Dertinger report the thymidine positive ion in powdered thymine crystals.[27] The resolution is sufficient to resolve only the large methyl proton splitting of 19 G. This value is in good agreement with that found in our previous work[8] considering the differences in molecular environment.

Reactions of the Cation and Anion Radicals of Several DNA Bases[1]

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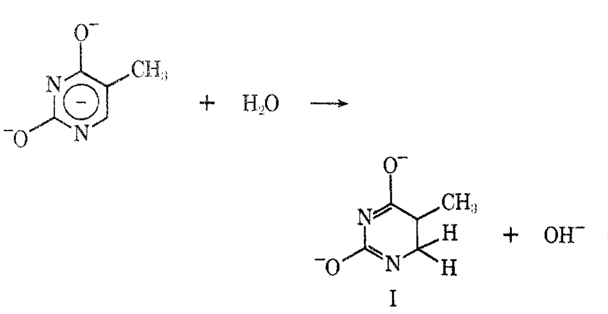
Publication costs assisted by Oakland University

The reactions of the π-cation and anion radicals of several DNA bases are investigated in a number of aqueous glasses. For thymine and 5-methylcytosine the π-cations initially formed at 77°K react upon warming in 8 N NaOH or 5 M K_2CO_3 by deprotonation of the methyl group to produce a $RCH_2\cdot$ radical. This is verified by the production of the $RCH_2\cdot$ radical by an independent method in the case of thymine. Results are found which indicate possible cation radical decomposition in the cases of thymidine and 5-methyldeoxycytidine. Computer simulations of the glassy spectra expected for the thymine $RCH_2\cdot$ radical where the nitrogens are either protonated or unprotonated suggest splittings near 17 G for the CH_2 group and 6 G for the 6-position proton in the unprotonated case. The anion of thymine in 12 M LiCl which is stable at low temperatures is found to protonate upon warming to form the 5,6-dihydro-5-thymyl radical. Identical results are found for thymidine. The results found for the reactions of cation and anion radicals are discussed in terms of results found in crystalline DNA bases and recent pulse radiolysis results. A mechanism of radiation damage to DNA is discussed.

Introduction

The study of the reactions of ion radicals of biological molecules is of importance to the understanding of the effect of radiation on these structures. Holroyd and Glass were the first to show that the thymine π-anion in an alkaline matrix reacts by protonation to form the 5,6-dihydro-5-thymyl or "thymyl" radical (reaction 1).[2] Since the esr spectra of irradiated DNA clearly show the thymyl radical, this reaction has been suggested to be significant in the radiolysis of DNA.[2] In a preliminary report Sevilla indicated that the thymine π-cation radical in an alkaline matrix decomposed by deprotonation of the methyl group to form radical II by reaction 2.[3] A number of other investigations have also found evidence for the role of positive ion radicals toward the production of more stable radical species.[4–6]



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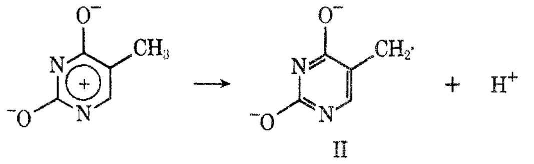

(2)

In our preceding work we reported an esr study of the π-cation radicals of several DNA bases.[3,7] In this work we report an esr investigation of the reactions of these ion radicals. Experimental evidence is given which confirms that the thymine π-cation undergoes the deprotonation reaction (reaction 1). In addition, the results suggest the π-cation radicals of 5-methyl-substituted DNA bases react by this deprotonation

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(1) This research was supported in part by the Division of Biology and Medicine of the U. S. Atomic Energy Commission.

(2) (a) R. A. Holroyd and J. W. Glass, *Int. J. Radiat. Biol.*, **14**, 445 (1968); (b) M. G. Ormerod, *ibid.*, **9**, 291 (1965).

(3) M. D. Sevilla, *J. Phys. Chem.*, **75**, 626 (1971).

(4) J. Hüttermann, J. F. Ward, and L. S. Myers, *Int. J. Radiat. Phys. Chem.*, **3**, 117 (1971).

(5) G. Hartig and H. Dertinger, *Int. J. Radiat. Biol.*, **20**, 577 (1971).

