RADIATION CHEMISTRY OF NUCLEIC ACIDS:

IDENTIFICATION OF THE MAJOR HYDROPEROXY THYMINE

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Summary: The major product from γ-radiation of thymine in aerated aqueous solution is shown to be cis-5-hydroxy-6-hydroperoxy-5,6-dihydrothymine by considering its uv, ir, nmr spectral data, the nmr deshielding effect on the OOH proton, and its reduction to cis-thymine glycol. These suggest that the earlier structural assignment [Cadet and Teoule, Biochem. Biophys. Acta., **238**, 8 (1971)] was in error. Furthermore, HOOH oxidation of either cis- or trans-thymine glycol in acidic condition gives this hydroperoxide in yields >90%. This result again directly contradicts that reported in the previous paper. Our present findings are readily explained by considering the chemistry of thymine glycols and the reaction of OH radicals with thymine. Also, contrary to the earlier notion that this peroxide should be unstable, we find that it is sufficiently stable for studying the thymine peroxide interaction with chromosomes, bacterial cells and nucleic acid components.

It has been shown[1] that radiolysis of aerated aqueous solutions of nucleic acids, pyrimidine nucleotides, Ura[2], or Thy[2] results in the formation of hydroxy-hydroperoxides. A similar suggestion was made from the observation[3] that organic peroxides are formed together with HOOH in these systems. Subsequently, the major Thy hydroperoxide was characterized as 5-hydroperoxy-6-hydroxy-5,6-dihydrothymine ($\mathrm{ho_2^5ho^6hThy}$, **I**) by chemical synthesis[4] and by measurement of rate constants for the liberation of iodine in the reaction of the hydroperoxide with iodide[5].

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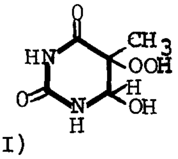
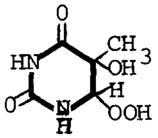
Furthermore, the radiation product was shown[6] to be a mixture of cis and trans isomers of I as was suggested previously.[4,5] The



(I)

