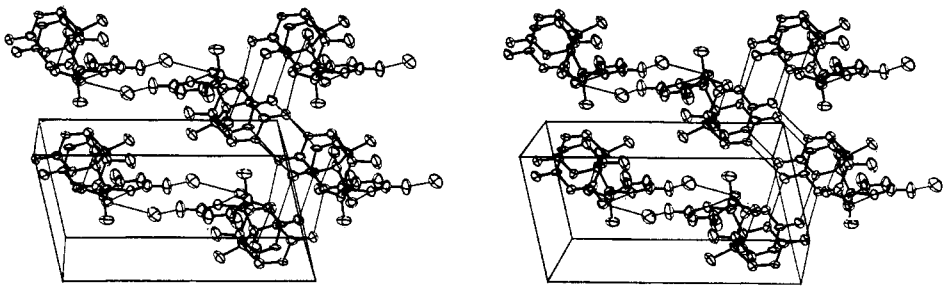

Figure 4. Another view of the packing, viewed at right angles to Figure 4. The axial directions are: c, ↑ ; b, →; and a, out of the paper.

in thymine·H_2O where each C=O is bonded to the NH from two adjacent molecules. However, these double strands occur in two layers, joining ring I to ring II′ and ring II to ring I′ of the molecules related by the centers of symmetry at $^1/_2$, 1, $^1/_2$ and $^1/_2$, 1, 1 as illustrated in Figures 3 and 4. Thus a continuous channel along the c axis is enclosed by the four independent bonds, N(1)H···O(12′), O(12′)···HN(3″), N(11)H···O(2′), O(2′)···HN(13″) and their symmetry equivalents.

In the trimer crystal there was no evidence of dissociation of the dimer moiety in the X-ray beam during the diffraction experiment. The final difference map was featureless in this area. Dissociation of the cis-syn dimer into monomers in the crystalline state upon X irradiation had been noted for the crystals of A,[6] dimethylthymine dimer,[4] and the Na salt of the *cis-syn*-thymine dimer.[26]

(26) C. H. Wei and J. R. Einstein, Abstracts, American Crystallographer's Association, Buffalo, N. Y., July 1968, paper L9, p 102.

Thymine Phototrimer[1]

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Contribution from the Department of Biochemistry, School of Hygiene and Public Health, The Johns Hopkins University, Baltimore, Maryland 21205. *Received September 23, 1970*

Abstract: Thymine phototrimer has been isolated from thymine irradiated in a frozen aqueous solution with 254-nm light. The new photoproduct, which presumably forms through the ring opening of an initial oxetane derivative, has been characterized by uv, ir, nmr, and mass spectra. The spectroscopic evidence confirms the results of X-ray diffraction analysis of a single crystal. It contains a 1,3-diol structural element, which is responsible for many of the intriguing reactions observed. One of these reactions is the formation of monomeric thymine from the trimer by acid or base catalysis. The possible importance of the 1,3-diol structure or the reversion reaction in the photochemistry and photobiology of nucleic acids is pointed out.

Observations from this laboratory showed that when a native DNA solution (40 μg/ml of 0.15 *M* NaCl) was irradiated (mainly 254-nm light) at a dose rate of 110 ergs/(mm² sec) for 30, 60, 90 sec, etc., there was a gradual decrease in the absorbancy at 260 nm with a simultaneous increase in the absorbancy in the 300–350-nm region (cited in ref 2). Dulbecco[3] reported that the absorption spectrum of phage T_2 particles undergoes complex changes with uv irradiation. He observed a general decrease, rather than an increase, in absorption in wavelengths longer than 320 nm. However, after the first hour of irradiation, a faint band was noticed; it became more evident during the second hour. This band has maximum absorption around 330 nm and extends to about 380 nm. Both experiments showed an apparent increase in absorbancy at wavelengths longer than 320 nm but their characteristics and temporal sequences were quite different. Moreover, the data obtained in this laboratory clearly showed that this spectral increase is the direct result of irradiation of native DNA; it cannot be an artifact resulting from the method of assay. The chemical event associated with such a change must assume some roles in biological systems. Thus, an investigation was undertaken of the chemistry responsible for this change. Earlier reports[4,5] suggested that thymine dimer (T=T) is the sole product of uv irradiation of DNA. Our study revealed that the *above defined* "thymine dimer" is in fact a mixture of cis-syn T=T (P_2A)[6] and the deaminated cytosine–

