Whitney Gallery of Western Art, Cody, initiated excavations supervised by R. Edgar. W. R. Wedel was consultant, and J. H. Moss undertook geological studies. In 1964, the National Geographic Society and private donors supported an expanded operation for which Dr. W. Mulloy provided guidance. In 1966, W. M. Husted studied the artifacts and records, and prepared the archeological report. In 1967, additional information was obtained from a trench dug to bedrock with power equipment financed by National Science Foundation grants to Franklin and Marshall College. No evidence of human occupation was found below layer 1. Pollen from the cave was studied by H. E. Wright; vertebrate remains by A. H. Harris.

11 March 1968

Thymine-Thymine Adduct as a Photoproduct of Thymine

Abstract. *A product isolated from thymine irradiated with ultraviolet light in frozen aqueous solution undergoes dehydration on heating with acids. As judged by elemental analysis, mass, ultraviolet, infrared, and nuclear magnetic resonance spectra, the most probable structures for this compound and its dehydration product, respectively, are 5-hydroxy-6-4′-[5′-methylpyrimidin-2′-one]-dihydrothymine and 6-4′-[5′-methylpyrimidin-2′-one]-thymine. Apparently, this compound is a thymine-thymine adduct and presumably is formed through the rearrangement of an initial photoproduct. Both compounds are closely related to 6-4′-[pyrimidin-2′-one]-thymine which has been isolated from acid hydrolyzates of ultraviolet-irradiated DNA and supposedly is derived from cytosine-thymine adduct. Formation of such adducts between pyrimidine bases is apparently a common photoreaction and may be important to the study of the photochemistry and photobiology of nucleic acids.*

A thymine-derived product having an absorbancy maximum of 316 nm has been separated from acid hydrolyzates of ultraviolet-irradiated DNA (*1*); 6-4′-[pyrimidin-2′-one]-thymine (PO-T) has been suggested as the most probable structure (*2*). We have isolated a closely related product from thymine irradiated, in frozen aqueous solution, with ultraviolet light. Elemental analysis and mass spectroscopy indicate that this compound is an adduct of thymine and thymine which is unstable and undergoes dehydration on heating with acids. This thymine-thymine adduct presumably is formed through the rearrangement of an initial photoproduct. Based on ultraviolet, infrared, nuclear magnetic resonance, and mass spectra, 5-hydroxy-6-4′-[5′-methylpyrimidin-2′-one]-dihydrothymine and 6-4′-[5′methylpyrimidin-2′-one]-thymine can be assigned as the most probable structures for the thymine-thymine adduct (T-T adduct) and dehydration product (MPO-T), respectively.

The isolation of T-T adduct was carried out by irradiation of twice-recrystallized thymine (2 mmole/liter) in frozen aqueous solution for 1 hour (*3*). The thawed solution was concentrated to dryness and dissolved in a minimum amount of 0.1*N* HCl; the solution was applied on Whatman No. 3 paper and developed with an *n*-butanol, acetic acid, and water system (80 : 12 : 30). The dried chromatogram showed by ultraviolet lamp a fluorescent band with an R_F value of 0.29, identical to that of thymine dimer (*4*). The fluorescent bands were cut out; the material was extracted three times with water. The extract was concentrated and applied to a column of Dowex 50W-X12 (H^+, 100 to 200 mesh). The column was eluted with water. The eluent (316-nm absorbancy maximum) was collected and evaporated to dryness. Two recrystallizations from water gave colorless cubes (about 2 percent); melting point, 265° to 270°C, with decomposition.

The T-T adduct was dehydrated by refluxing it (25 mg) for 90 minutes in 50 ml of 0.5*N* HCl. The solution was concentrated and chromatographed on Whatman No. 3 paper with an *n*-butanol, acetic acid, and water system (80 : 12 : 30) as eluent. The major product (R_F=0.5) was rechromatographed with a *t*-butanol, methyl ethyl ketone, ammonia, and water system (40 : 30 : 10 : 20), and it appeared as a single band with the R_F value of 0.45; this material was extracted with absolute methanol. After recrystallization with absolute ethanol, the product (16 mg) melted, with decomposition, above 300°C. The dehydration product can also be obtained by heating the T-T adduct for 1 hour at 170°C with trifluoroacetic acid in a sealed tube, or by heating it above its melting point.