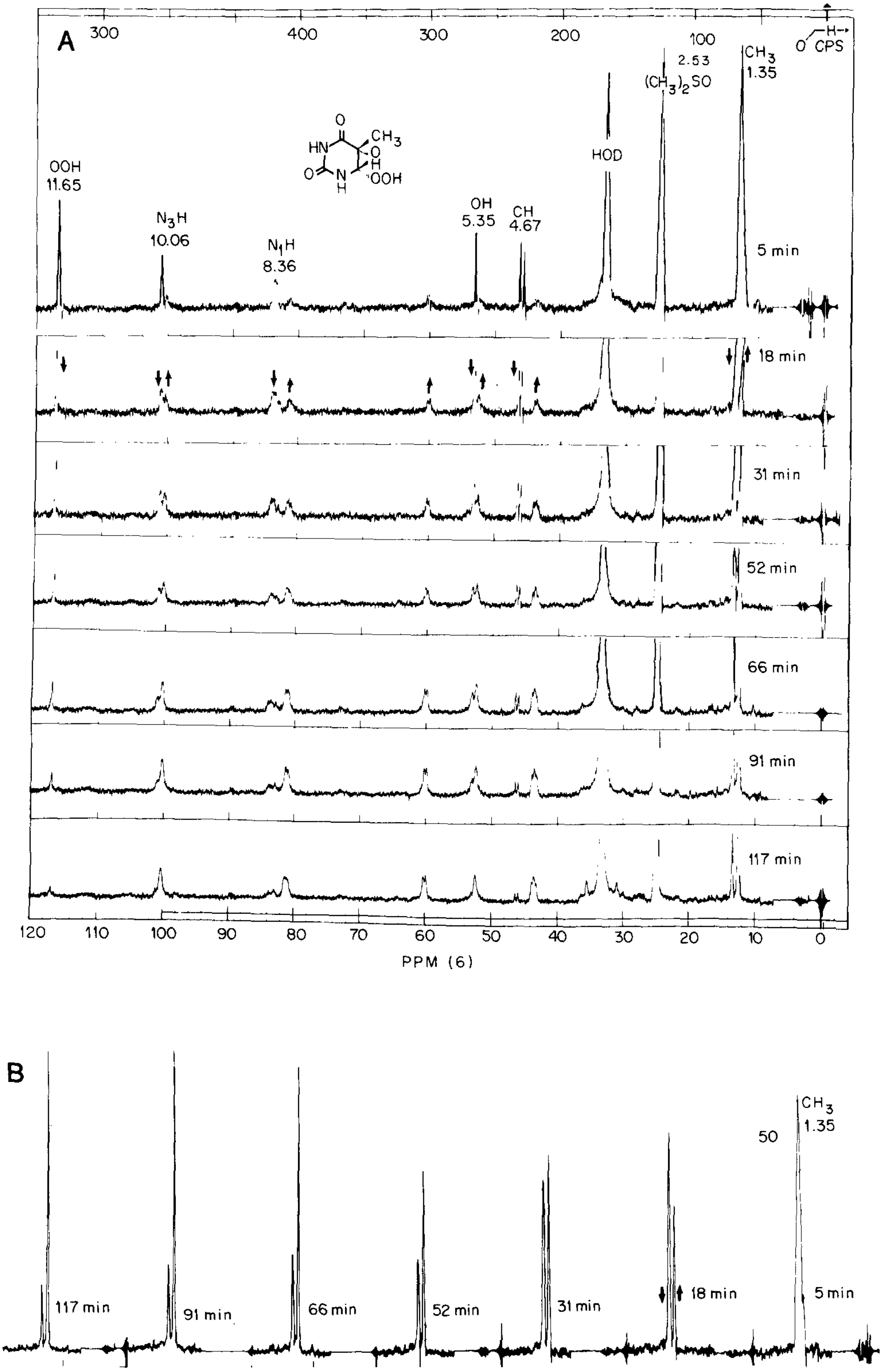


(II)

positional isomer, ho^{5}ho$_{2}^{6}$hThy (II) was ruled out as a major radiation product of Thy. Therefore, subsequent research efforts on I have focused on quantitative measurements[7] rather than on identification. Recently, Cadet and Teoule[8] supported the original structural analysis by reexamining the chemical synthesis of these hydroperoxides and by studying their IR and NMR spectra. Since the delineation of this major radiation product is essential to the understanding of the radiation chemistry of nucleic acids, we undertook a routine confirmation of these reports. Surprisingly, we found that cis-ho^{5}ho$_{2}^{6}$hThy (II) rather than I is the major product. Experimental evidence and chemical criteria are presented to support our finding.

The compound studied is probably the same major radiation product obtained by Cadet and Teoule[8], based on the R_f values on MN Silica gel S-HR tlc with eluent A[9]. Although their R_f values varied[8], R_{thy} calculated from these values is consistent, and agree with our findings. Since the three reported hydroxy-hydroperoxy-hThy isomers are easily separated with R_{thy} of 0.69, 0.83 and 1.12 (major product, G 0.83)[8] respectively, their separation did not present a problem. This major product was isolated from a 10 mM aerated aqueous solution of Thy irradiated in the usual manner[10] and was characterized as cis-II according to the following criteria. The product was reduced to cis-Thy glycol (III)[11] in $(CD_3)_2SO$ as evidenced by the NMR changes (Figure 1). Since this conversion involves no C-O cleavage, the parent compound must also have had a cis configuration. What remains unclear is the location of the C-OOH and C-OH moieties. A consideration of the changes in chemical shifts (Δ) of the corresponding proton nmr signals between cis-II and III will favor the C_6OOH assignment. The signal for the OOH is downfield (-5.65 ppm) from that for the OH indicating a considerable deshielding effect on the OOH proton.[12] This effect decreases with distance but should influence other protons.[12] The downfield shift trend is C_6H (Δ-0.33)> N_1H (-0.21)> N_3H (-0.11)>CH_3 (-0.07) for cis-II in comparison with III suggesting the C_6OOH assignment. This also accounts for Δ -0.07 for C_5OH. If the assignment were C_5OOH, a Δ +0.65 would have to be attributed to C_6OH. But it is highly improbable that an upfield shift would occur in a proton neighboring a strong deshielding group. Although one may argue that NMR chemical shifts for hydroxy and hydroperoxy do vary to some extent, this change will not alter the effects of -OOH on the trend of downfield shifts discussed above.

