Trapping of Radicals Formed in the Photochemical Reaction between Hydrogen Peroxide and Some Pyrimidine Bases, Nucleosides, Nucleotides, and Yeast Nucleic Acid

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Abstract: The short-lived radicals formed in the photochemical reaction between hydrogen peroxide and a number of pyrimidine bases, nucleosides, nucleotides, and yeast nucleic acid have been trapped as nitroxide radicals. The secondary splittings in the esr spectra of the nitroxides formed with the pyrimidine bases with no substitution on N-1 of the pyrimidine ring exhibited a dominant ^{14}N splitting. Provided the pyrimidine bases reacted in their common tautomeric forms, the primary radicals were considered to be formed by the addition of photochemically generated OH radicals to the double bond between C-5 and C-6. The nitrogen atom of the nitroxide group was believed to be attached at C-6. The nitroxides obtained in the reaction with nucleosides, nucleotides, and yeast nucleic acid indicated that the primary radicals had been formed in a reaction which involved the abstraction of a hydrogen atom from the carbohydrate part of the molecule by OH radicals.

In addition to a direct interaction, high-energy irradiation and uv light are injurious to pyrimidine bases, nucleosides, nucleotides, and nucleic acids, when present in liquid solutions, through ions and radicals formed in the radiolysis of solvent water. Radicals derived from the parent compounds are formed in a secondary reaction with primary radical species such as H· and ·OH. The radicals produced in these reactions cannot be detected directly by the technique of esr spectroscopy since their lifetimes are too short except when continuously generated in highly material-consuming flow systems.[1]

The nitroxide method[2] has been applied in the present work to the study of the free radicals produced in water solutions in the photochemical reaction between hydrogen peroxide and some pyrimidine bases, nucleosides, nucleotides, and yeast nucleic acid. The primary radicals derived from these compounds were considered to be formed in the reaction with OH radicals produced by the photochemical cleavage of H_2O_2.

Experimental Section

The parent compound, about 10 μM, was dissolved together with the scavenger, *tert*-nitrosobutane (0.5 mg, ~5 μM), in 0.5 ml of H_2O, D_2O, or an alkaline solution of these solvents. One drop of 30% H_2O_2 was added and the reaction mixture was then transferred to an aqueous solution cell. The sample was irradiated *in situ* in the esr cavity with light from a high-pressure mercury lamp (Osram HBO, 200 W) with no special light filters but equipped with a movable shutter and an adjustable diaphragm, so that the duration and intensity of the light could be easily varied.

The pyrimidine bases, nucleosides, nucleotides, and nucleic acids were commercial preparations obtained from Sigma Chemical Co. and Fluka A.G. 3-Methyluracil was prepared by N-methylation of uridine followed by removal of the carbohydrate part. *tert*-Nitrosobutane was prepared as described by Pedersen and Torssell.[3]

The esr spectra were obtained with a Varian 100-kHz spectrometer and a 9-in. magnet. Exchange of protons for deuterium was checked by a Varian A-60 nmr spectrometer.

Results

Generally, the spectra were taken at ambient temperature but most of the nitroxides were stable enough to be recorded even at a temperature of 70°. The coupling constants were rather independent of the temperature except that of the doublet splitting assigned to H-6 (see below), the value of which was found to increase about 30% with the temperature in the range from 2 to 70°.

Coupling constants of the nitroxide radicals are collected in Table I, and the esr spectra of some of the nitroxides are shown in Figures 1–6. The lines of the symmetrical di-*tert*-butyl nitroxide derived exclusively from the scavenger are marked with an S in some of the figures. No unsymmetrical nitroxides could be detected in the absence of hydrogen peroxide, or without irradiation with uv light.

