Radiolysis of Thymine in Aqueous Solutions: Change in Site of Attack with Change in pH

Abstract. *In the radiolysis of thymine in neutral air-saturated solutions the principal site of attack is at the 5,6 double bond; in strongly alkaline solutions the attack shifts to the 5-methyl group. The shift correlates well with a model based on two changes in the reaction path as the* p*H is increased.*

Interest in the radiolysis of thymine in dilute aqueous solutions arises from the possibility that radiation reactions on the thymine component of DNA may play an important role in radiobiological phenomena and also from intrinsic interest in the radiation chemistry of pyrimidine compounds and their heterocyclic unsaturated 6-membered rings. Thymine, in acid solution, has a double bond in the 5,6-position of the pyrimidine ring, and this double bond is conjugated with one of two oxo groups. As the *p*H is increased by addition of NaOH, the thymine isomerizes from the lactam to the lactim form and ionizes as a dibasic acid. Values of the negative logarithms of the ionization constants are $pK_1 = 9.9$ and $pK_2 > 13$ (*1*). Each of the combined isomerization-ionization steps introduces a double bond into the ring; after the second step the ring is essentially aromatic. Thus by changes in *p*H, the reactivities of three related heterocyclic structures toward the active species generated by irradiation of water may be compared (*2*).

On irradiation of weakly acidic or neutral air-saturated solutions (in which thymine is uncharged) 5,6-dihydroxythymine or 6-hydroxy,5-hydroperoxythymine is formed (*3*). The reaction is initiated by addition of hydroxyl free radical (OH·) to the 6-position of the ring, and completed by reactions at the 5-position which saturate the 5,6 double bond. Since this bond is an essential part of the chromophoric group, its saturation is accompanied by a proportionate decrease in ultraviolet absorption, and this change provides a convenient means of following the reaction. We present here the results of irradiations over a wide *p*H range extending into strongly alkaline solutions (in which most of the thymine molecules have a charge of −2). The effects were studied mainly by the techniques of ultraviolet spectroscopy and paper chromatography combined with radioautography.

Air-saturated solutions of thymine ($\sim 2 \times 10^{-4} M$) containing enough Na_2SO_4 to maintain a constant ionic strength of 1.5, and NaOH or H_2SO_4 to give the desired *p*H, were irradiated with cobalt-60 γ-rays. Dose rates were 6×10^{19} to 4×10^{20} ev per liter-minute, and total doses ranged from 7×10^{20} to 2.5×10^{21} ev per liter. Ultraviolet absorption spectra were measured on a Cary Model 11 recording spectrophotometer, and the change in peak height with radiation dose was determined for each *p*H from points on the linear portion of dose-response curves. The extinction coefficient of the thymine absorption peak for the particular *p*H was used to convert these values to initial yields for the disappearance of the chromophoric group, G(−chromophore) (points in Fig. 1). The yields are expressed in units of molecules per 100 ev absorbed. In the *p*H range of 4 to 9 there is an uncertainty of as much as ±0.5 of a *p*H unit because of a decrease in *p*H during irradiation. As the *p*H is raised above 9 this effect becomes progressively smaller. The uncertainty in the values of G(−chromophore) is about ± 0.1 unit from *p*H 4 to 12. At higher *p*H values, presumably because of the rapid change in yield with *p*H, it was extremely difficult to obtain reproducible results, and the uncertainty is ± 0.2. Fortunately this difficulty does not prevent observation of an obvious large decrease in G(−chromophore) as *p*H is increased toward and beyond 14.

Table 1. R_F values of thymine, alkaline irradiation products, 5-hydroxymethyluracil, and urea. All chromatograms except the last were run on Whatman No. 4 paper by the descending technique. The last was run on Whatman No. 1 paper by the ascending technique. UV, ultraviolet.

Thymine	UV-absorbing product	HMU	Second product	Urea
Propanol, water (86:14)				
	0.64	0.64		
Propanol, water (80:20)				
0.70	0.50	0.49	0.50	0.51
n-*Butanol, propionic acid, water (92:47:61)*				
0.72	0.51	0.51	0.65	0.66
n-*Butanol, ammonium hydroxide, water (86:9:5)*				
	0.17	0.17		
t-*Butanol, methyl ethyl ketone, water, ammonium hydroxide (40:30:20:10)*				
0.67	0.53	0.53		
Ethyl acetate, formic acid, water (70:20:10)				
0.76	0.57	0.57		
0.62	0.32	0.32		

Attempts to relate this decrease to changes in the yields (*4*) or to generally accepted values for ionization constants of primary products of water radiolysis were unsuccessful. However, there is an excellent correlation with the change in structure of thymine due to its ionization, as shown by the curve in Fig. 1. The curve was constructed as follows: the fraction of each species of thymine present was calculated as a function of *p*H; pK_1 was taken as 9.7, a value derived from the results of Shugar and Fox (*1*) by application of an approximate correction for ionic strength (*5*), and pK_2 was taken as 13.2 or 13.5, values arbitrarily selected so that the curve would pass through the point at *p*H 13.1 or the one at 14. Values of G(−chromophore) were taken as 2.1 for undissociated thymine, the observed value at *p*H 4 to 6 where thymine is undissociated; 1.3 for singly ionized thymine, the observed value at *p*H 11.6 where 98 percent of the thymine is singly ionized; and zero for doubly ionized thymine, in agreement with the trend of the data and measurements in 2*N* NaOH. Multiplication of these yields by the fraction of the corresponding species gives the contribution of the species to G(−chromophore), and addition of the contributions gives the total G(−chromophore) shown in the figure. The curve fits the points well, but a cause-effect relationship is not proven. Other sets of two processes with the same effective constants would give an equally good fit.

