Pulse Radiolysis of Nucleic Acid Constituents and Related Compounds. I. Optical Spectrum and Reactivity of the 5,6-Dihydrothyminyl Free Radical[1]
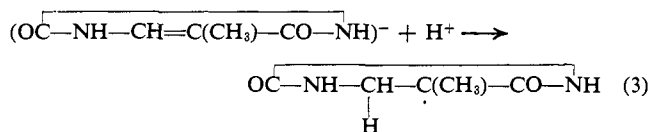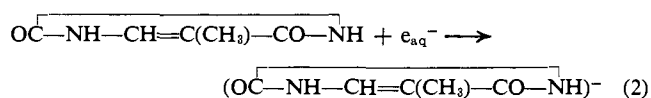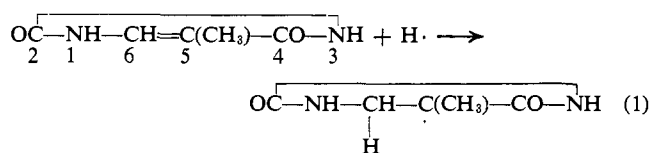
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Abstract: Dilute aqueous solutions of dihydrothymine, saturated with N_2O, were irradiated at pH 6.8 and 1.0 with submicrosecond pulses of 10 MeV electrons, and changes in optical absorbance were observed for periods up to 800 μsec in the wavelength range 300–600 nm. A broad absorption band with a maximum at 400 nm was assigned to the 5,6-dihydrothyminyl (probably the 5-yl) free radical. This absorption was also observed on reaction of H· with thymine, but not on reaction of e_{aq}^- with thymine and subsequent protonation of the anion radical. The dihydrothymin-5-yl radical is stable with respect to intramolecular reactions and decays by second-order kinetics with $2k = 2.35 \times 10^9$ M^{-1} sec^{-1} at pH 6.8 and 1.3×10^9 at pH 1.0. The slower decay in acid solutions is consistent with partial protonation of the radical.

Damage to nucleic acids is generally believed to be an important cause of the biological effects of radiation in lower organisms and possibly in mammalian cells as well. Many studies have shown that one of the results of nucleic acid radiolysis is destruction of pyrimidine bases.[4] Investigations of the reactions which lead to this result are therefore needed to provide a basis for understanding the biological effects of radiation. Such studies are also of interest because they contribute to our understanding of the reactions of heterocyclic compounds with free radicals, and of some of the properties of heterocyclic free radicals.

In this paper we consider the spectrum and reactivity of a radical believed to be the 5,6-dihydrothymin-5-yl radical. This radical might be expected to be formed from thymine (T) in irradiated air-free aqueous solutions by reaction 1 or by the sequence 2 and 3. Pre-

$$\text{OC—NH—CH}{=}\text{C(CH}_3)\text{—CO—NH} + \text{H·} \longrightarrow$$
$$\overset{}{2}\ \overset{}{1}\ \ \overset{}{6}\ \ \overset{}{5}\ \ \ \ \ \overset{}{4}\ \ \overset{}{3}$$

$$\text{OC—NH—CH—}\underset{\text{H}}{\overset{\cdot}{\text{C}}}\text{(CH}_3)\text{—CO—NH} \quad (1)$$

$$\text{OC—NH—CH}{=}\text{C(CH}_3)\text{—CO—NH} + e_{aq}^- \longrightarrow$$

$$(\text{OC—NH—CH}{=}\text{C(CH}_3)\text{—CO—NH})^- \quad (2)$$

$$(\text{OC—NH—CH}{=}\text{C(CH}_3)\text{—CO—NH})^- + \text{H}^+ \longrightarrow$$

$$\text{OC—NH—CH—}\underset{\text{H}}{\overset{\cdot}{\text{C}}}\text{(CH}_3)\text{—CO—NH} \quad (3)$$

