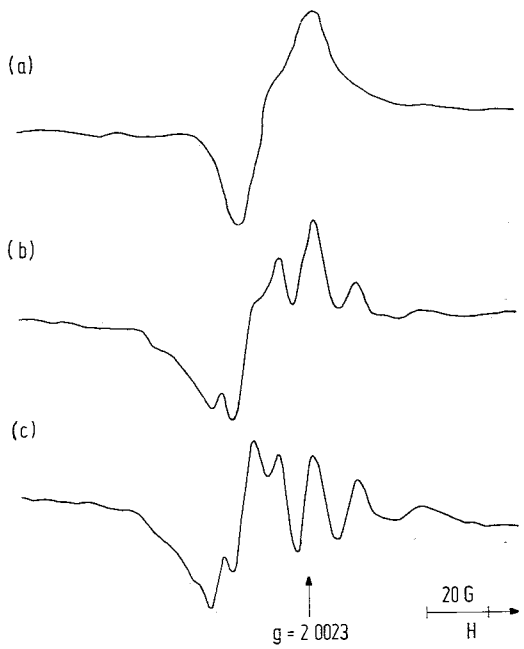


Figure 7. First derivative of the E.P.R. spectrum of calf thymus DNA after irradiation at 77°K, measured at (*a*) 77°K (gain 8), (*b*) 300°K (gain 50), and (*c*) 300°K DNA spectrum after subtraction of the 77°K spectrum. Microwave power approx. 1 μW.

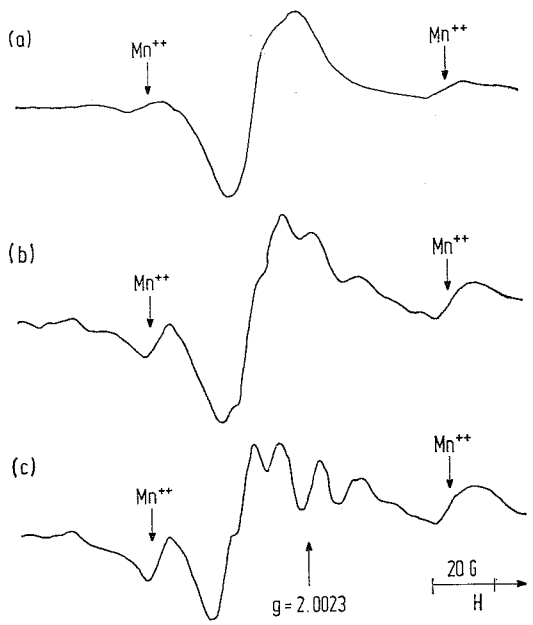


Figure 8. First derivative of the E.P.R. spectrum of bacteriophage T1 DNA after irradiation at 77°K, measured at (*a*) 77°K (gain 16), (*b*) 300°K (gain 100), and (*c*) 300°K DNA spectrum after subtraction of the 77°K spectrum. Microwave power approx. 1 μW.

and nucleotides revealed that the hyperfine structure in our DNA samples at room temperature is almost entirely due to the thymine X$^{\bullet}$ radical (cf. table).

4. Discussion

From the results presented in §3 the radical transformation processes occurring in thymine-containing compounds can easily be derived. First we consider the reaction of the thymine cation XH^+ which is best illustrated by figure 1. It has been outlined that the 300°K spectrum represents the radical X$^{\bullet}$ quoted together with the other thymine radicals in the table (disregarding for the moment the anion resonance at $g = 2{\cdot}0022$). The most probable explanation accounting for the observed cation transformation is clearly a deprotonization reaction:

$$XH^+ \longrightarrow X^{\bullet} + H^+. \qquad (1)$$

This reaction is supported by the observation that the overall radical concentration in figure 1 is not reduced upon warming up (cf. the equal gain settings in figure 1 (*a*) and (*b*)) indicating transformation rather than decomposition of the primary thymine cation. The radical X$^{\bullet}$ interpreted as being produced by abstraction of atomic hydrogen from the methyl group of the thymine moiety (Hüttermann 1970) is thus formed by the loss of a proton from the cation followed by a rearrangement process. A dissociative formation of the X$^{\bullet}$ radical is also ruled out by the fact that we could not find the E.P.R. signals of hydrogen atoms trapped in irradiated thymine, thymidine and dTMP in striking contrast to other DNA constituents (Dertinger and Hartig, manuscript in preparation) and DNA itself (Müller and Dertinger 1968, and this work). As shown in §3, the

radical $X^{\bullet}$ is also found in thymidine (figures 3 and 4), dTMP (figures 5 and 6) and in DNA (figures 7 and 8). In addition, the results obtained with thymidine and dTMP reveal that the deprotonization has already taken place at low temperature in these compounds (excluding DNA).