(I) Uracil (1). The secondary splittings of the nitroxide formed with uracil consisted of 3 × 2 × 2 × 2 lines (Figures 1a and b). The dominant splitting was a ^{14}N triplet caused by one of the nitrogen atoms of the pyrimidine ring. The smallest of the three doublet splittings, *i.e.* that of 0.26 G, was absent when the reaction was carried out in D_2O (Figure 1c). The smallest doublet splitting was still present in 0.1 *N* NaOH but absent in 0.2 *N* NaOH in H_2O (Figure 1d). These findings indicated that the splitting in question was caused by an easily exchangeable proton located on a nitrogen atom or on a hydroxyl group. When a solution of uracil in 0.2 *N* NaOD in D_2O was kept at 90° for 6 days, the two doublet splittings of 0.66 and 0.26 G had disappeared (see Figure 1e). The nmr spectrum of the sample showed that the two nmr doublet splittings originating from H-5 and H-6 of uracil had been replaced by a single line at the position of the low-field doublet.[4] These results indicated that the largest of the esr doublets, *i.e.* that of 1.42 G, originated from H-6, and that of 0.66 G, which had been exchanged in alkaline D_2O, was caused by H-5.

(1) (a) Cl. Nicolau, M. McMillan, and R. O. C. Norman, *Biochim. Biophys. Acta*, 174, 413 (1969); (b) H. Taniguchi, *J. Phys. Chem.*, 74, 3143 (1970); (c) H. Dertinger and Cl. Nicolau, *Biochem. Biophys. Acta*, 199, 316 (1970); (d) B. B. Singh and Cl. Nicolau, *Progr. Biophys. Mol. Biol.*, 23, 23 (1971); (e) J. K. Dohrmann and R. Livingston, *J. Amer. Chem. Soc.*, 93, 5363 (1971).

(2) (a) M. J. Perkins, *Chem. Soc., Spec. Publ.*, No. 24, 97 (1970); (b) E. G. Janzen, *Accounts Chem. Res.*, 4, 31 (1971); (c) C. Lagercrantz, *J. Phys. Chem.*, 75, 3466 (1971).

(3) J. A. Pedersen and K. Torssell, *Acta Chem. Scand.*, 25, 3151 (1971).

(4) S. R. Heller, *Biochem. Biophys. Res. Commun.*, 32, 998 (1968).

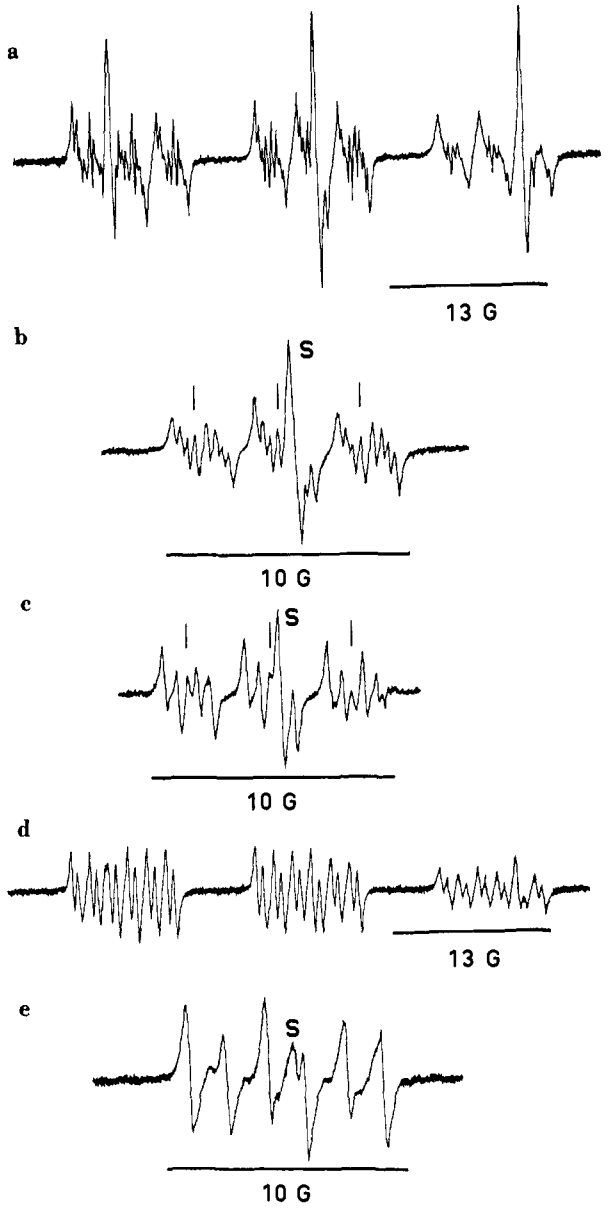
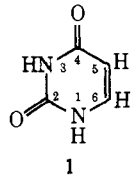


Figure 1. Esr spectra of the nitroxide radicals formed in the reaction between photochemically generated OH radicals and uracil (**1**): (a) in H_2O; (b) main center component $M_I = 0$, in H_2O; the indices mark the lines of an additional overlapping radical species; (c) main center component $M_I = 0$, in D_2O; (d) in 0.2 *N* NaOH in H_2O; (e) main center component $M_I = 0$, after incubation of the substance at 90° for 6 days in 0.2 *N* NaOD in D_2O.