vious work[5] has shown however that the two reaction

paths give different products. One product, with $\lambda_{max} \approx 400$ nm, was observed by pulse radiolysis of an air-free 0.93×10^{-2} M thymine solution, pH 0.65. Under these conditions the reaction of hydroxyl free radical (OH·) with T has a half-life of 10^{-8} sec, hydrated electrons (e_{aq}^-) are converted to hydrogen atoms (H·) in less than 10^{-9} sec, and a spectrum attributed to reaction of H· with T (path 1) grows over a period of several hundred nanoseconds. The other product, with $\lambda_{max} \approx 330$ nm, was observed in air-free T solutions, pH 4, containing 0.9 M ethanol as an OH· scavenger. Under these conditions e_{aq}^- is the principal reactive species, and a relatively intense spectrum appears within a few nanoseconds which was attributed to the thymine anion radical formed by reaction 2. This spectrum decreases to a lower intensity during a period of several hundred nanoseconds, with little change in position of λ_{max}, at a rate which depends on the hydrogen ion concentration. The decrease is believed to be a protonation reaction, but the product cannot be the same as that formed by the H· reaction. A similar result was obtained using sodium formate as an OH· scavenger.[6]
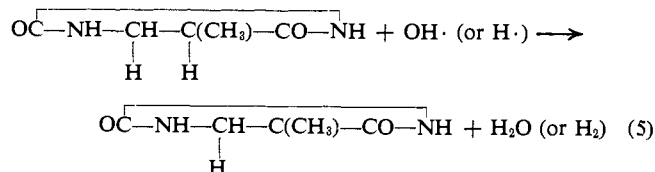
The spectrum with $\lambda_{max} \approx 400$ nm was tentatively assigned to the H· adduct radical shown in reaction 1 and the weak 330-nm absorption to the protonated anion with the H+ added to the C-4 carbonyl. These assignments were based on general considerations, and are by no means certain.

$$(\text{OC—NH—CH}{=}\text{C(CH}_3)\text{—CO—NH})^- + \text{H}^+ \longrightarrow$$

$$\text{OC—NH—CH—C(CH}_3){=}\underset{\underset{\text{H}}{\overset{|}{\text{O}}}}{\overset{\cdot}{\text{C}}}\text{—NH} \quad (4)$$
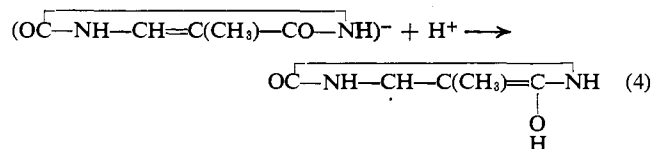
The reactions of dihydrothymine with OH· and H· provide a means of determining which, if either, of these spectra is correctly assigned to the dihydrothyminyl radical. Dihydrothymine (DHT) is the saturated analog of thymine, and OH· and H· are believed to

abstract hydrogen atoms from it according to reaction 5 to give the same product as reaction 1.[7] These re-

$$OC—NH—CH—C(CH_3)—CO—NH + OH \cdot \; (or\; H \cdot) \longrightarrow$$
$$\quad\quad\quad\; | \quad\quad | $$
$$\quad\quad\quad H \quad\quad H$$

$$OC—NH—CH—C(CH_3)—CO—NH + H_2O \; (or\; H_2) \quad (5)$$
$$\quad\quad\quad\quad\quad | $$
$$\quad\quad\quad\quad\quad H$$

actions, and reaction 1 as well, may also give the 6-yl radical

$$OC—NH—CH—C(CH_3)—CO—NH$$
$$\quad\quad\quad\quad\quad\quad\; . \quad\quad | $$
$$\quad\quad\quad\quad\quad\quad\quad\quad H$$
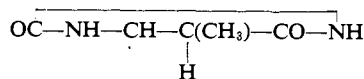
Stable products isolated after radiolysis of dihydrouracil (which differs from dihydrothymine only in that it has a 5-hydro instead of a 5-methyl group) are consistent with an initial abstraction reaction.[8] We have therefore carried out a pulse radiolysis study of the reactions of DHT with OH· and H· with the aims of (a) identifying the spectrum associated with the 5,6-dihydrothyminyl free radical and (b) investigating the reactivity of this radical.