Any real variation in the total decomposition of thymine of the magnitude apparently observed is unlikely; it is much more reasonable that at high *p*H the direct proportionality between chromophore and thymine disappearance does not hold, and that reaction paths are being followed which have little or no effect on the chromophoric group. Accordingly, a search was made for products which have absorption spectra similar to that of thymine and, hence, which would not have been detected by our measurements.

Solutions of thymine, 2 to $20 \times 10^{-4} M$ and labeled with C^{14} in the 2-carbon or 5-methyl positions, were prepared as described, but without the addition of Na_2SO_4, and irradiated.

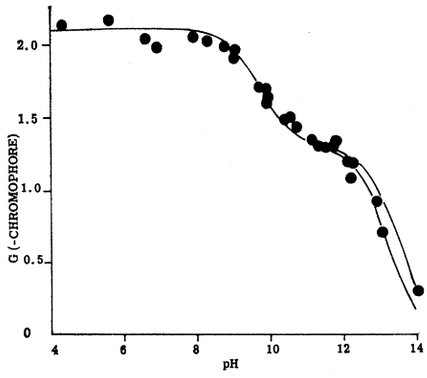

Fig. 1. Dependence of the disappearance yield of the chromophore of thymine on *p*H at an ionic strength of 1.5. Upper curve, $pK_2 = 13.5$; lower curve, $pK_2 = 13.2$. Air-saturated solutions. Units for G(—chromophore) are molecules changed per 100 ev absorbed.

Portions were chromatographed on paper (*6*) in one or two dimensions, and the distribution of radioactivity was determined by counting with a paper chromatogram scanner or by radioautography. Prior to chromatography, sodium hydroxide was removed from the 1.0 and 2.0*N* NaOH solutions by one of two procedures. In one, the samples were acidified to *p*H 1 with HCl and shaken with Norite A. Adsorbed thymine and irradiation products were eluted with a mixture of ethanol, water, and ammonium hydroxide (50:45:5 by volume), evaporated to dryness, and dissolved in water (*7*). Alternatively, solutions were neutralized by addition of the hydrogen form of the cation-exchange resin, Dowex-50.

Radioautographs of chromatograms of strongly alkaline irradiated solutions developed in two dimensions, first by a mixture of propanol and water (80:20) and then by a mixture of butanol, propionic acid, and water (92:47:61), revealed two products, neither of which is formed on irradiation of neutral air-saturated solutions. One accounts for more than half of the product radioactivity and absorbs light when illuminated by a long wavelength ultraviolet source; it moves precisely with authentic 5-hydroxymethyluracil (HMU) in cochromatographic experiments, and, when eluted from the paper, has an ultraviolet spectrum indistinguishable from that of HMU. Further evidence of its identity was obtained by chromatography with mixtures of *t*-butanol, methyl ethyl ketone, water, and ammonium hydroxide (40:30:20:10), and ethyl acetate, formic acid, and water (70:20:10). In each solvent the unknown behaved as expected for HMU, and there was no indication of uracil-5-carboxylic acid or 5-formyluracil. Formation of HMU is evidently initiated by attack on the 5-methyl group of thymine by the OH· free radical, or by $O^{\cdot -}$, the oxygen radical ion which results when OH· ionizes. 5-Hydroxymethyluracil has also been reported as a product of irradiation in the absence of air in solutions of neutral thymine (*8*) and as a product of thymine metabolism in liver slices and *Neurospora* (*9*).

The lesser product, which accounts for the bulk of the remaining product activity, moves precisely with urea in cochromatographic experiments (Table 1). It is probably formed by a series of reactions starting with addition of a free radical and possibly oxygen to the double bond in the ring and ending with alkaline hydrolysis. Supporting this idea are the observations that the saturated hydroxyhydroperoxide and dihydroxy derivatives of thymine give urea on standing in alkaline solution and that the reaction is accelerated by resin or charcoal. Ureido compounds were not found in appreciable amounts on any of the chromatograms of alkaline solutions.

In the range *p*H 10 to 13, the amounts of HMU and of compounds formed by addition to the double bond are intermediate between those found in neutral and in strongly alkaline solutions.

These results show clearly that as the *p*H is increased the major site of attack in the radiolysis of thymine in air-saturated aqueous solutions shifts from the double bond in the ring at neutral *p*H to the 5-methyl group in alkaline solutions. This shift can be correlated with the ionization of the thymine molecule or with other two-step changes in the system. Ionization is an important possibility for at least one of these steps because it leads to an increase in aromatic character of the pyrimidine ring and therefore to a decrease in reactivity of the 5,6 double bond activation of the methyl group. Free radicals and radical ions consequently would tend to react with the methyl group when thymine is ionized.

The possible significance of these findings to radiobiology should not be overlooked. The electron configuration around thymine in a DNA molecule is not known and radiation-induced reactions characteristic of the enol form may well occur and lead to formation of the 5-hydroxymethyl derivative. The presence of this group would probably not necessitate any change in the configuration of the DNA molecule, but might well interfere with or modify attempted replication (*10*).

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Immunoadsorbent for the Isolation of Purine-Specific Antibodies

Abstract. *An immunoadsorbent for the isolation of purine-specific antibody has been synthesized. The technique is applicable for isolating antibody to any purine or pyrimidine capable of being converted to a derivative which can be coupled to amine groups. The recovery of antibody from a serum sample is better than 82 percent, and the precipitability of the isolated antibody is as high as 89 percent.*

Since 6-halomethylpurines and pyrimidines have been synthesized (*1*), these bases have been coupled to carrier proteins, and antibodies specific to these groups which show cross-