(6) A. Gräslund, A. Ehrenberg, A. Rupprecht, and G. Ström, *Biochim. Biophys. Acta*, **254**, 172 (1971).

reaction. Work is also reported which shows the protonation of the anions of thymine and thymidine to form the thymyl radical is not pH dependent.

Experimental Section

The details of the experimental procedure can be found in our previous publication.[7] In this work a new aqueous glass is employed. It is found that 12 *M* LiCl is compatible with the photooxidation of ferrocyanide technique for generating electrons. The electron is stable in this glass at 77°K. Although we know of no previous report of the use of this glass with the ferrocyanide method, there are investigations of the radiolysis of the glass in which its characteristics are described.[8]

The γ irradiations were performed using a ^{60}Co source.

Results

Reactions of Cation Radicals. Thymine. In previous work it was shown that photolysis of thymine (0.25 mg/ml) in 8 *N* NaOH glass at 77°K produces the well-resolved esr spectrum of the π-cation radical and the electron.[3] Photobleaching the trapped electron produces the π-anion and π-cation radicals in about equal concentrations.[3] We find that warming the glass containing the cation and anion to about 140°K results in the loss of the π-cation radical esr spectrum. Further warming to 190°K results in loss of the π-anion spectrum and production of the spectrum shown in Figure 1A. This spectrum is considered to be the overlapped spectrum of the thymyl radical and the radical produced by the decomposition of the π-cation. The eight-line spectrum of the "thymyl" radical is clearly present. The reaction of the anion to form this species has been reported by other workers and shown to be a reaction of the anion with water in the aqueous solvent.[2a] The dotted curve in the spectrum shown in Figure 1A is the thymyl radical in an 8 *N* NaOH glass in the absence of the π-cation decomposition product. This species is produced in a separate experiment by electron attachment to thymine and subsequent protonation of the π-anion by warming to 190°K. The electrons are generated by the photooxidation of ferrocyanide ion. Subtraction of the thymyl radical spectrum from the overlapped spectrum results in the spectrum shown in Figure 1B. This spectrum is considered to be the π-cation decomposition product. The spectrum extends 53 G, consists of eight components, is anisotropic in nature, and cannot be interpreted directly. However, the spectrum appears as an overall triplet which is further split. This radical species is believed to be radical II since it has been observed in irradiated crystalline thymine.

To verify that radical II is the decomposition product of the π-cation radical, the same radical was produced by an independent means. It is known that at high pH hydroxyl radicals ($\cdot$O$^-$) attack the methyl group of thymine to produce radical II.[9,10] To produce this species only, an alkaline D_2O glass containing 1 m*M* thymine and saturated with N_2O at low temperature was irradiated with ^{60}Co γ rays (dose 2.4×10^{18} eV/g) at 77°K. This produces $\cdot$O$^-$ and electrons which are both stabilized at this temperature. Upon warming to 180°K the electrons react with N_2O to produce $\cdot$O$^-$ and N_2. The predominant attacking species is then $\cdot$O$^-$. Figure 1C shows the spectrum of the irradiated sample at 190°K after $\cdot$O$^-$ attack. The large central peak is background signal resulting from the irradiated quartz sample tube. Except for increased resolution of the hyperfine components and the background signal in Figure 1C the spectra in Figures 1B and 1C are identical. The increased resolution is due to the fact that the irradiated sample was prepared in D_2O while that which yielded spectrum 1B was prepared in H_2O.