There was a distorting overlap present in the spectra of the nitroxides obtained with uracil both with H_2O and with D_2O; see the vertical indices in Figures 1b and c. This overlap was believed to be caused by the presence of an additional, unknown radical species, the spectrum of which was not further analyzed.



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(II) Cytosine (2). The secondary splittings of the nitroxide spectra obtained with this substance were rather similar to those observed with uracil and consisted of 3 × 2 × 2 × 2 lines also in this case. The resolution of the smallest doublet was poorer than in

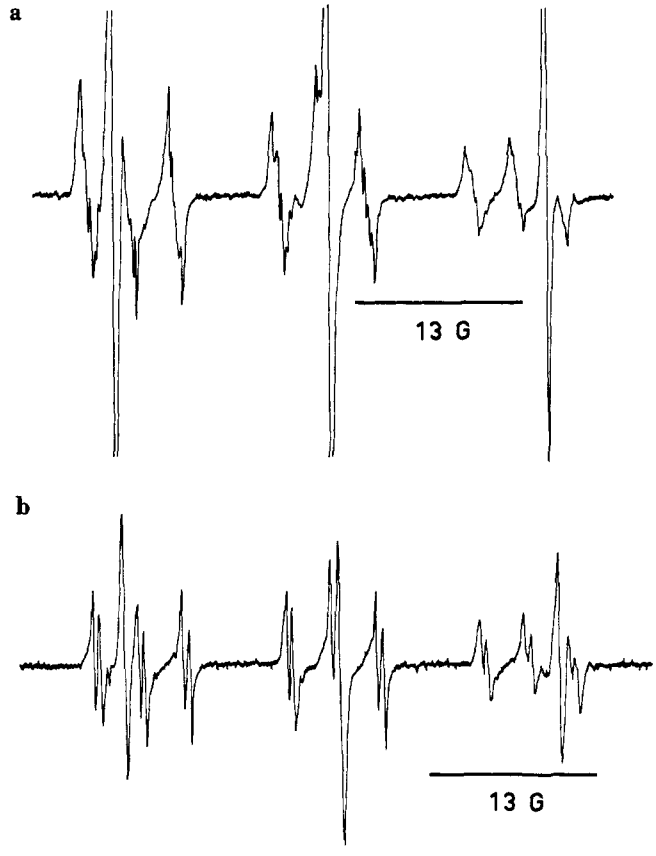


Figure 2. Esr spectra of the nitroxide radicals formed in the reaction between photochemically generated OH radicals and thymine (**3**): (a) in H_2O; (b) in D_2O.

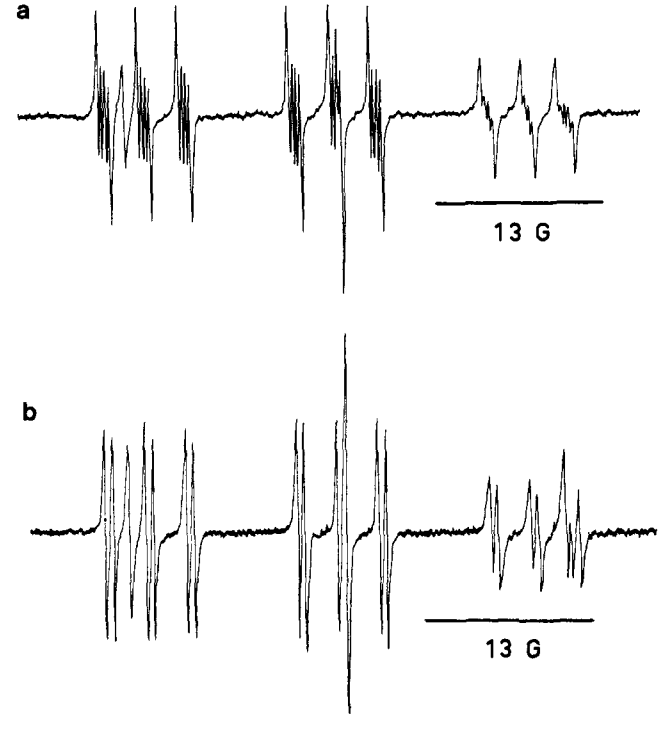


Figure 3. Esr spectra of the nitroxide radicals formed in the reaction between photochemically generated OH radicals and isoorotic acid (**5**): (a) in H_2O; (b) in D_2O.