Experimental Section

Aqueous solutions of DHT (2×10^{-3} M) were made from commercially available material (Sigma Chemical Co.) and triply distilled water. pH was adjusted with NaOH and HClO$_4$ solutions, and solutions were saturated with N$_2$O at room temperature by bubbling for at least 20 min before (to remove CO$_2$) and after adjusting the pH. The N$_2$O was presaturated with water to minimize concentration changes during bubbling.

A general description of the pulse radiolysis equipment has been published.[9] In this work samples were transferred to a 2-cm spectrophotometer cell by remote control, and exposed to single 4–20 nsec pulses of 10 MeV electrons from a linear accelerator. Fresh solution was used for each pulse. Doses varied with the tuning of the accelerator and the pulse length, but generally ranged from 500 to 5000 rads per pulse. Relative dose was determined for each pulse by collecting the electrons on an aluminum block after they had passed through the sample. The integrated charge was read on an electrometer which was calibrated against dose using the hydrated electron absorbance as a standard. Changes in the optical absorbance of the samples were observed by monitoring intensity of the analyzing light by a photomultiplier (4-pass cell, 8-cm path length). The optical system consisted of a 450-W mercury-xenon lamp, a glass filter (absorbance = 0.3 at 312 nm, 1.0 at 295 nm) to decrease photolysis of the solutions by ultraviolet light, a Bausch and Lomb monochromator, and an RCA 1P 28 photomultiplier tube. The light was projected from the irradiation room into an adjacent room containing the monochromator, photomultiplier, and associated cables in a low-noise environment. Output from the photomultiplier was fed directly into an oscilloscope, and data were recorded by photographing the traces.

Under the conditions used in these experiments e_{aq}^- is converted almost entirely to OH· or H·. The rate constant for reaction of e_{aq}^- with DHT has not been reported, but is probably close to the value of 4.5×10^9 M^{-1} sec^{-1} reported for dihydrouracil.[10] If so, at pH 6.8 about 6% of e_{aq}^- reacts with DHT and the remainder is converted to OH· by reaction with N$_2$O ($\sim$0.025 M, $k_{e_{aq}^- + N_2O}$ = 5.6×10^9 M^{-1} sec^{-1}).[11] The absorption maximum of the

(7) (a) H. C. Heller, S. Schlick, and T. Cole, *J. Phys. Chem.*, **71**, 97 (1967); (b) L. S. Myers, Jr., M. L. Hollis, and L. M. Theard, "Radiation Chemistry," Advances in Chemistry Series, No. 81, American Chemical Society, Washington, D. C., 1968, p 345.

(8) G. Vencendon, A. Cier, and C. Nofre, *Compt. Rend.*, **260** (2), 711 (1965).

(9) L. M. Theard, F. C. Peterson, R. L. Willson, J. F. Ward, L. S. Myers, Jr., and R. B. Ingalls, *Rad. Res.*, **31**, 581 (1967).

(10) C. L. Greenstock, M. Ng, and J. W. Hunt, "Radiation Chemistry," Advances in Chemistry Series, No. 81, American Chemical Society, Washington, D. C., 1968, p 396 ff.

(11) J. P. Keene, *Rad. Res.*, **22**, 1 (1964).
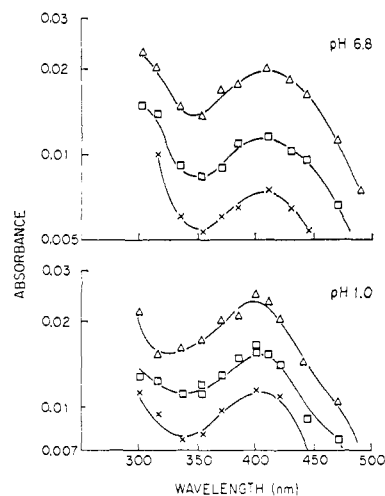
Figure 1. Absorption spectra of radicals formed by reaction of OH· and H· with dihydrothymine at pH 6.8 (upper curves) and 1.0 (lower curves). Absorbances are adjusted to a dose of 1000 rad/pulse, 8-cm light path. Time after pulse in μsec: —△—, 10; —□—, 50 at pH 6.8, 60 at pH 1.0; —×—, 100.