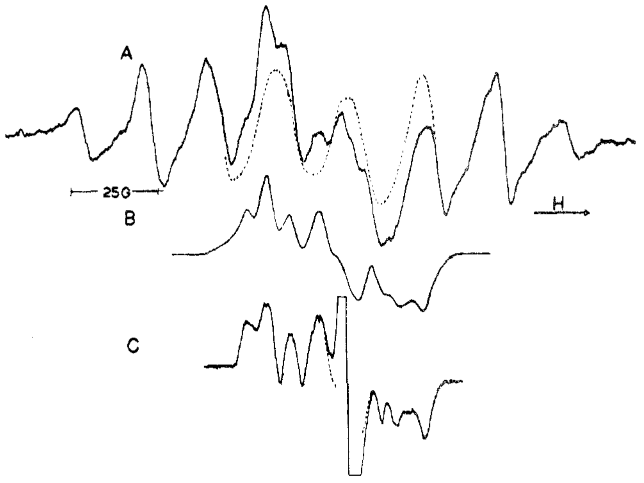

Figure 1. (A) Esr spectrum of the 5,6-dihydro-5-thymyl radical and the thymine π-cation decomposition product at 190°K in an 8 *N* NaOH–H_2O glass. The dotted curve is the "thymyl" radical alone. (B) Spectrum of the π-cation decomposition product resulting from a subtraction of the two curves in A. (C) Spectrum of radical II at 190°K produced by $\cdot$O$^-$ attack on thymine in 8 *N* NaOD–D_2O. The central peak is a background signal. The nearly identical spectra in B and C confirm the identity of the π-cation decomposition product.

A second method was used to separate the π-cation decomposition spectrum from the thymyl radical. Samples of thymine (1 m*M*) in 8 *N* NaOD containing 1 m*M* ferricyanide ion were photoionized with uv light. The ferricyanide ion acts as an electron scavenger. Photobleaching the electron in these samples results

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(7) M. D. Sevilla, C. Van Paemel, and C. Nichols, *J. Phys. Chem.*, **76**, 3571 (1972).

(8) L. Kevan in "The Chemical and Biological Action of Radiations," M. Haissinsky, Ed., Masson, Paris, 1969.

(9) L. S. Myers, J. F. Ward, W. T. Tsukamoto, D. E. Holmes, and J. R. Julca, *Science*, **148**, 1234 (1965).

(10) N. Nazhat and J. Weiss, *Trans. Faraday Soc.*, **66**, 1302 (1970).

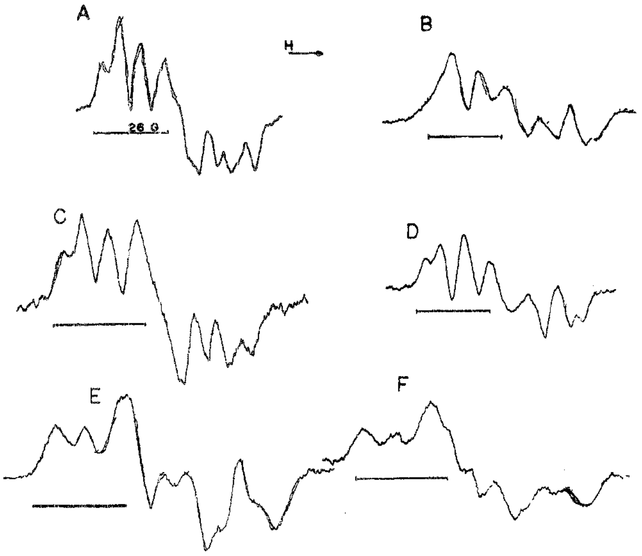
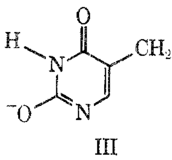


Figure 2. Esr spectra of radicals produced by warming the π-cation radicals of several DNA bases to 190°K in 8 N NaOD–D_2O or 170°K in 5 M K_2CO_3–D_2O in the presence of an electron scavenger (1 mM ferricyanide ion): A, thymine in 8 N NaOD; B, thymine in 5 M K_2CO_3; C, cytosine in 8 N NaOD; D, cytosine in 5 M K_2CO_3; E, thymidine in 5 M K_2CO_3; F, 5-methyldeoxycytidine in 5 M K_2CO_3.

in an esr spectrum of the cation and a small signal due to the anion radical. The electron is thus effectively scavenged. Warming this sample to 190°K results in the spectrum shown in Figure 2A. This spectrum is virtually identical with Figure 1C and is more resolved than Figure 1B as would be expected due to the deuterated matrix. From these results we conclude that the ferricyanide ion is an effective electron scavenger which does not take part in the reaction. From the overall results in this section we can conclude that the thymine π-cation radical deprotonates to form radical II.