Table I. Nitroxide Radicals Formed at Room Temperature by Trapping of the Radicals Produced in the Reaction between Photochemically Generated OH Radicals and Some Pyrimidine Bases, Nucleosides, and Nucleotides[a]

Parent compound	Solvent	Main ^{14}N splitting of the nitroxide group, a_N, G	Secondary splittings, G
Uracil (**1**)	H_2O, D_2O	15.0	3.37 (t, N-1), 1.43 (d, H-6), 0.66 (d, H-5), 0.26 (d, OH, NH)
Cytosine (**2**)	H_2O, D_2O	14.9	3.37 (t, N-1), 1.53 (d, H-6), 0.66 (d, H-5), 0.30 (d, OH, NH)
Thymine (**3**)	D_2O	15.1	3.42 (t, N-1), 0.50 (d, H-6)
5-Fluorouracil	D_2O	15.0	3.07 (t, N-1), 0.50 (d, H-6)
5-Hydroxymethyluracil	D_2O	15.0	3.07 (t, N-1), 0.50 (d, H-6)
Pseudouridine[b]	D_2O	14.8	3.07 (t, N-1), 0.50 (d, H-6)
Orotic acid (**4**)	H_2O, D_2O	15.5	3.58 (t, N-1) + incompletely resolved lines
Isoorotic acid (**5**)	H_2O, D_2O	15.0	3.09 (t, N-1), 0.72 (d, H-6), 0.31 (d, OH, NH)
2,4-Dimethoxypyrimidine (**6**)	H_2O, D_2O	15.3	0.77, 12 equally spaced lines (see text)
1,3-Dimethyluracil (**7**)	D_2O	14.7	2.25 (q, N-1 and H-6), 0.48 (d, H-5)
3-Methyluracil (**8**)	H_2O, D_2O	15.0	3.37 (t, N-1), 1.43 (d, H-6), 0.66 (d, H-5), 0.26 (d, OH, NH)
Uridine[c]	H_2O, D_2O	14.8	5.01 (d, H-5′), 1.43 (d, H-4′)

[a] Scavenger, *tert*-nitrosobutane; d = doublet, t = triplet, q = quartet. [b] An additional radical species derived from the carbohydrate part was also present. [c] Identical spectra were obtained with 2′-deoxyuridine, uridylic acid, thymidine, and thymidylic acid.

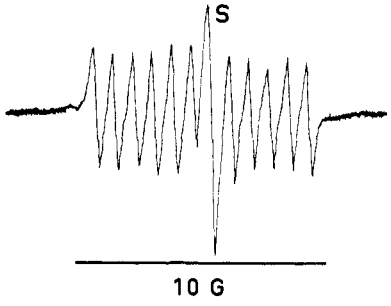


Figure 4. Esr spectrum of the nitroxide radicals formed in the reaction between photochemically generated OH radicals and 2,4-dimethoxypyrimidine (**6**); main center component $M_I = 0$; solvent, H_2O.

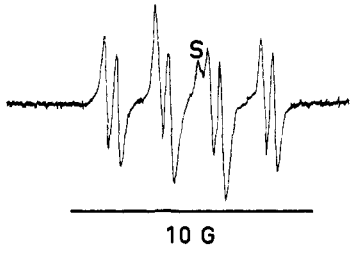
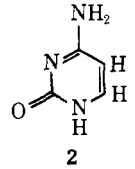


Figure 5. Esr spectrum of the nitroxide radicals formed in the reaction between photochemically generated OH radicals and 1,3-dimethyluracil (**7**); main center component $M_I = 0$; solvent, D_2O.

the corresponding spectra of uracil. The distorting overlap observed for uracil was present. but was much less prominent in this case.