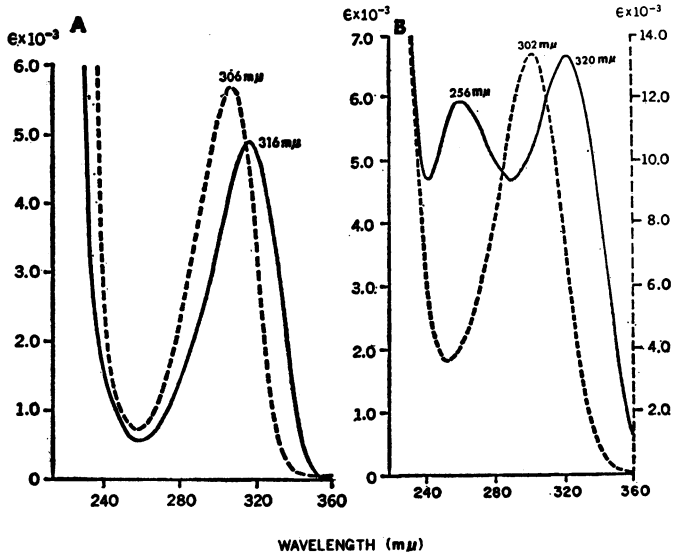


Fig. 1. The ultraviolet spectra (A) of the T-T adduct and (B) of the dehydration product of T-T adduct.

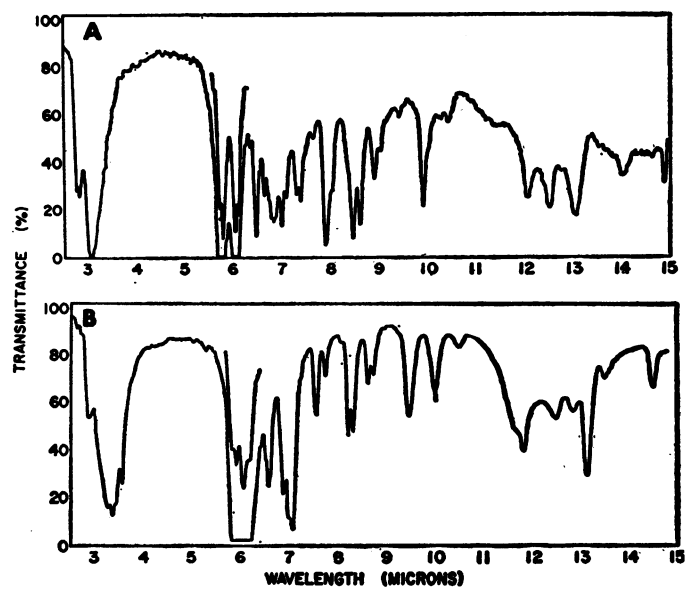


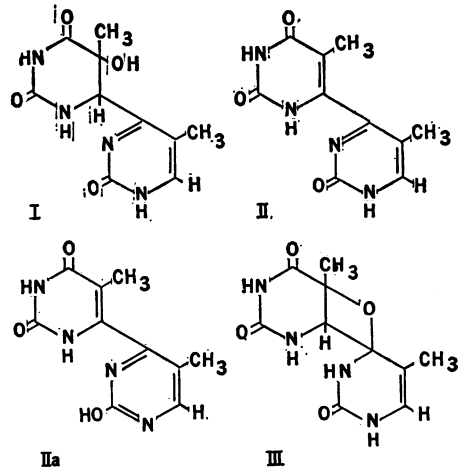
Fig. 2. The infrared spectra (A) of the T-T adduct and (B) of the dehydration product of the T-T adduct.

The ultraviolet spectrum of the T-T adduct has absorbancy maximums at 316 nm (E=4940) in aqueous solutions at neutral pH and at pH 2, and 306 nm (E=5680) at pH 12 (Fig. 1A); these maximums are characteristic of the chromophore of pyrimidin-2-one (PO) and its derivatives (*5*). The dehydration product (MPO-T) has two absorbancy maximums in water and in 0.1N HCl, one at 256 nm (E = 6000) and the other at 320 nm (E = 6650), and only one in 0.1N NaOH, at 302 nm (E = 13,300) (Fig. 1B). This suggests that the new chromophore formed by dehydration may not interact with the chromophore of PO, the two components of the molecule being in a skew conformation.