Since the decomposition mechanism could be matrix dependent, it was of interest to perform experiments at a lower OH^- concentration. Photolysis of thymine (1 mM) in 5 M K_2CO_3/D_2O with 1 mM ferricyanide ion produced the esr spectrum of the π-cation and electron. Photobleaching and warming to 170°K resulted in the spectrum shown in Figure 2B. This spectrum is 56 G in width and is similar to that found in the NaOH glass. We associate this spectrum with radical III. These results are good evidence that the mechanism is the same in NaOD and K_2CO_3 matrices.



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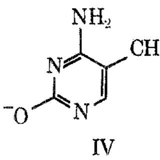

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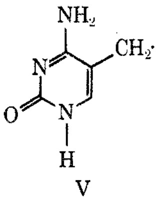
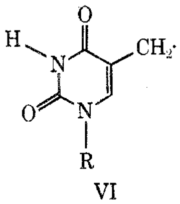


V

5-Methylcytosine. Samples of 5-methylcytosine (1 mM) in 8 N NaOD containing 1 mM ferricyanide were photoionized with uv light. Warming the sample containing the π-cation to 190°K results in the esr spectrum found in Figure 2C. In 5 M K_2CO_3–D_2O a similar procedure produced the spectrum shown in Figure 2D. These spectra are virtually identical with those found for thymine and suggest an identical mechanism of decomposition to form radical IV in NaOH[11] and V in K_2CO_3.[12]

Thymidine and 5-Methyldeoxycytidine. Neither the thymidine or 5-methyldeoxycytidine π-cations gave evidence for decomposition in 8 N NaOD. In fact, thymidine π-cation was observed at temperatures as warm as 195°K. However, warming the π-cations of these nucleotides in 5 M K_2CO_3 to 190°K produced esr spectra shown in Figures 2E and 2F. These spectra differ from the virtually unresolved spectra originally found for the π-cations. This change in spectra was found to be irreversible and may suggest a further reaction. The total width of both of these spectra is approximately 60 G and is somewhat larger than the 56-G width found for the thymine and 5-methylcytosine π-cation decomposition radicals. In addition, line components for the nucleosides are not in agreement with those found for the pyrimidines. Results of spin density calculations and spectrum simulations (discussed later) suggest that such differences in spectra may be expected for a radical of the same form as radical II when substitution occurs at the nitrogen. However, owing to the fact that the positive ions were stable in 8 N NaOD and that the π-cation spectra would appear as similar quartets, we can only tentatively associate the radicals which give rise to the spectra in Figures 2E and F to radicals VI and VII.



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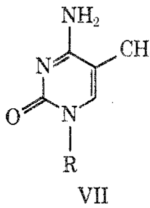

VII

Reactions of Thymine and Thymidine Anion Radicals. Photolysis of 10 mM ferrocyanide ion in 12 M LiCl–H_2O glasses containing *ca.* 1 mM thymine or thymidine produces the anion radicals (see Figure 3A). The results found for thymine and thymidine are virtually identical. The spectra of these anions which consist of 14-G doublets are similar to those found in our previous work in 8 N NaOD glasses.[3,13] Both anion spectra show the presence of a small amount of the thymyl radical which is probably explained by the

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(11) In view of our results for the 5-methylcytosine π-cation in 8 N NaOH (ref 7) a structure with one amino proton is also possible.

(12) This structure is considered most likely in 5 M K_2CO_3 glasses since production of this radical in concentrated D_3PO_4 by ·OD attack produces an identical spectrum.

(13) M. D. Sevilla and C. Van Paemel, *Photochem. Photobiol.*, **15**, 407 (1972).

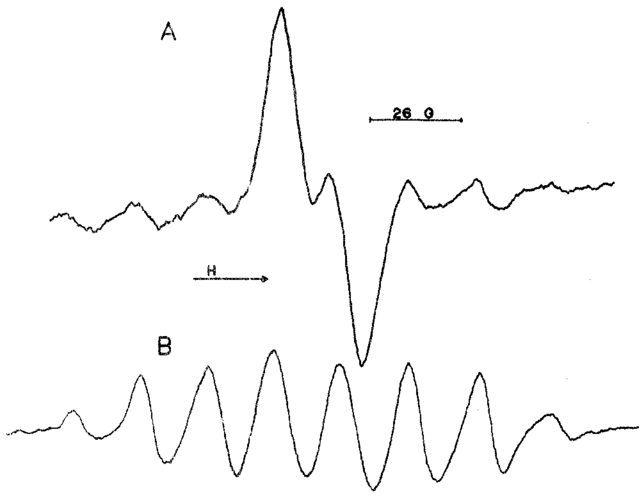


Figure 3. (A) Esr spectrum of the thymine anion produced by electron attachment at 110°K in 12 M LiCl. (B) Esr spectrum of the 5,6-dihydro-5-thymyl radical produced by warming the anion in A to 170°K.

