Photochemistry of Thymidine in Ice*

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ABSTRACT: Ultraviolet (λ 254 nm) irradiation of thymidine in frozen aqueous solution produces three dimeric products referred to as Td_1, Td_3, and Td_4, in addition to the cyclobutane-type dimers. Both Td_1 and Td_4 have ultraviolet absorption maxima at 316 nm; Td_3 has an absorption maximum at 267 nm. These dimeric products are not converted into thymidine by irradiation in aqueous solution at 254 nm. Mild acid hydrolysis of Td_1 gives 5-hydroxy-6-4′-[5′-methylpyrimidin-2′-one)dihydrothymine (T–T adduct). Acid hydrolysis of Td_3 gives 5-thyminyl-5,6-dihydrothymine (TDHT). On heating in alkaline solution Td_4 is converted into thymidine and a product having ultraviolet absorption spectra and chromatographic mobility identical with that of 5-methylpyrimidin-2-one deoxyribonucleoside. Td_1 and Td_3 are probably the deoxyribosides of T–T adduct and TDHT, respectively. Ultraviolet absorption spectra of Td_4 and its acid hydrolysis product suggest that it is an isomer of Td_1. The presence of sodium chloride or sodium hydroxide in the irradiation solution favors formation of Td_1, Td_3, and Td_4 while hydrochloric acid prevents their formation.

The isolation of cyclobutane-type thymine dimer (Beukers and Berends, 1960; Wang, 1961) from thymine irradiated with ultraviolet light opened up a new era in photobiology. A major fraction of damage produced in a number of biological systems by ultraviolet light has been attributed to cyclobutane-type dimers formed between adjacent pyrimidines in DNA. Recently this has been reviewed in detail by a number of authors (Setlow, 1967; Smith, 1966). However, there is evidence that photoproducts other than cyclobutane-type pyrimidine dimers have considerable biological importance. Formation of these different products depends on the environment of the DNA during irradiation (Smith, 1967). Thus, although thymine cis-syn dimer (Blackburn and Davies, 1966; Varghese and Wang, 1967b) is the major photoproduct of DNA or of bacterial cells irradiated in aqueous solution, 5-thyminyl-5,6-dihydrothymine (TDHT)[1] (Varghese, 1970) is the major thymine-derived photoproduct of frozen DNA. In addition to the cis-syn thymine dimer, a cyclobutane-type dimer of uracil and thymine (Weinblum, 1967), presumably derived from cytosine and thymine residues, and the deamination product of a cytosine–thymine adduct (Varghese and Patrick, 1969) have also been isolated from DNA irradiated in aqueous solution. The biological importance of the cytosine–thymine adduct has been recently investigated (Patrick, 1970; Ikenaga *et al.*, 1970).

Since all these products can be isolated from thymine or thymidine irradiated with ultraviolet light under suitable conditions, we have investigated the nature of all the thymidine photoproducts formed under different conditions. Such an identification might help considerably in elucidating the nature of unidentified thymine photoproducts in DNA. The isolation and properties of four isomeric cyclobutane-type dimers from thymidine irradiated in the frozen state (Weinblum and Johns, 1965) and in solution in the presence of a photosensitizer (Ben-Hur *et al.*, 1967) have previously been reported by others. These investigators have also reported evidence for the formation of photoproducts other than cyclobutane-type dimers. In this paper we describe the isolation and characterization of three products in addition to the cyclobutane-type dimer from thymidine irradiated in frozen aqueous solution. In a recent similar study with thymine, the isolation of a T–T adduct (Varghese and Wang, 1968b) and a trimeric product (Varghese and Wang, 1968a) was reported.

Materials. Thymidine, obtained from Sigma Chemical Co., was used without further purification. Tritiated thymidine was purchased from Schwarz BioResearch, Inc. The T–T adduct and its dehydration product were prepared following the procedure described by Varghese and Wang (1968).

Irradiation. Portions of thymidine solution (200 ml, 2 mmoles) were frozen (−78°) in enamel pans (40 × 25 cm) over a Dry Ice–ethyl alcohol mixture and irradiated for 1 hr at a distance of 8 cm from a bank of six germicidal lamps (15-W General Electric). For labeled samples, the solution contained 0.5 μCi/ml of thymidine-*methyl-t* (6.2 Ci/mmole).