This C_6OOH assignment is further strengthened by IR spectra.[13] The two bands attributed[14] to νOOH (3451 cm^{-1}) and νC_6O-O (842) in cis-II are absent in III. While the two C_5-O bands have similar patterns and intensities for cis-II and III, C_6-OO (1181) has a greatly reduced intensity and (1018) shifts to a longer


A
300
400
300
200
100
O CPS
H
OOH
11.65
N_3H
10.06
N_1H
8.36
OH
5.35
CH
4.67
HOD
2.53
$(CH_3)_2SO$
CH_3
1.35
5 min
18 min
31 min
52 min
66 min
91 min
117 min
120
110
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90
80
70
60
50
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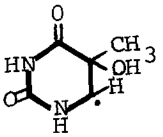
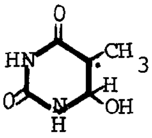
30
20
10
0
PPM (δ)
B
CH_3
1.35
50
117 min
91 min
66 min
52 min
31 min
18 min
5 min

wavelength as compared to the corresponding C_6-O bands (1081;1051) for III as is expected between C-OOH and C-OH resonances.[14]

This structural assignment is also corroborated mechanistically. When Thy was reacted with HO• generated chemically or photochemically, ho^6hThy-5• was the predominant species at pH 1.[15] However, above pH 2, ho^5hThy-6• is dominant[15] and a subsequent reaction in the presence of O_2 would yield II as the major product. The earlier mechanistic notion[5] in favor of I was based on the assumption that a tertiary carbon radical is more stable than a secondary one. Recent results[15] indicate that this notion is not universally applicable.

(ho^5hThy-6•)





(ho^6hThy-5•)

The most crucial evidence was obtained from the study of the synthesis of cis-II, which was carried out according to the method of Ekert and Monier.[4] When 10 mg of III or IV was treated with 4 ml 10% HOOH and 15 µl conc. HCl (pH 1.4) at room temperature for 24 hr, the residue, after lyophilization at <-25° and methanol recrystallization, was cis-II [both yields >90%, mp 122-124° dec.]. This synthetic material and the major radiation

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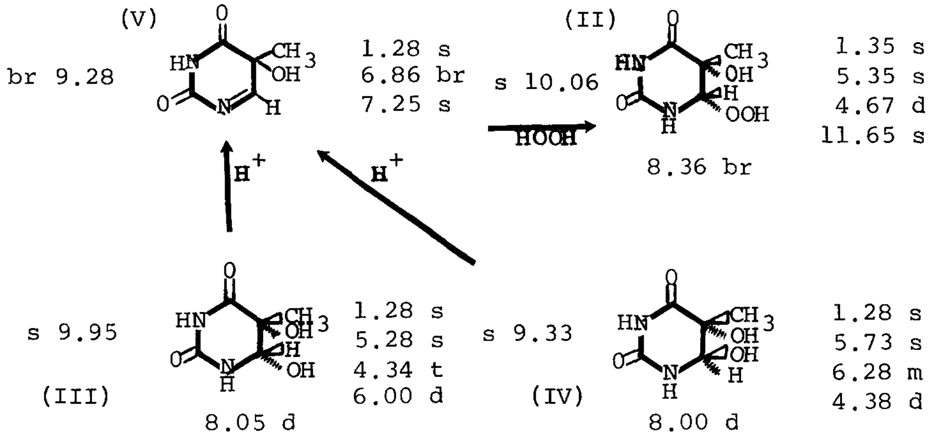
Figure 1. The sequential changes of nmr signals of cis-5-hydroxy-6-hydroperoxy-hydrothymine to those of cis-5,6-dihydroxy hydrothymine in $(CD_3)_2SO$. Part. A, the complete spectra excludes the CH_3 signals which are reproduced in Part B.

product are identical. A logical explanation for obtaining *cis*-II from either III or IV is that both Thy glycol isomers convert to a common intermediate with the assymmetry at C_6 eliminated for further reaction steps. Such an intermediate (V) has been isolated[10] and its formation should be facilitated by acid. In mild acidic media, HOOH is a powerful nucleophile and should react nonstereospecifically with unsaturated compounds.[16] Yet, only *cis*-II was obtained. This may be explained in terms of steric effects; the bulkier HOOH attacks the side with the small OH group rather than the CH_3 yielding *cis*-II. For a small attacking group such as HOH on V, both Thy glycols form albeit in unequal proportions.[10] The reported[8] formation of I from III in acidic HOOH is unlikely since IR, NMR, etc., show no loss of C_5OH. Formation of I from III is additionally unlikely since C_5OH is more stable than C_6OH, e.g. $\mathrm{ho}^6\mathrm{hPyr}$ derivatives dehydrate readily while $\mathrm{ho}^5\mathrm{hPyr}$ derivatives fail to react even under forcing conditions.[17]



Since only two IR spectra[18] were available and the nmr chemical shifts given[19] were incomplete for the four possible isomers, a direct comparison with previous results is impossible. However, the earlier structural assignment would give unlikely Δ values considering the -OOH deshielding effect on its neighboring protons as discussed above.