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(2) A. J. Varghese and S. Y. Wang, *Science*, 156, 955 (1967) (also cited by K. C. Smith in "Photophysiology," Vol. II, A. C. Giese, Ed., Academic Press, New York, N. Y., 1964, p 329).

(3) R. Dulbecco, *J. Bacteriol.*, 59, 329 (1950).

(4) R. Beukers and W. Berends, *Biochim. Biophys. Acta*, 41, 550 (1960); A. Wacker, H. Dellweg, and E. Lodeman, *Naturwissenschaften*, 47, 477 (1960).

(5) R. B. Setlow, *Science*, 153, 379 (1966).

(6) A. J. Varghese and S. Y. Wang, *Nature (London)*, 213, 909 (1967).

thymine adduct (P_2B).[7] The ratio of P_2A/P_2B was approximately 9/1 at all doses (4800–28,800 ergs/mm^2) as assayed from the acid hydrolysates of uv-irradiated *Escherichia coli* 15 T$^-$ cells.[2] The characterization study[7] indicated that deaminated cytosine–thymine adduct actually results from vigorous conditions of acid hydrolysis of uv-irradiated DNA and is not the product formed directly upon irradiation. Therefore, our effort was engaged in the isolation and characterization of its elusive precursors from the irradiation of a uracil–thymine mixture[8] and a deoxycytidine–thymidine mixture (unpublished results) in a frozen state. These precursors have absorbancy maxima in the 300–350-nm region, as does the thymine–thymine adduct.[9] However, these adducts provide no structural element for their direct reversion to the monomers. We are interested in this reversion reaction because it resembles and might account for the biologically important "repair processes"[10] of radiation lesions in DNA or biological systems. The new thymine phototrimer has a 1,3-diol moiety, a structural element which can give rise to a monomer thymine. This diol structure, therefore, may be of great importance in photo- and radiation biology. Since a trimeric photoproduct of this kind is uncommon, it should also be of general interest in organic photochemistry.

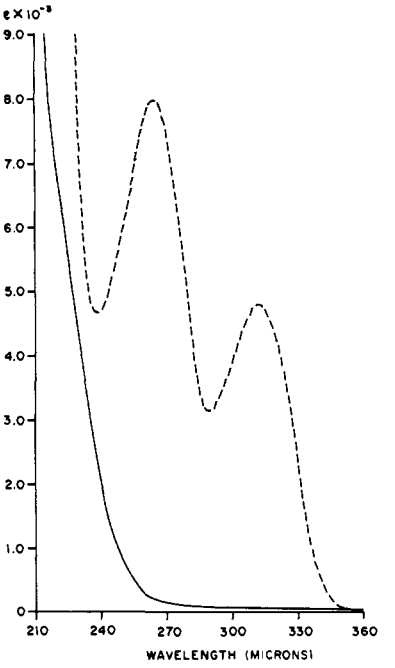


Figure 1. Uv spectra of thymine phototrimer in water (——) and after irradiation with 254-nm light (– – – –).