Paper Chromatography. The samples were concentrated, streaked on Whatman No. 3MM paper (46 × 57 cm, about 50 mg/sheet), and chromatographed by the descending technique. The following solvent systems were used: (A) *sec*-butyl alcohol saturated with water, (B) *tert*-butyl alcohol–methyl ethyl ketone–formic acid–water (40:30:15:15, v/v), (C) *n*-butyl alcohol–methanol–ammonium hydroxide–water (40:20:1:20, v/v), (D) isopropyl alcohol–ammonium hydroxide–water (70:10:20, v/v), (E) *tert*-butyl alcohol–methyl ethyl ketone–water–ammonium hydroxide (40:30:20:10, v/v), (F) *n*-butyl alcohol–acetic acid–water (80:12:30, v/v), and (G) *n*-butyl alcohol–95% ethanol–ammonium hydroxide–propyl alcohol (40:10:20:10, v/v)

Detection and Isolation of Products. From a chromatogram of irradiated thymidine, strips (1 cm wide) were cut and eluted with water. The ultraviolet absorption spectrum of each eluent was taken; 3-ml aliquots from each of these fractions

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[1] Abbreviations used are: TDHT, 5-thyminyl-5,6-dihydrothymine;

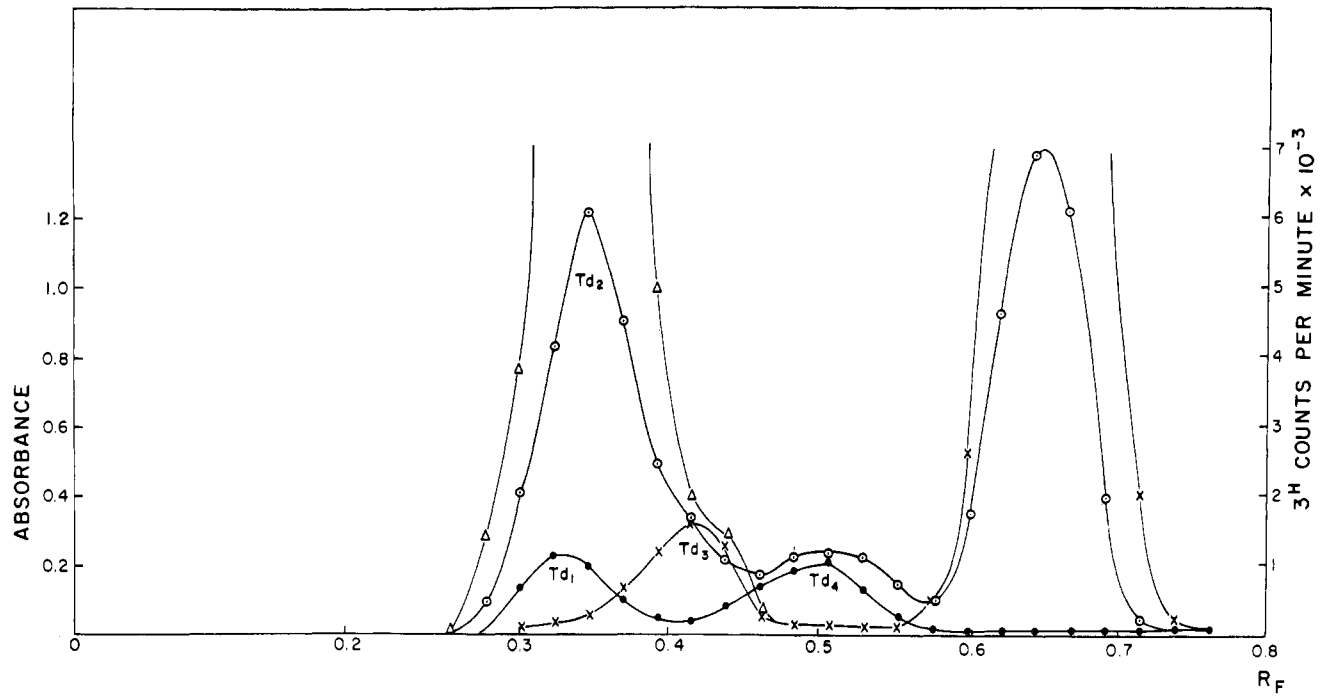


FIGURE 1: Distribution of thymidine photoproducts on paper chromatograms developed in *sec*-butyl alcohol saturated with water. Thymidine was irradiated in frozen aqueous solution. The chromatograms were cut into 1-cm strips and eluted with water. Aliquots of each were analyzed as described in Methods. Distribution of radioactivity (⊙); optical density at 265 nm (×); optical density at 315 nm (●); optical density at 265 after irradiation at 254 nm (△). The peaks are Td_1, Td_2, Td_3, Td_4, and thymidine (R_F 0.65).

were reirradiated for approximately 3 min at 2 cm from two germicidal lamps and any increase in absorbance at 267 nm was recorded. (If cyclobutane-type dimers are present, the absorbance after reirradiation at 267 nm will increase because of reversal to the monomer.) Aliquots from each eluent were counted for radioactivity. When viewed under an ultraviolet lamp, photoproducts other than cyclobutane-type dimers appeared as dark or fluorescent bands. For isolation, strips containing the desired product were cut out and extracted thoroughly with water. The extracts were concentrated and subjected to rechromatography in different solvent systems as required.