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(III) Thymine (3). The nitroxide spectra obtained with thymine in H_2O exhibited a dominant secondary ^{14}N splitting. Each of these lines was incompletely split into a number of narrow lines (Figures 2a). When the reaction was carried out in D_2O the incompletely resolved lines were replaced by a doublet splitting (Figure 2b). Evidently the spectrum had been simplified by the exchange of one or two protons located on a nitrogen atom and/or a hydroxyl group. The doublet splitting was considered to be caused by H-6. A small amount of a further radical species could also be seen in these spectra.

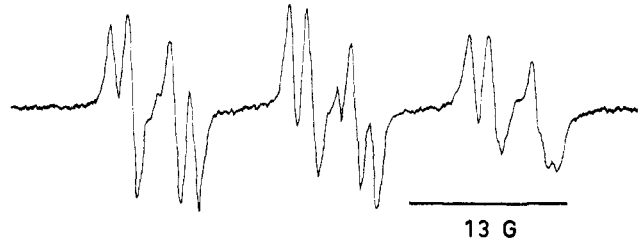
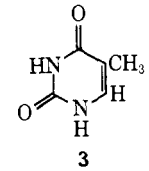


Figure 6. Esr spectrum of the nitroxide radicals formed in the reaction between photochemically generated OH radicals and uridine; solvent, H_2O.



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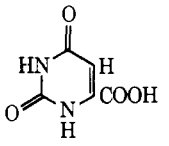
(IV) 5-Fluorouracil, 5-Hydroxymethyluracil, and Pseudouridine. The nitroxide spectra obtained with these substances were very similar to those observed with thymine. The incompletely resolved splittings of the secondary ^{14}N triplet lines were replaced by a doublet splitting (H-6) in these cases when the reactions were carried out in D_2O. No splittings were observed with 5-fluorouracil which could be traced to the fluorine atom. Pseudouridine, in which the carbohydrate part of the molecule is attached to C-5 of the pyrimidine ring, gave rise to a spectrum exhibiting the presence of an additional radical species, very probably formed by the trapping of a primary radical derived from the carbohydrate part of the parent compound.

(V) Orotic Acid (4). The nitroxide spectra obtained with this substance were similar to those observed with thymine, 5-fluorouracil, 5-hydroxymethyluracil, and pseudouridine. However, the spectra showed essentially no change when the reaction was carried out in

D_2O, a result which was consistent with the presence of the carboxy group on C-6.



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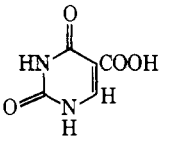
(VI) Isoorotic Acid (5). In addition to the dominant secondary ^{14}N splitting, the nitroxide spectra obtained with isoorotic acid exhibited two secondary doublet splittings (Figure 3a). When the reaction was carried out in D_2O, the smaller of the two doublet splittings disappeared (Figure 3b). The larger of the doublet splittings was considered to be caused by H-6, and the smaller one by an easily exchangeable proton on a nitrogen atom or on a hydroxyl group.



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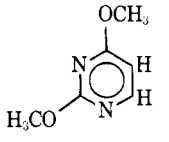
(VII) 2,4-Dimethoxypyrimidine (6). The secondary splittings of the nitroxide spectra obtained with this substance consisted of 12 almost equally spaced lines of equal intensity (Figure 4). These secondary hyperfine splittings (hfs) were caused by one ^{14}N nucleus and two nonequivalent protons, *i.e.* H-5 and H-6, giving rise to the $3 \times 2 \times 2$ lines, but it could not be settled whether the largest splitting originated from an interaction with the ^{14}N nucleus or with one of the protons. No change of the spectrum was found when the reaction was carried out in D_2O.



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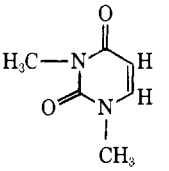
(VIII) 1,3-Dimethyluracil (7). The secondary splittings observed in this case consisted of a quartet with the intensity ratio rather close to 1:2:2:1. Each of the four lines was further incompletely split into at least two lines. When the reaction was carried out in D_2O the spectrum became clearer by an exchange of protons located on OH groups, and each of the quartet lines was now split into a doublet (Figure 5). The quartet was believed to originate from the combined interaction with one ^{14}N nucleus and one proton with nearly identical coupling constants. The proton contributing to the quartet and that giving rise to the doublet were evidently H-5 and H-6 of the pyrimidine ring.