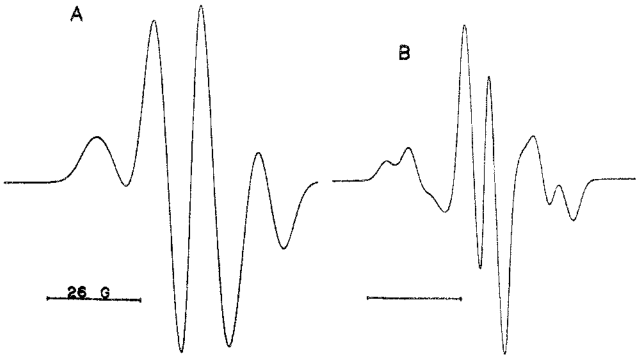
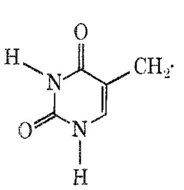


Figure 4. (A) Computer simulation of the glassy spectrum of radical VIII based on parameters from single crystal work. (B) Computer simulation of the glassy spectrum of radical II based on parameters described in text.

fact that reaction 1 can be induced by uv light.[14] Upon warming the samples to 170°K a loss of the anion signal occurs with a concurrent buildup in the characteristic eight-line spectrum of the thymyl radical. Figure 3B shows the spectrum for thymine after the reaction has gone to completion. Analysis of the final spectrum for thymine yields a_{CH_3} = 20.3 G and a_{CH_2}(av) = 39.6 G at 110°K. For thymidine we find a_{CH_3} = 20.2 G and a_{CH_2}(av) = 37.6 G at 110°K. These results are similar to those found by Holroyd and Glass in NaOH glasses.[2a] Through computer simulations these workers estimated the difference between the two methylene proton splittings to be 9 G for the thymidine case.

Spectrum Analysis by Computer Simulations. Simulations of the polycrystalline spectrum of π-cation decomposition radical (radical II) are possible from previous work on thymine single crystals. Hüttermann reports the hyperfine and g tensors and their direction cosines for radical VIII.[15] This radical



VIII

differs from radical II only by the protonation of the nitrogens. The reported isotropic components are 15 G for the CH_2 protons and 11.3 G for the 6-position proton. Transforming the hyperfine tensors into the g tensor axis system and utilizing a computer program which simulates the polycrystalline spectrum to first order[16] results in the spectrum in Figure 4A. In the simulation the line components were assumed to be gaussian and the line width was 3.5 G for each component. The resultant spectrum consists of a quartet of unequal spacing. Although this spectrum is similar to that found in polycrystalline thymine by Hartig and Dertinger[5] which they assigned to radical VIII, it is unlike that found here for radical II. This suggests a relatively large effect due to the differences in protonation between radicals II and VI. Neta has produced the monoprotonated radical by O^- attack at high pH in a steady-state radiolytic technique.[17] This radical is found to have isotropic hyperfine splittings of approximately 16 G for the CH_2 protons and 8 G for the 6-position proton. Thus a small increase in the CH_2 splitting and a significant reduction in the 6-position splitting is induced by the deprotonation of position 1. A greater change in hyperfine splittings may be expected in 8 N NaOH where both nitrogens at position 1 and 3 are deprotonated. McLachlan MO calculations of the spin density distribution employing usual parameters[7] for radical II indicate an increase in the spin density at the CH_2 group and a decrease at position 6 over that calculated for radical VIII. We have simulated the esr spectrum of radical II by employing the same g and hyperfine tensor elements found by Hüttermann for radical VIII but have altered the tensor elements proportionately so that the isotropic components correspond to the theoretically indicated values of 17 G for CH_2 and 6 G for the 6-position proton splitting (see Figure 3B). Gaussian lines with 1.5-G line width are assumed. The resolution of individual components and peak heights in Figure 3B is not in good agreement with experiment; however, the line positions are in relatively good agreement, *i.e.*, the average of the magnitudes of the deviations of theoretical positions from experiment is 1 G.