DHT electron adduct, (DHT)$^-$, should be at shorter wavelengths than that of the (T)$^-$adduct ($\sim$330 nm) because DHT lacks conjugated double bonds, and hence the small yield of (DHT)$^-$ should make a negligible contribution to spectra in the wavelength range observed. At pH 1.0 DHT may be partially protonated, and may react with e_{aq}^- much more rapidly than in neutral solutions. If a rate constant[12] equal to $k_{e_{aq}^- + H^+}$ = 2.3×10^{10} M^{-1} sec^{-1} is assigned to this reaction, 2% of the e_{aq}^- reacts with DHT and 98% is converted to H·. We may thus conclude that the spectra we report below can be attributed almost entirely to organic radicals formed by reaction of OH· or H· with DHT.

Results

Spectra of radicals formed at pH 6.8 and 1.0 are given in Figure 1. The data were obtained with a system rise time of 300 nsec and an oscilloscope sweep speed of 10 μsec per division. At this speed the later stages of radical growth as well as the early stage of decay can be observed. No major changes in absorbance occurred in any of the oscillograms during this period and the rates of growth were consistent with the rate constant reported for the reaction of OH· with DHT[7b] and a somewhat smaller rate constant for the reaction of H· with DHT. It follows that formation of a product at a faster rate is unlikely, that rapid reactions immediately following radical growth did not occur, and that barring extraordinarily rapid reactions which take place within the system response time, the spectra observed within a few μsec after the pulse are correctly attributed to the radicals formed by the initial reactions of OH· and H· with DHT. This conclusion cannot be taken for granted. Radicals formed by some of the pyrimidine bases under certain conditions do undergo secondary reactions with half-times of the order of 1 μsec. These will be discussed in a later paper.

The spectrum obtained at pH 6.8 has a broad maximum at 410 nm, a minimum at 350 nm, and an increasing absorption toward shorter wavelengths. Radical decay takes place without change in spectral shape until the absorbance becomes too low to measure accurately. Similar results were obtained at pH 1.0, except that the

(12) (a) S. Gordon, E. J. Hart, M. S. Matheson, J. Rabani, and J. K. Thomas, *J. Amer. Chem. Soc.*, **85**, 1375 (1963); (b) L. M. Dorfman and I. A. Tomb, *ibid.*, **35**, 2370 (1963).
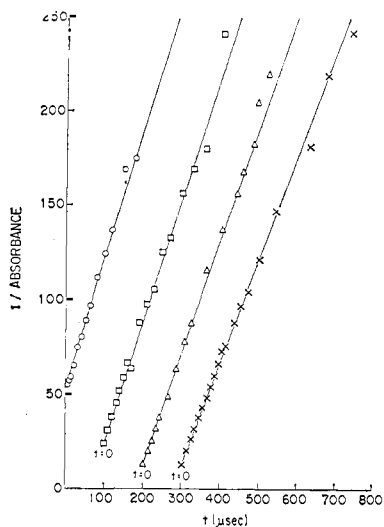
Figure 2. Decay of absorbance of radicals formed by reaction of OH· and H· with dihydrothymine at pH 6.8. The time scale is shifted 100 μsec to the right for each successive curve. Doses per pulse in rads: —O—, 800; —□—, 1830; —△—, 3340; —X—, 4000.

maximum is at a slightly shorter wavelength, 400 nm, and the minimum, initially at 320 nm, moves toward longer wavelengths as decay progresses.

Graphs of 1/OD vs. time (OD = absorbance, 8-cm light path) at wavelengths near the absorption maximum are linear to the longest times for which significant measurements could be made (Figure 2). At pH 6.8, values of the second-order rate constant for radical decay show only a slight change over a fivefold range of dose (Table I).