Experimental Section

Detection and Percentage Yields of Trimer from Thymine Irradiated in Frozen Aqueous Solution. Twice-recrystallized thymine was dissolved in distilled water (0.5 mg/ml) and thymine-2-C^{14} was added to give ~1.5 × 10^7 cpm/ml [50 μCi in 6 ml of thymine solution]. Each 0.2-ml portion of the solution was frozen in a depression of a porcelain spot plate placed on a Dry Ice bath. These samples were irradiated for 30 min at a dose rate of ~20 ergs/(mm^2 sec). The thawed solution was applied on a 1.5-in. wide strip of Whatman no. 3 paper and was developed with eluent A (*n*-butyl alcohol–acetic acid–water, 80:12:30). The dried chromatograms were analyzed for distribution of radioactivity with a Vanguard Autoscanner 880. Three radioactive peaks were detected having R_f values of 0.60 (A), 0.29 (B), and 0.15 (C), corresponding to thymine, a mixture of T=T and T—T adduct, and trimer, respectively. Peak areas corresponding to B and C were rechromatographed. Each peak area was then cut out and eluted with water. The eluate of B was diluted to 50 ml and the two others to 20 ml. Samples of 0.2 ml of A, 0.1 ml of B, and of C were evaporated in the vials until dry. The entire sample was taken up in each vial with 10 ml of scintillation solution (ref 11 without ethylene glycol). Triplicate samples of each were counted in the vials for intervals of 1 or 2 min in a Nuclear-Chicago Model 723 scintillation spectrometer. The counting efficiency was 75% determined by internal standardization using standard toluene samples.

Effect of 254-nm Light on Trimer. The above obtained trimer (1.5 × 10^7 cpm/ml) in aqueous solution was irradiated for 5 min in a cuvette. Portions of 0.2 ml were applied on paper for chromatography and then radioactive counting of A, B, and C, as in the above procedure. For isolation of T–T adduct, a solution of the trimer (14 mg/300 ml) was irradiated for 10 min with 254-nm light. The solution was concentrated, applied on a 1 × 12 cm Dowex 50 W-X12 (H^+, 100–200 mesh) column, and eluted with distilled water. Fractions of 20 ml each were collected and uv spectra were taken. Fractions 13–20 gave spectra typical of the T–T adduct and were combined. The residue (4.8 mg), after recrystallization twice from water, gave colorless cubes. The ir spectrum of this product in a KBr pellet was identical with that of the known T–T adduct.[9]

Isolation of Thymine Phototrimer. An aqueous solution of thymine (1 g in 4 l.) was irradiated with G.E. germicidal lamps in an irradiator[12] for 60 min in a frozen state (8 mm in thickness). The thawed solution was evaporated at below 40° to a volume of ~2 ml with periodic filtration to remove insoluble precipitate, thymine dimer. This concentrated solution was applied on Whatman no. 3 paper and developed with 90% methanol. The dried chromatograms were cut into sections and eluted with water. The trimer fraction displayed the characteristic spectral changes before and after irradiation as shown in Figure 1. The material of this fraction was eluted with 90% methanol for 2 days and the eluate was concentrated to ~1 ml. After filtration, 4 ml of absolute methanol was added. The resulting solution was allowed to stand at 5° overnight. Pale yellow crystals (~15 mg) were obtained and were purified by rechromatography. Upon concentration of the eluate, colorless needlelike crystals formed.

Stability of Trimer in Water. Trimer in crystalline form is stable. However, a 10^{-4} *M* aqueous solution of trimer showed an increase in uv absorption at 316 and 264 nm, again indicating the formation of T–T adduct and thymine. There was about 10 and 14% conversion, as estimated with the extinction coefficient, when the solution was allowed to stand for 2 and 3 days, respectively.

Effect of 2 *N* HCl on Trimer at Room Temperature. A solution of trimer in 2 *N* HCl gave a uv absorption maximum at 285 nm. Upon irradiation, the absorption maxima at 316 and 264 nm appeared in an identical manner as observed with trimer in water, described above. After neutralization, similar procedures were used for the identification of compounds.

Effect of 1 *N* NH_4OH on Trimer. Trimer containing ^{14}C labeling was dissolved in 1 *N* NH_4OH (~1 × 10^6 cpm/ml) and was allowed to stand at room temperature for 48 hr. Portions of 0.2 ml of the solution were applied on paper for chromatography as described above. Three radioactive peaks were detected having R_f values of 0.60, 0.29, and a very broad one (R_f 0.1–0.2) which somewhat overlapped the 0.29 peak. The peak areas of R_f 0.60 and 0.29 were about 10% of the total.