Column Chromatography. Dowex 50W-X12 (H^+, 100–200 mesh) and Bio-Gel P-2 (100–200 mesh, Bio-Rad Laboratories, Richmond, Calif.) were used. An aqueous solution of the sample (10–15 mg) to be chromatographed was applied to a column (100 × 3 cm). The column was eluted with water and 20-ml fractions were collected. The ultraviolet absorption spectrum of each fraction was taken and those containing the product were combined and evaporated to dryness, *in vacuo* at low temperature.

Acid Hydrolysis. Trifluoroacetic acid hydrolysis was carried out in sealed tubes at 165° for 90 min. The hydrolysate was streaked on paper and chromatographed as described above. For mild acid hydrolysis, the samples were heated at 80° for 15 min in 4 N hydrochloric acid. The hydrolysates were evaporated to dryness, redissolved in water, and chromatographed on paper.

Infrared and Ultraviolet Absorption Spectra. Infrared spectra were recorded on a Perkin-Elmer 337 grating spectrophotometer in potassium bromide pellets. Ultraviolet absorption spectra were recorded using a Hitachi Perkin-Elmer double-beam spectrophotometer equipped with a Sargent SRG recorder.

TABLE I: R_F Values of Thymidine Photoproducts.

Compound	Solvents				
	A	B	C	D	E
Td_1	0.32	0.35	0.25	0.60	0.57
Td_2	0.36				
Td_3	0.42	0.25	0.26	0.52	0.49
Td_4	0.50		0.32	0.49	0.65
Thymidine	0.65	0.62	0.62	0.70	0.72

Results

Analysis of the chromatograms of thymidine irradiated in frozen aqueous solution with ultraviolet light, as described in Methods, revealed the presence of four products (Figure 1). In the order of chromatographic mobilities in solvent A, they were denoted as Td_1, Td_2, Td_3, and Td_4, having R_F values 0.31, 0.34, 0.43, and 0.51, respectively. When viewed under an ultraviolet lamp, Td_1 and Td_4 appeared as fluorescent bands while Td_3 and thymidine (R_F 0.63) appeared as dark bands. The R_F values of the various products are presented in Table I.

Cyclobutane-Type Thymidine Dimers (Td_2). Eluent of the Td_2 band has only negligible absorbance above 250 nm and showed an increase in absorbance at 267 nm on irradiation