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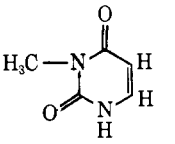
(IX) 3-Methyluracil (8). This substance gave rise to nitroxide spectra identical with those obtained with uracil both when the reaction was carried out in H_2O and in D_2O.



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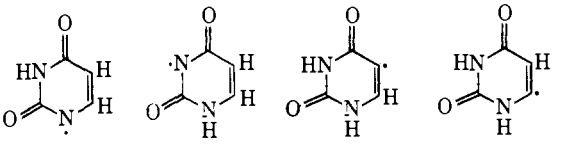
(X) Nucleosides and Nucleotides. Nearly identical nitroxide spectra were obtained with uridine, 2′-deoxyuridine, uridylic acid, thymidine, and thymidylic acid. The esr spectra showed secondary splittings consisting of rather broad doublets of doublets indicating an interaction with two nonequivalent hydrogen atoms (Figure 6). It is suggested that the radicals trapped in these cases were produced by the abstraction of a hydrogen atom from the carbohydrate part of the molecule, possibly one of the two hydrogen atoms on C-5′. Accordingly, the large doublet of about 5 G originated from the remaining hydrogen atom on C-5′ and the smaller one from the hydrogen atom on C-4′.

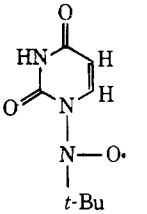
(XI) Nucleic Acids. A preparation of yeast nucleic acid gave rise to an esr spectrum almost identical with that obtained with the nucleosides and nucleotides. A DNA preparation gave rise to a spectrum indicating the trapping of radicals as nitroxides but no resolved secondary splittings could be seen. This result was perhaps to be expected in view of the large molecular weight of DNA, which indirectly gives rise to broad spectral lines by the slow tumbling of the nitroxides formed.

Structure of the Nitroxides and the Primary Radicals. Radicals Formed after the Abstraction of a Hydrogen Atom by OH Radicals from Common Tautomeric Forms. If the common dominant oxo–oxo tautomer constituted the reacting form of uracil, four different radical species could be formed by the abstraction of a hydrogen atom, *i.e.* **9–12**.



9 10 11 12

In view of the relatively large secondary ^{14}N splitting of about 3 G observed in the spectra of the nitroxides obtained with the pyrimidine bases unsubstituted at N-1, it was tempting to consider N-1 or N-3 to be attached on the nitrogen atom of the nitroxide group. In such a case the nitroxide would have been formed by trapping of the primary radical **9** or **10**. As identical nitroxides were obtained with uracil and 3-methyluracil, any trapping of **10** could be ruled out. Therefore, the formation of nitroxide **13** was expected by the



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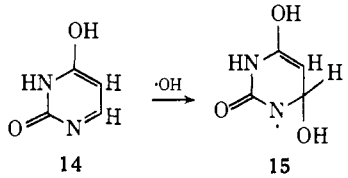
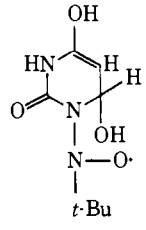
trapping of **9**. The analogous primary radicals and the corresponding nitroxides derived from the other pyrimidine bases unsubstituted at N-1 were easily found by use of the pertinent common tautomer.

However, the third doublet splitting, *i.e.* that of about 0.3 G present in the nitroxide spectra obtained with substances such as uracil, cytosine, and isoorotic acid, required a structure allowing for an easily exchangeable hydrogen atom. The only easily exchangeable one present in a nitroxide such as **13** was the hydrogen atom located on the second nitrogen atom of the pyrimidine ring, namely H-3. Provided an interaction with this hydrogen atom was the origin of the doublet splitting concerned, the adjacent nitrogen atom was expected to also make a contribution to the secondary splittings. No such interaction could be detected in any of the nitroxide spectra here reported. In view of these considerations, and the fact that an easily exchangeable hydrogen atom was also present in the nitroxide obtained with 3-methyluracil, structure **13** could very probably be ruled out.