Early structural assignments were mainly based on the comparison with the synthetic material whose identification is now shown to be incorrect. This finding further strengthened our contention that _cis_-II is the major radiation product, and furthermore, suggests that the earlier structural assignments for various isomers of II[8] should be reinvestigated. Also, contrary to the previous notion[8] that _cis_-II is unstable, we found it to be sufficiently stable for studying the Thy peroxide interaction with chromosomes, bacterial cells, and nucleic acid components.

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11. A pure sample in $(CD_3)_2SO$ resulted in the appearance of the nmr signals which corresponded to cis-Thy glycol only. Hydroperoxy compounds reported in Ref. 8 gave signals for several products at the temperature of the nmr probe.
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13. This compound is stable in KBr pellet for spectral measurements in contrast to that reported in Ref. 8. Compound II has peaks νOOH 3450 cm-1, νOH 3170, νC=O imide 1690, νC=O ureide 1660, νCH_3 1462, νC-N 1220, νC_5-O 1134, νC_5-O 1111, νC_6-OO 1181 νC_6-OO 1018 and νC_6O-O 842. Compound III has peaks νOH 3300 cm-1, νOH 3220, νC=O imide 1730, νC=O ureide 1655, νCH_3 1475, νC-N 1225, νC_5-O 1175, νC_5-O 1111, νC_6-O 1081 and νC_6-O 1051.
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18. There are only two ir spectra recorded in the Thesis of R. Teoule (1970) for the four possible isomers. The resolutions are such that no major differences can be specified. Our two spectra of cis-II were reproduced for characterization, one in Nujol for the purpose of comparison with Teoule's and the other in a KBr pellet for demonstrating its stability.

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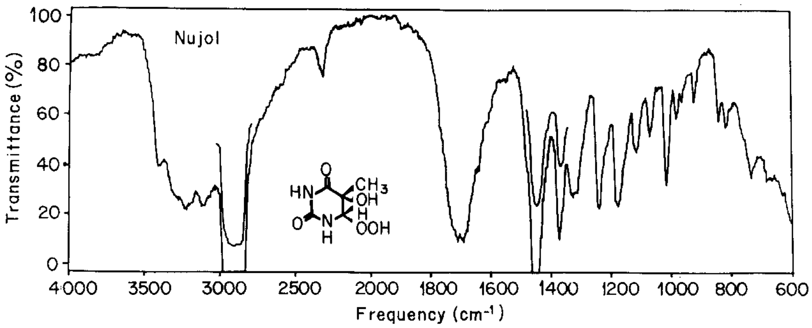

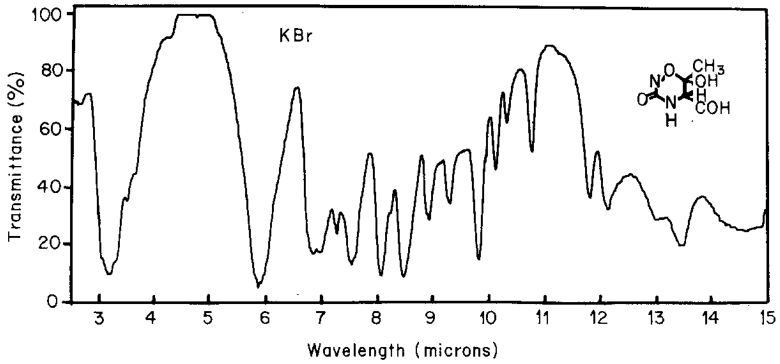

19. The nmr chemical shifts given in Ref. 8 are as follows: for II is δ 1.34 (CH_3); for <u>cis</u> I are δ 1.34 (CH_3), δ 4.55 (C_6H) and δ 8.18 (N_1H) and for <u>trans</u> I are δ 1.34 (CH_3), δ 4.62 (C_6H) and δ 8.27 (N_1H). Unfortunately, the tell-tale signals were not reported.