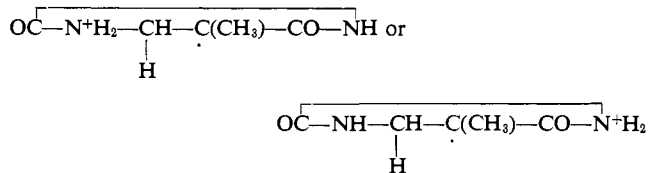Table I. Rate Constants for Decay of Transient Absorbance

pH	λ (nm)	Dose (rads)	Initial absorbance (8-cm path length)	$2k/\epsilon$
6.8	411	800	0.0185	5.4×10^6
		1830	0.0417	5.1
		3340	0.0769	4.7
		4000	0.091	4.4
			Average	$(5.0 \pm 0.4) \times 10^6$
1.0	400	1540	0.0357	2.4×10^6
		1640	0.0357	2.8
	411	1390	0.0333	2.6
			Average	$(2.6 \pm 0.13) \times 10^6$

Discussion

The spectra of Figure 1 show conclusively that at least one of the 5,6-dihydrothyminyl radicals has an absorption maximum at about 400 nm. The 5-yl radical has an unpaired electron which is conjugated with the C=O double bond on carbon atom number 4, and should absorb at longer wavelengths than the 6-yl radical

in which the electron is separated from the double bond by a saturated carbon atom. Many aromatic radicals in which conjugation is important have their longest wavelength absorption bands between 400 and 500 nm.[13] It is therefore likely that the absorption at ~400 nm is associated with the 5-yl radical.

The small differences in the spectra at pH 6.8 and 1.0 are probably caused by protonation of the radical at low pH to give

$$\overset{\begin{array}{c}\lceil\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad\rceil\end{array}}{O\overset{|}{C}—N^+H_2—CH—\overset{\cdot}{C}(CH_3)—CO—NH}\text{ or}$$
$$\overset{|}{H}$$

$$\overset{\begin{array}{c}\lceil\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad\rceil\end{array}}{O\overset{|}{C}—NH—CH—\overset{\cdot}{C}(CH_3)—CO—N^+H_2}$$
$$\overset{|}{H}$$

Thymine and uracil undergo protonation with pK values of ~0 and 0.5, respectively. The pK value for the radical should be similar. Protonation has been reported to have little effect on the spectrum of radicals[13] in the absence of tautomeric changes or changes in amount of conjugation.

The spectrum of the 5,6-dihydrothyminyl radical at pH 1 is almost identical with the spectrum reported elsewhere for the product of the reaction of thymine with H· in acid solutions. It follows that reaction 1 must give the 5,6-dihydrothyminyl (probably the 5-yl) radical as the principal absorbing species. This result confirms the previous assignment of the 400-nm absorption to this radical and shows almost certainly that electron capture by T, followed by protonation, does not give the 5-yl radical.

Values of the molar extinction coefficient of the 5-yl radical cannot be calculated from the data because the proportions of 5-yl and 6-yl radicals formed are unknown, and need not be the same for reactions 1 and 5.

The absence of any change in spectral shape during decay and the second-order decay kinetics indicate that 5,6-dihydrothymin-5-yl radicals are stable with respect to rapid intramolecular reactions and that they decay by radical-radical reactions. The rate constants for decay, calculated from values of $2k/\epsilon$ in Table I and apparent extinction coefficients of 480 at pH 6.8, and 500 at pH 1.0, give $2k = (2.35 \pm 0.15) \times 10^9\ M^{-1}\text{sec}^{-1}$ and $(1.3 \pm 0.1) \times 10^9$, respectively. Values calculated from the initial radical concentration (given by $g(\text{H·}) + g(\text{OH·}) + g(e_{aq}^-)$) and the length of the first half-period agree well. The smaller value in the acidic solution is consistent with partial protonation of the radicals because like-charged ion radicals should interact more slowly than neutral radicals.

(13) E. J. Land, *Progr. Reaction Kinetics*, 3, 369 (1965).