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(14) Y. Lion and A. Van de Vorst, *Int. J. Radiat. Phys. Chem.*, **3**, 513 (1971).

(15) J. Hüttermann, *Int. J. Radiat. Biol.*, **17**, 249 (1970).

(16) M. Si Swin Wei, Ph.D. Thesis, The University of Michigan, 1970.

(17) P. Neta, *Radiat. Res.*, **49**, 1 (1972).

The line positions are even in better agreement (0.8-G average deviation) with those found for the 5-methylcytosine radical in Figure 2C. These results indicate that the isotropic splittings of 17 and 6 G used in the reconstruction are near the true isotropic splittings of radicals II and IV.

The results found for thymidine and perhaps 5-methyldeoxycytidine may now be explainable in terms of the effects of ribose substitution at position 1. The expected effect of this substitution would be to increase the 6-position splitting so as to produce a spectrum which appeared as a quartet. This is what is found experimentally in Figure 2E.

Discussion

The results found here suggest that the mechanism of decomposition of the π-cation radicals of 5-methyl-substituted DNA bases in aqueous glasses is deprotonation of the methyl group. These results are in good agreement with recent results obtained on irradiated crystalline DNA bases. Hüttermann, *et al.*, in work with irradiated 5-methylcytosine single crystals suggest that the positive and negative ions first produced react to form more stable secondary radicals by mechanisms analogous to reaction 1 and 2.[4] Hartig and Dertinger report results for irradiated dry powders of thymine, thymidine, and DNA.[5] For thymine they find that the positive ion initially formed deprotonated to form radical VIII. For thymidine and even DNA, radicals of the form of radical VIII are observed in the esr spectra. They suggest that deprotonation is the mechanism of formation of these radicals as well.

In recent results on irradiated single crystals of 6-azathymine Herak and Schoffa suggest a radical analogous to radical VIII is formed by hydrogen atom abstraction.[18] Results found here would suggest the deprotonation of the positive ion as an alternative explanation.

Our results for the reactions of the thymine and thymidine anions have a bearing on a point of disagreement between esr and pulse radiolytic experiments. Esr investigations show that thymine anion undergoes irreversible protonation in alkaline matrices to produce the 5,6-dihydro-5-thymyl radical.[2a,14] Pulse radiolysis of aqueous solutions of thymine at pH 7 indicate the thymine anion protonates at an oxygen.[19-21] Theard, *et al.*, suggest that a possible reason for this apparent discrepancy is that the pH affects the protonation mechanism.[19] The results found here in 12 *M* LiCl glasses do not support a pH dependence. The fact that there is evidence that "thymine" anions in irradiated wet DNA protonate upon warming also argues against this explanation.[22] Another possible explanation for this difference in results is that the ionic strength, not pH of the glasses, affects the mechanism of protonation. The results found in DNA would argue against this. In addition, Verma, *et al.*, report that thymine anions protonate in mixtures of methanol and 0.5 *M* NaOH where the ionic strength is much lower.[23] A likely explanation for this difference is that the 6-protonation is a slow step not observed in the pulse radiolysis experiment.