Isolation of cis-syn T=T was carried out with a trimer solution (15 mg /500 ml of 1 *N* NH_4OH) having stood at room temperature for 72 hr. The solution had an absorption peak appearing at 244 nm. Upon concentration, the solution was applied on Whatman no. 3 paper, and was developed with eluent A. The dried chromatogram was cut into 1-in. strips and the material was eluted with water. The eluate obtained from the R_f 0.60 area gave uv spectra characteristic of thymine. The eluates from strips 4, 5, and 6 (area between 3 and 6 in.) were combined and rechromatographed.

(7) S. Y. Wang and A. J. Varghese, *Biochem., Biophys. Res. Commun.*, 29, 543 (1967).
(8) D. F. Rhoades and S. Y. Wang, *Biochemistry*, **9**, 4416 (1970).
(9) A. J. Varghese and S. Y. Wang, *Science*, **160**, 186 (1968).
(10) J. Jagger, "Ultraviolet Photobiology," Prentice-Hall, Englewood Cliffs, N. J., 1967.
(11) G. A. Bray, *Anal. Biochem.*, **1**, 279 (1960).
(12) S. Y. Wang, *Nature (London)*, **190**, 690 (1961).

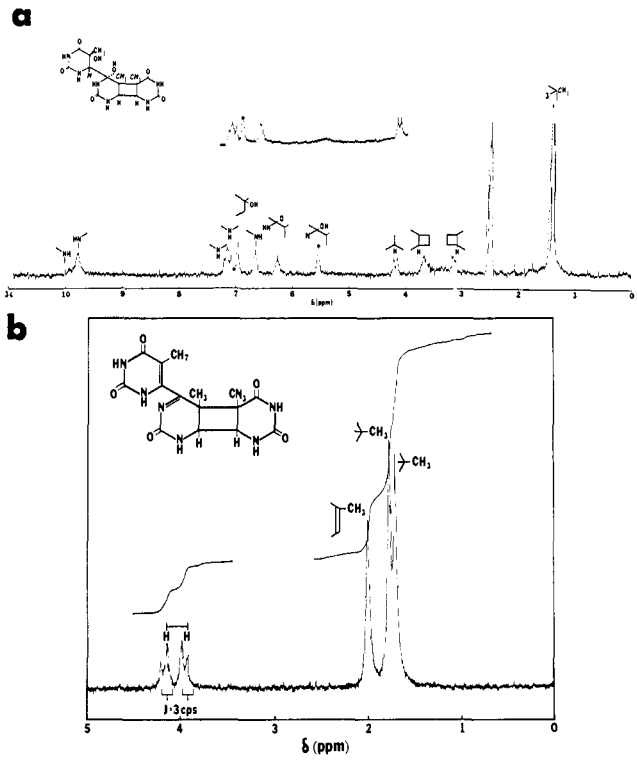


Figure 2. Nmr spectra of (a) thymine phototrimer and its dehydration product VII and (b) compound VIII.

Then, the area corresponding to R_f 0.29 was cut from the chromatogram and eluted with water. After evaporation, the dried residue (0.7 mg) in a KBr pellet gave an ir spectrum identical with that of known cis-syn T=T.

Effect of 2 *N* HCl on Trimer at Refluxing Temperature. Trimer was dissolved in 2 *N* HCl (15 mg/25 ml) and was refluxed for 15 min. The solution was evaporated until dry and the residue was pink in color. A small amount of the residue was redissolved in water and applied on Whatman no. 3 paper. After developing with ether–acetic acid–water (13:3:1) solvent system, the dried chromatogram was cut into 2-cm strips. The material was eluted with water and the uv spectra of the eluates were taken. The following uv-absorbing products were detected: R_f 0.34 (λ_{max} 267 nm), 0.42 (267 and 325 nm), 0.60 (267 nm), and 0.72 (267 and 325 nm). The main product had an R_f 0.34. The products with R_f 0.60 and 0.72 had the same characteristic uv spectra as thymine and dehydrated T–T adduct, respectively. The remainder of the residue was chromatographed on paper with the *tert*-butyl alcohol–methyl ethyl ketone–formic acid–water (40:30:15:15) solvent system. The main product again had an R_f of 0.35. This portion was cut from the dried chromatograms, eluted with water, concentrated, and rechromatographed. The compound was then extracted with water and evaporated until dry. The residue was recrystallized from absolute methanol as a faint yellow powder. It melts at 260° dec and is stable when irradiated with 254-nm light. The nmr spectrum was taken and the molecular weight found to be 360 (mass spectrum).