Radicals Formed by the Addition of OH Radicals to Rare Tautomers. Still considering a direct attachment of N-1 to the nitrogen atom of the nitroxide group as a prerequisite for the explanation of the relatively large value of the secondary ^{14}N splitting, and at the same time allowing for an easily exchangeable hydrogen atom, it must be assumed that the primary radical had been formed by the addition of an OH radical to double bonds of a rare tautomer. As an example let us consider the addition of an OH radical to the double bond between N-1 and C-6 of the uracil tautomer **14**, a reaction which leads to the primary radical **15** (eq 1) and to the formation of the nitroxide **16**. The structure of



14 15 (1)



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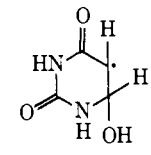
16 is wholly consistent with the secondary splittings of the nitroxide spectra obtained with uracil, including the dominant ^{14}N triplet caused by N-1 and the two doublets originating from an interaction with H-6 and H-5. The third doublet, which was absent in D_2O, originated from an easily exchangeable hydrogen atom on the hydroxy group of C-6. The disappearance of the third doublet in 0.2 *N* NaOH in H_2O might be taken as evidence for the presence of a dissociable proton located on a hydroxy group.

A crucial question concerns the extent to which rare tautomers really are present and constitute a reacting form such as **14**. The common oxo–oxo and oxo–amino tautomers are the dominant species, but small amounts of the rare tautomers might be of general importance for a number of reactions of the pyrimidine bases, including those of mutagenesis and miscoupling of nucleic acids. The importance of small amounts of rare tautomers for the radical reactions studied in the present work might be supported by the fact that the samples were irradiated with uv light, and that calculations have shown the stability of some of the rare tautomers to increase when the substance was present in an excited state.[5]

Radicals Formed by the Addition of OH Radicals to Common Tautomers. Even if the nitroxide spectra seemed to be satisfactorily explained by the trapping of radicals derived from the rare tautomers, it was perhaps more realistic to assume an interpretation involving only the common dominant tautomers. Evidently, the common tautomer was the unique reacting form of 1,3-dimethyluracil in which the oxo–oxo form was locked by the presence of the methyl group on the nitrogen atoms.

The addition of an OH radical to the double bond between C-5 and C-6 of the common tautomer of uracil would give rise to the primary radicals **17** and **18** and to the corresponding nitroxides **19** and **20**. In view



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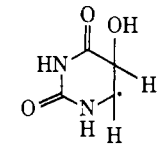

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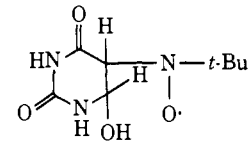

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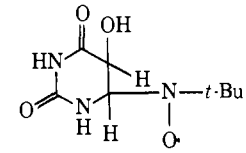

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of the relatively large secondary ^{14}N splitting, structure **20** seemed to be the most probable of the two alternatives. The crucial point was evidently whether the large ^{14}N splitting caused by N-1 could be consistent with a structure such as **20** in which N-1 was no longer adjacent to the nitrogen atom of the nitroxide group.

The hyperconjugative transmission of spin density from the π orbital of the nitrogen atom of the nitroxide group to the β atoms is generally expressed by eq 2.[6]

$$a^{\beta} = (A + B\cos^2\theta_{\beta})\rho_N{}^{\pi} \qquad (2)$$

In this expression a^{β} is the coupling constant of the appropriate β atom, A is the parameter of the π–σ interaction independent of the dihedral angle, B is the parameter of the angle-dependent π–σ interaction, and θ_{β} is the dihedral angle, *i.e.* the angle between the axis of the π orbital of the nitrogen atom and the bond axis between the α atom and the β atom in a plane perpendicular to the bond between the nitrogen and the α atom. $\rho_N{}^{\pi}$ is the spin density of the π orbital of the nitrogen atom. Generally, A is much smaller than B. In the case of a free rotation around the bond between the nitrogen and the α atoms we have $\cos^2\theta_{\beta} = \langle\cos^2\theta_{\beta}\rangle = {}^1/_2$.

(5) B. Pullman and A. Pullman, *Progr. Nucl. Acid Res. Mol. Biol.*, 9, 327 (1969).

(6) (a) C. Heller and H. M. McConnell, *J. Chem. Phys.*, 32, 1535 (1960); (b) A. D. McLachlan, *Mol. Phys.*, 1, 233 (1958); (c) M. C. R. Symons, *J. Chem. Soc.*, 277 (1959).