The results found here and the results of a number of other workers leads to a mechanism of the direct effect of radiation on DNA. Gräslund, *et al.*, have shown that the initial effect of radiation is to produce ions which are stabilized on the DNA bases at low temperature.[6] The DNA strand can then be viewed as a semiconductor for excess charge and holes. At low temperatures the electrons and holes are immobile while as the temperature increases the charges can migrate through the π-orbital systems of the stacked DNA bases. In the migration process the bound electrons and holes can recombine, transfer out of the DNA strand if the DNA is in contact with another molecule,[24] or induce reactions at the most reactive DNA bases. Ion recombination would explain the loss of signal intensity observed when samples of DNA irradiated at low temperatures are warmed.[5] The results found for irradiated DNA clearly suggest that the electron would be expected to react at the thymine base to form the thymyl radical.[22,25-28] A number of workers have suggested this reaction to explain the appearance of the thymyl radical spectrum only when wet DNA initially irradiated at 77°K is warmed to temperatures above 200°K.[2] The fact that irradiated samples of dry DNA do not produce the thymyl spectrum is in accord with the anion protonation mechanism.[5,22] The results found here would suggest the deprotonation of the methyl group of thymine base in DNA as a possible reaction for the positive hole. Hartig and Dertinger's findings that irradiated *dry* DNA shows a predominant signal due to a deprotonated base thymine radical is good evidence that this reaction of the positive ion in DNA may be preferential over other possible reactions.[5] Thus it is likely that electrons and holes in wet DNA react predominantly with thymine base to produce radicals *via* mechanisms analogous to those in reactions 1 and 2.

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Acknowledgment. We are indebted to Julien Gendell and John Fritz for their aid in the spectrum simulation. We also would like to thank Anthony Ervin for his experimental aid.

Studies of the Ester Bond. II.[1] Nuclear Magnetic Resonance Studies of *tert*-Butyl Formate

by Torbjörn Drakenberg* and Sture Forsén

Division of Physical Chemistry, The Lund Institute of Technology, 220 07 Lund 7, Sweden *(Received July 3, 1972)*

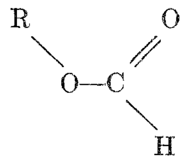
Publication costs assisted by The Lund Institute of Technology

The s-cis, s-trans interconversion barrier and s-cis, s-trans population ratio of *tert*-butyl formate in various solvents have been determined by means of nmr total line shape analysis. The free energy difference between the two conformers ($\Delta G°$) is found to vary linearly with the Onsager parameter $(\epsilon - 1)/(2\epsilon + 1)$. Extrapolation to $\epsilon = 1$ (gas phase) results in $\Delta G° = 6$ kcal/mol. For a solution of *tert*-butyl formate in dimethylformamide-d_7–dimethyl ether (1:1) the free energy of activation was found to be $\Delta G^{\ddagger}_{183} = 8.75$ and 9.32 kcal/mol for the two isomeric forms. It has also been possible to estimate the population ratio at room temperature from the coupling constant ${}^5J_{\mathrm{H-H}}$. These values show reasonable agreement with results derived from the total line shape data.

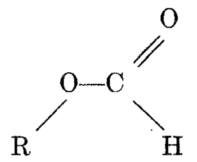
Introduction

It is now well known, from nmr studies, that there exist large barriers to internal rotation about the formal single bonds in amides,[2] nitrites,[3] and nitrosamines.[4] Due to the chemical similarity it is to be expected that the C–O bond of the ester group should possess a torsion barrier of a similar order of magnitude; this has, however, not been confirmed by nmr measurements. There do exist however, ir[5] and ultrasonic relaxation[6] data, showing that the barrier to hindered rotation in methyl formate is *ca.* 10 kcal/mol.

Chiefly on the basis of dipole moment measurements[7] and microwave spectroscopy[8] it has mostly been assumed that the conformation of formates is almost exclusively s-trans. In small ring lactons, however, the ester group is forced to be in the s-cis conformation, and it has by means of dipole moment measurements been found that in lactons with 8 or 9 atoms in the ring there is an equilibrium between the s-cis and s-trans conformers and for larger rings the s-trans conformer dominates.[9] In a recent *ab initio* calculation[1] the energy difference between the two conformers of methyl formate was calculated to be 9 kcal/mol with the s-cis conformer having the higher energy, in agreement with the microwave data.[8] These data show that the energy difference between the s-cis and s-trans conformers of the ester group is normally very high. This can probably explain the fact that the cis–trans isomerism of esters has, to date, not been observed by means of nmr spectroscopy.



s-trans



s-cis

Recently it has been shown that the formyl proton pmr signal from *tert*-butyl formate splits into an unequal doublet at low temperature (below −90°) in DMF solution,[10] showing that the population ratio in *tert*-butyl formate is not as far from unity as is that for methyl

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