Results and Discussion

Ultraviolet irradiation of [2-^{14}C]- or [5-*methyl*-^{3}H]-thymine (I) in a frozen aqueous solution produces two detectable bands[13-15] in addition to that of thymine. One band has R_f 0.15 in the *n*-butyl alcohol–acetic acid–water (80:12:30, by volume) paper chromatogram; the other has R_f 0.29.[14] Contrary to the general belief that the material of R_f 0.29 band consists only of T=T,[4,5] it is in fact a mixture of cis-syn T=T (II) and thymine–thymine adduct (T–T adduct, III).[9] The eluate from the R_f 0.15 band, however, has been isolated as a single compound (designated as trimer) with intriguing properties.[16] It has the spectral characteristics of the T–T adduct when treated with acid and base, the photoreversibility of T=T (see reviews[17-19]), and the instability of thymine photohydrate IV.[20]

In a frozen state, a 4 m*M* aqueous solution of thymine irradiated with a dose of 3.6×10^4 ergs/mm^2 yielded 6% of trimer (average total counts per 2 min, 1.8×10^6) and 80% of R_f 0.29 mixture (25×10^6); the unreacted thymine amounted to 14% (4.5×10^6). When trimer (1.5×10^7 cpm/ml) was irradiated for 5 min in aqueous solution, about 50% of trimer (average total counts per 2 min, 3×10^6 at R_f 0.15) was treated to give T–T adduct (2×10^6 at R_f 0.29) and thymine (1×10^6 at R_f 0.60). The radioactivity counting data indicated that the T–T adduct and thymine were formed in equimolar proportions.

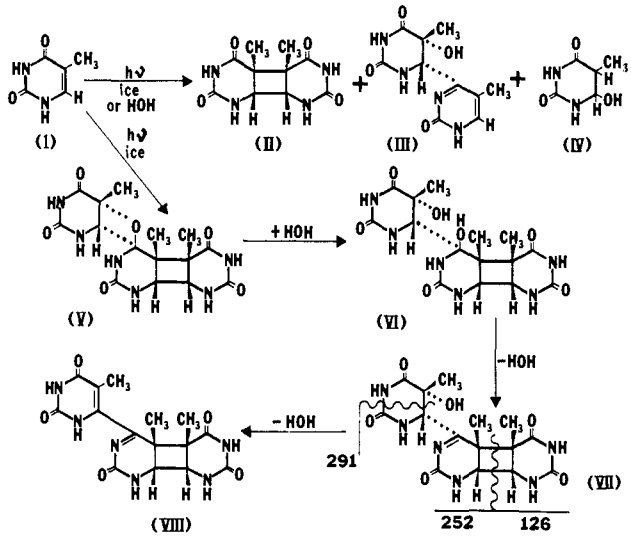
In order to further our understanding of the trimer and also the actual identification of the T–T adduct obtained from the above experiment, the isolation of thymine phototrimer was carried out. The T–T adduct obtained from the irradiation of a 1.2 m*M* solution of trimer was found to be identical with that of the known isomer. Thus, a trimer molecule is a combination of the cis-syn T=T II and T–T adduct III. The X-ray crystal-structure analysis[21,22] has established the molecular structure and stereoconfiguration of this compound as a "hydrated trimer of thymine" with the additional OH and H moieties as shown in VI.