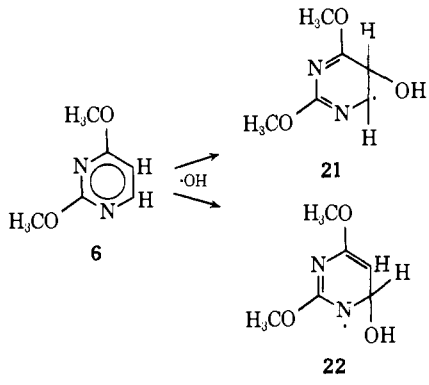
In nitroxide **20** C-6 constituted the α atom and N-1, H-6, and C-5 the three β atoms. If there is free rotation around the bond between the nitrogen atom and C-6, $a_{H\text{-}6}$ was expected to be about 10–12 G, whereas the observed value was between 0.5 and 1.5 G for the nitroxides derived from the free pyrimidine bases. These findings could be plausibly rationalized by suggesting the existence of a hindered rotation locking the nitroxide into a conformation with $\theta_{H\text{-}6}$ rather close to 90°. Such a value implied that $\theta_{C\text{-}5}$ and $\theta_{N\text{-}1}$ would be about 30°. A dihedral angle of about 30° was certainly not incompatible with the observed $a_{N\text{-}1}$ value of about 3 G. This was supported by the result obtained with the nitroxide formed on trapping the primary radical derived from *N*,*N*-dimethylformamide by the abstraction of a hydrogen atom from one of the methyl groups.[7] In this nitroxide, $HC(O)\text{–}N(CH_3)\text{–}CH_2\text{–}N(O\cdot)\text{–}t\text{-Bu}$, the two hydrogen atoms and the nitrogen atom of the amide group constituted the β atoms. There was restricted rotation around the bond between the nitrogen atom of the nitroxide group and the α-carbon atom, which led to a conformation with nonequivalent hydrogen atoms. One of the coupling constants a_H was large, *i.e.* about 20 G, whereas the other one was too small to be observed. The coupling constant of the β-nitrogen atom was 2.44 G. These values implied an angular arrangement with $\theta_{H\text{-}1} \sim 90°$, and $\theta_{H\text{-}2}$ and θ_N about 30°.

In view of these arguments we conclude that the values of the coupling constants $a_{N\text{-}1}$ observed for the nitroxides obtained with the pyrimidine bases unsubstituted at N-1 were wholly consistent with a structure such as **20**. This statement implied that the primary radicals were formed by the addition of an OH radical to the double bond between C-5 and C-6 of the common tautomers. Such an interpretation was also consistent with the results obtained with flow methods.[1]

The results obtained with the flow techniques indicated that the majority of the primary radicals derived from the pyrimidine bases under study were formed by the addition of an OH radical to the double bond between C-5 and C-6 of the common tautomers. The radical center was considered to be located on C-5 or C-6, and only exceptionally did the interpretation involve a radical center on N-1, or a radical formed by the abstraction of a hydrogen atom from the methyl group of thymine.

(7) C. Lagercrantz, manuscript in preparation.

Radicals Formed with 2,4-Dimethoxypyrimidine. The reacting form of this substance corresponded to the hydroxy–hydroxy tautomer, which was locked in this structure by the methyl groups on the oxygen atoms. The primary radicals were considered to be formed by the addition of an OH radical to the double bond between C-5 and C-6, or between N-1 and C-6 (eq 3).



(3)

In view of the discussion given above for β atoms, it is possible that the nitroxide observed had been formed on trapping of **21**. Thus, a primary radical with the center on C-6 might also be formed in the case of a hydroxy–hydroxy tautomer.

Generally, only one radical species was trapped as a nitroxide in the reaction with a particular pyrimidine base, whereas several radical species had been observed simultaneously in several of the corresponding flow experiments. These findings might be connected with a selective survival of a particular radical species during the diffusion from the sites of formation to the scavenger, with steric hindrance for the scavenger to reach the radical center of the primary radical, or with an extraordinary stability of the observed nitroxide. Such factors might also be responsible for the rather selective trapping of the radicals formed from the carbohydrate part in the reaction of nucleosides and nucleotides.

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