That the infrared spectrum of the T-T adduct (Fig. 2A) has OH bands at 2.77 and 2.83 μ, whereas MPO-T (Fig. 2B) has only one, at 2.88 μ, suggests the loss of water on dehydration. The bands at 5.73 and 5.89 μ, usually observed in saturated thymine derivatives (*6*), are present in the T-T adduct. These bands are absent in the spectrum of MPO-T, which has bands at 5.75 and 5.90 μ, as observed in the thymine spectrum (*7*). The bands at 6.05 and 6.12 μ are characteristic of PO (*5*) and remain unchanged for both compounds.

The mass spectrum of the T-T adduct shows a low-intensity molecular-ion peak at 252, corresponding to the molecular weight of $C_{10}H_{12}O_4N_4$. The relatively high intensity peak at m/e (mass/charge) 234 suggests that the T-T adduct loses fragments of mass 18 (H_2O) quite readily. Indeed, the mass spectrum of the dehydration product shows a molecular-ion peak at 234, corresponding to the molecular weight of $C_{10}H_{10}O_3N_4$, and resembles that of the T-T adduct. The peaks at m/e 219 (M-15) and m/e 217 (M-17) and the metastable ions at m/e 205 and m/e 201.2 suggest cleavage of a methyl group and a hydroxyl group from an aromatic moiety. Two pathways, one from 219 and the other from 217, can be discerned in the complicated fragmentation pattern, each of which is perfectly analogous to that known for a 5,6-disubstituted uracil (*8*). Furthermore, the fragment ion at m/e 109, its demethylated fragment ion at m/e 94, and the dehydroxylated-ion peak at m/e 92 support the presence of the PO moiety.

Thus, the foregoing evidence supports the structures 5-hydroxy-6-4′[5′-methylpyrimidin-2′-one]-dihydrothymine for T-T adduct (I) and 6-4′-[5′-methylpyrimidin-2′-one]-thymine for dehydration product (II).



I II IIa III

The nuclear magnetic resonance spectrum of T-T adduct in deuterated dimethyl sulfoxide $(CD_3)_2SO$ at 100 Mc/sec shows strong singlets at δ (ppm from TMS) 1.848 (3H) and δ 2.356 (3H), indicating the presence of two methyl groups. A peak at δ 4.696 (1H, doublet) which is coupled with an NH proton at δ 7.900 (1H, doublet) with spin-spin coupling constant equal to 2 cycle/sec is due to the proton at C-6. This signal becomes a singlet at δ 5.332 in deuterated trifluoroacetic acid. A sharp peak at δ 7.967 (1H, singlet) indicates a vinyl proton. A signal at δ 5.996, which disappears in deuterated trifluoroacetic acid, is probably due to an OH proton. Two NH protons appear as singlets at δ 10.37 and at δ 11.94. Therefore, the data agree with the structure I. The spectrum of MPO-T in $(CD_3)_2SO$ at 100 Mc/sec shows that the strong singlets at δ 1.905 (3H) and at δ 2.264 (3H) are from two methyl groups. The presence of a vinyl proton is indicated by a singlet (1H) at δ 8.468. Two broad peaks at δ 11.32 and 11.60 are due to two NH groups. Thus, this spectrum conforms to structure II. The absence of a third peak for the NH group suggests that one of the three lactam groups exists as a lactim. This is supported by the presence of an OH band at 2.88 μ in the infrared spectrum of II. Also, the cleavage of an OH group from the PO moiety is indicated in the mass spectrum, as discussed above. These data suggest that PO probably exists as an aromatic nucleus (structure IIa).

To explain the formation of a T-T adduct from thymine, one may consider the possibility of an oxetane derivative (III) or related compound, formed by bimolecular photocycloaddition of simple carbonyls to olefins (*9*) or of two α,β-unsaturated keto derivatives (*1*, *10*). Such intermediates form various rearranged products by acid catalysis (*11*).

The T-T adduct should be responsible for the absorbancy at 320 nm observed in the thawed solution of thymine irradiated in ice (*4*). The photoproduct of thymidylyl-thymidine (TpT) described as TpT[4] by Pearson *et al.* (*12*) is probably a derivative of T-T adduct.

The observed increase in absorbancy at 320 nm in ultraviolet-irradiated DNA without further treatment and the isolation of PO-T in relatively large amounts under certain conditions suggest the formation of the photoinduced cytosine-thymine adduct in DNA (*1*, *2*). Now, the characterization of the T-T adduct (analogous to cytosine-thymine adduct) from ultraviolet-irradiated thymine and its dehydration product (analogous to PO-T) emphasizes that the formation of such adducts between various pyrimidine bases is a common photoreaction and may be of considerable significance in the study of the photochemistry and photobiology of nucleic acids.

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