The uv spectrum of the trimer in water exhibits only end absorption as expected. Its ir spectrum in potassium bromide pellets lacks OH bands characteristic of C(5)–OH (2.95 μ) or C(6)–OH (2.99 μ) of saturated pyrimidines[23] or the strong OH band at 2.83 μ observed of the T–T adduct.[9] Accordingly, the OH group is stereochemically favored to form a strong H bond with another, resulting in a shift to the 3.1–3.5-μ region. This shift has been observed with several other photoproducts recently identified.[8] This finding conforms with that of the X-ray analysis.[21,22] The data from the nmr spectrum at 100 Mcps (Figure 2a) [($(CD_3)_2SO$) 1.33 from TMS (s, CH_3), 1.35 (s, CH_3), 1.36 (s, CH_3), 3.11 and 3.64 (broad, two cyclobutyl protons), 4.15 (s, CH), 5.51 (s, OH), and 6.92 ppm (s, OH), and six NH signals] comply with structure VI. After prolonged standing the proton signals at 5.51 (OH) and 6.32 ppm (NH) nearly disappeared, indicating that dehydration of the diol VI takes place to give VII. In other aspects, the two spectra are identical. Dehydration of VI in trifluoroacetic acid or 2 *N* HCl at refluxing temperature yields VIII. Its nmr data (CF_3COOD) (Figure 2b) are

(13) K. C. Smith, *Photochem. Photobiol.*, 2, 503 (1963).
(14) S. Y. Wang, A. J. Varghese, M. Patrick, and C. S. Rupert, *Proc. Nat. Acad. Sci. U. S.*, 57, 465; 58, 2483 (1967).
(15) R. O. Rahn and J. L. Hosszu, *Photochem. Photobiol.*, 10, 131 (1969).
(16) A. J. Varghese and S. Y. Wang, *Biochem. Biophys. Res. Commun.*, 33, 102 (1968).
(17) S. Y. Wang, *Fed. Proc., Fed. Amer. Soc. Exp. Biol.*, 24, S-71 (1965).
(18) H. L. Gunther and W. H. Prusoff, *Methods Enzymol., Part A*, 12, 19 (1967).
(19) E. Fahr, *Angew. Chem., Int. Ed. Engl.*, 8, 578 (1969).
(20) S. Y. Wang, *Nature (London)*, 184, B.A. 59 (1969).
(21) J. L. Flippen, I. L. Karle, and S. Y. Wang, *Science*, 169, 1084 (1970).
(22) J. L. Flippen and I. L. Karle, *J. Amer. Chem. Soc.*, 93, 2762 (1971).
(23) S. Y. Wang, *ibid.*, 78, 4180 (1956); 80, 6196 (1958).

as follows: 1.72 (s, CH_3), 1.78 (s, CH_3), 2.01 (s, vinyllic CH_3), and 3.96 and 4.18 ppm (d, with J = 3 Hz (two cyclobutyl protons)). They are consistent with structure VIII, which is an asymmetric molecule with a syn or 1,2 arrangement of the cyclobutyl ring.[24,25] The mass spectrum of the trimer was obtained using the field ionization technique.[26] The molecular ion peak appears at *m/e* 378. The additional peak at *m/e* 379 is compatible with the occurrence of relatively high intensity M + 1 peaks under the conditions required to obtain mass spectra in this class of compounds;[27] it complies with structure VII. Dehydration of VI to form VII is to be expected at the elevated temperatures (>200°) used for the determinations. The detailed analysis is reported elsewhere[26] but the main features of the fragmentation patterns are shown in Scheme I.

Scheme I



Some of the intriguing reactions observed with the trimer can now be readily explained in terms of the assigned structure VI. Examples follow.