While deprotonization is the characteristic of cation transformation, protonization is seen to be involved in the transformation of the thymine anion yielding the thymyl radical (cf. table):

$$XH^{-} + H^{+} \longrightarrow XH_2^{\bullet} \qquad (2)$$

The role of the thymine anion as the precursor of the thymyl radical is reflected in figure 1 showing the anion resonance to persist after warming up without the formation of the thymyl octet in measurable concentration. On the other hand, the octet is prominent in the thymidine spectra (figures 2 and 3) but with the anion resonance being absent. To see the anion in this case we had to go down to 25°K (figure 4) where we are obviously faced with the onset of reaction (2). In dTMP the situation is not so clear since the interpretation of the 77°K doublet (figures 5 (*a*) and 6 (*b*)) is not unambiguous as outlined in §3.3. When the assumption is made, however, that the dTMP doublet does represent the anion spectrum in this substance or at least does contain the thymi(di)ne-like anion resonance (cf. figure 4) then the complete disappearance of the anion spectrum together with appearance of the octet at 300°K (figure 5 (*b*)) is indicative of reaction (2). As shown by Holroyd and Glass (1968) in frozen solutions of thymine derivatives, dTMP is in fact not an exception concerning the occurrence of reaction (2). Clearly, reaction (2) also explains the deuteration effects observed with the thymyl radical by these authors as well as the observed concentration dependence of this radical on the water content in irradiated DNA (Pershan *et al.* 1964) and in crystalline thymine (Henriksen and Snipes 1970). Obviously the proton in reaction (2) comes from a site easily interchanging H^{+} by D^{+}, e.g. from hydrogen bridges between thymine moieties or between thymine and the water molecules, the former situation being probably responsible for the deuteration effects in thymidine single crystals which are known to be anhydrous (Herak 1970). Furthermore, the observation that the shape of the thymine E.P.R. spectrum is strongly dependent on the crystalline properties of the thymine sample used (Müller 1964) can be explained on the basis of protonization and deprotonization of the thymine ion radicals. Certainly these processes are influenced by matrix effects which we also believe to account for our failure to detect measurable thymyl radical concentrations in our thymine samples (cf. figure 1). Taken together, our results rule out the participation of atomic hydrogen in the formation of the radical $X^{\bullet}$ and the thymyl radical $XH_2^{\bullet}$, the arguments being mainly the observed reactions (1) and (2) and the absence of trapped atomic hydrogen at 77°K as has already been stated above.

It would be of interest to see whether reactions (1) and (2) are also found in DNA (figures 7 and 8). Unfortunately the situation in DNA is complex since it is, for example, not clear to what extent the primary radicals generated at low temperatures are involved in the production of secondary radicals after warming up. Obviously, many of the radicals observed at 77°K (e.g. the ions of the bases and deoxyribose signals) decay after warming up (the gain has to be enhanced by a factor of 6 in figures 7 (*b*) and 8 (*b*)!) most probably by release of

atomic hydrogen which we find to be trapped in large amounts at 77°K. However, the appearance of the thymine X• lines in the room temperature spectra of DNA (figures 7 (*b*), (*c*), and 8 (*b*), (*c*)) is noteworthy as it indicates reaction (1) to proceed in DNA, too. The observation of the thymyl octet in DNA by other authors, and the pronounced deuteration effects observed with this signal suggest reaction (2) to take place too.

Hence, the 300°K spectra of DNA (figures 7 (*b*) and 8 (*b*)) do not allow the conclusion that atomic hydrogen originating from the formation or decay of the primary radicals is involved in the production of secondary radicals in significant amount. This is in line with the failure to detect the thymyl radical in DNA after exposure to thermal hydrogen atoms from a hydrogen discharge (Heller and Cole 1965), whereas large concentrations of this radical have been found after exposure of the thymine. Furthermore, Dertinger and Carpy (1971) have obtained quite the same results after bombardment with discharge-excited argon. None of these findings support the assumption that reactions of atomic hydrogen are a significant source of secondary radical formation in dry DNA. But further investigations are necessary for complete elucidation of the processes following ionization and dissociation and leading to the formation of secondary DNA radicals.

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We are grateful to Professor Dr. K. G. Zimmer for his continued interest in this work. We would also like to thank Dr. H. Jung for clarifying discussions.

L'irradiation de la thymine, thymidine, thymidine-5′-monophosphate et de l'ADN par des électrons de 2 MeV provoque la formation de radicaux libres. Le comportement de ces radicaux a été étudié par résonance paramagnétique électronique à 35 GHz. Les substances ont été irradiées à 77°K, en phase solide, et les spectres hyperfins enregistrés à 77° et à 300°K. Les changements, survenent dans les spectres, ont été analysés à l'aide de techniques faisant appel à l'ordinateur. Il en résulte que le radical de la 5,6-dihydro-5-thymyle se forme par protonisation de l'anion de la thymine. D'autre part, le cation de la thymine peut facilement perdre un proton. Les radicaux participant à ces transformations ont été identifiés dans les substances analysées.

Die Bildung und Umwandlung freier Radikale in Thymin, Thymidin, Thymidin-5′-Monophosphat und DNS nach Elektronenbestrahlung wurde mit Hilfe der Elektronspin-Resonanz (ESR) bei 35 GHz analysiert. Trockene Proben dieser Substanzen wurden bei 77°K bestrahlt und die ESR-Spektren anschließend bei 76°K und bei Zimmertemperatur in Abwesenheit von Sauerstoff registriert. Die dabei auftretenden Veränderungen des ESR-Spektrums wurden mit Hilfe eines Computers aufgeschlüsselt. Es ziegte sich, daß das Thymin-Kation leicht deprotonisiert, während andererseits das Anion durch Protonisierung in das bekannte 5,6-Dihydro-5-Thymyl-Radikal übergeht. Es konnte gezeigt werden, daß diese Umwandlung in allen untersuchten Substanzen vorkommen.

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