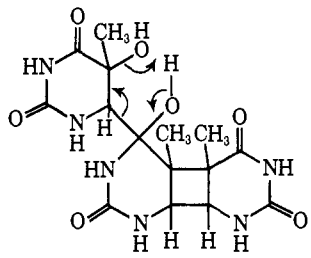
$$\text{VI} \xrightarrow[\text{HOH}]{h\nu} \text{T-T adduct III} + \text{T I} \qquad (1)$$

$$\text{VI} \xrightarrow[\text{room temp}]{2\ N\ \text{HCl}} \lambda_{max}\ 285\ \text{nm VII} \xrightarrow[\text{HOH}]{h\nu} \text{III} + \text{I} \qquad (2)$$

$$\text{VI} \xrightarrow{1\ N\ NH_4OH} \text{II} + \text{I} + \text{one other product} \qquad (3)$$

$$\text{VI} \xrightarrow[\text{refluxing}]{2\ N\ \text{HCl}} \lambda_{max}\ 267\ \text{nm VIII} + \text{I} + \text{III} \qquad (4)$$

In reaction 1, the trimer is irradiated with 254-nm light in aqueous solution. It results in the cleavage of the cyclobutane ring. Therefore, thymine and the hydrated T-T adduct should be formed. However, the latter is unstable and dehydration occurs spontaneously to give the T-T adduct. In reaction 2, acid-catalyzed dehydration converts the trimer to VII, which has an absorbancy maximum at 285 nm. Upon irradiation, VII gives thymine and the T-T adduct directly. In reaction 3, base-catalyzed decomposition of the trimer yields T=T and thymine as shown. In reaction 4, three separate chemical events must take place concurrently. First, the trimer is further dehydrated to VIII at refluxing temperature as compared to eq 2; VIII has an absorbancy maximum at 267 nm. Second is the cleavage of the cyclobutyl ring to give the T-T adduct. The third event is the possible acid-catalyzed decomposition of the trimer to form thymine, as shown.



Apparently, the 1,3-diol structure is important in many of these reactions. However, this trimer, with the diol structure, is probably not the photoproduct formed directly upon irradiation of thymine. The initial product might be an oxetane V. Derivatives of oxetane are particularly susceptible to attack by reagents, which results in ring opening.[28] The formation of such a diol by the attack of solvent molecules on dimeric adducts has been considered.[8]

The possible significance of this diol type of compound in photobiology is currently under study. Such a compound has a unique structure, which would conceivably allow the reversion of an adduct to its original monomers. If such a reaction involves a cytosine moiety as one of its bases (as in P_2B), deamination may occur, resulting in the transformation of a cytosine to a uracil moiety. Such a change should be examined in relation to the observed biological mutation induced by radiation.

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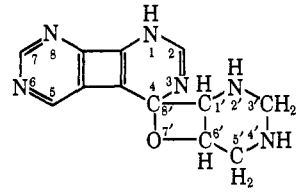

[1,2-*d*:4,3-*d'*]dipyrimidine-4(1*H*),8'-[7]oxa[2,4]diazabicyclo[4.2.0]octane]; the oxetane derivative V, tetrahydro-4a,4b,6'-trimethylspiro[cyclobuta[1,2-*d*:4,3-*d'*]dipyrimidine-4(1*H*),8'-[7]oxa[2,4]diazabicyclo[4.2.0]octane-2,3',5,5'7(3*H*,4b*H*,6*H*)-pentone; the diol derivative VI, 5-(hexahydro-5-hydroxy-5-methyl-2,6-dioxo-4-pyrimidinyl)octahydro-5-hydroxy-4a,4b-dimethylcyclobuta[1,2-*d*:4,3-*d'*]dipyrimidine-2,4,7(3*H*)-trione, etc.

(24) D. P. Hollis and S. Y. Wang, *J. Org. Chem.*, **32**, 1620 (1967).
(25) R. Anet, *Tetrahedron Lett.*, **42**, 3713 (1965).
(26) C. Fenselau, S. Y. Wang, and P. Brown, *Tetrahedron*, **26**, 5923 (1970).
(27) C. Fenselau and S. Y. Wang, *ibid.*, **25**, 2853 (1969).
(28) L. A. Paquette, "Principles of Modern Heterocyclic Chemistry," W. A. Benjamin, New York, N. Y., 1968, p 89.