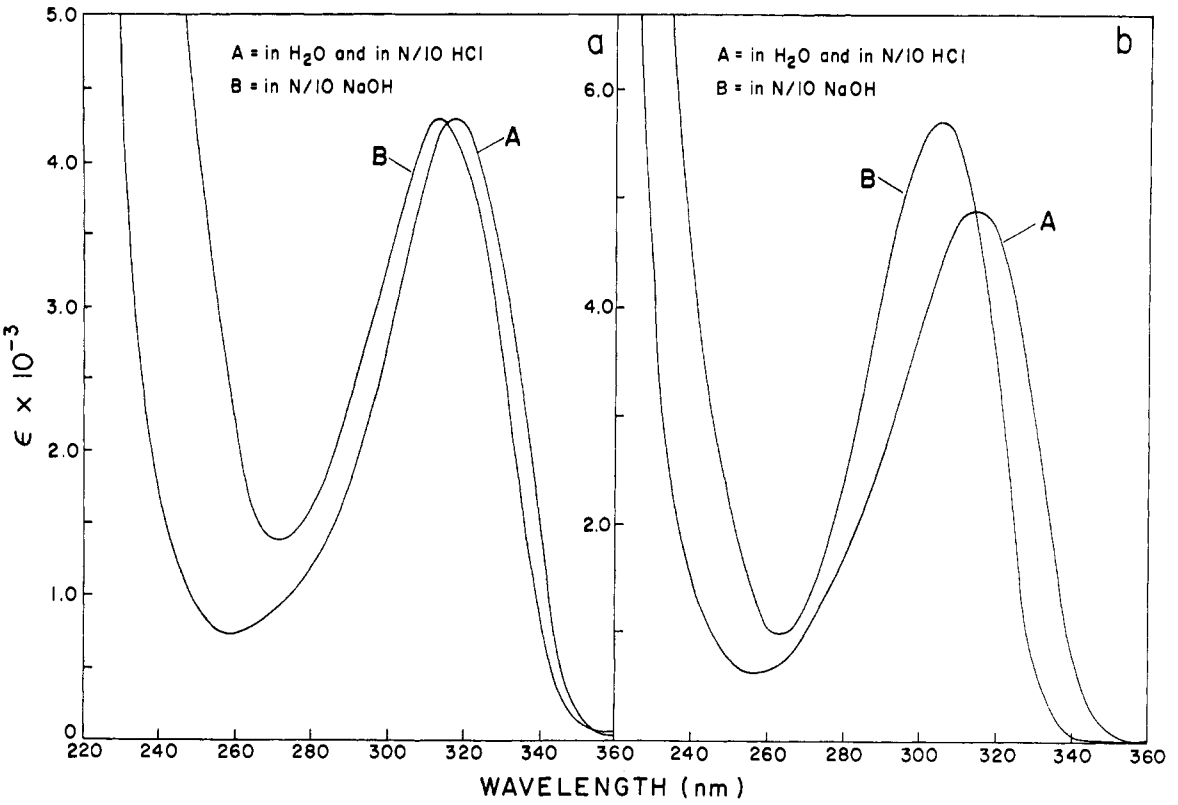


FIGURE 2: Ultraviolet absorption spectra of (a) Td_1 and (b) T–T adduct isolated from Td_1 after acid hydrolysis.

at 254 nm. Both these properties are characteristic of cyclobutane-type dimeric products of pyrimidine derivatives. Following the procedure of Weinblum and Johns (1965), we have also isolated four isomeric cyclobutane-type dimers from eluent of the Td_2 band. The assignment of the individual structures was on the basis of acid stabilities, chromatographic mobilities, and infrared spectra of thymine dimers as described by Weinblum and Johns (1965). The cis-anti(+), cis-anti(−) (I), cis-syn (II), and trans-anti (III) isomers were present in the ratio 16:43:32:9. No trans-syn isomer was detected. These results were also in agreement with that reported by Ben-Hur *et al.* (1967).

Isolation and Characterization of Td_1. When eluted from chromatograms developed in solvent A, Td_2 was found to be the major impurity associated with Td_1. After rechromatography in a number of solvent systems, the partially purified Td_1 was irradiated in aqueous solution with 254-nm radiation to monomerize any dimer present as contaminant. Subsequent chromatography of the irradiated mixture in solvent A yielded pure Td_1. The dose given to monomerize the dimer associated with Td_1 did not significantly affect the spectroscopic and chromatographic properties of the latter. Ultraviolet absorption spectra of Td_1 in acid and base are shown in Figure 2a. Td_1 is almost as stable as thymidine to ultraviolet irradiation in aqueous solution.

All attempts to crystallize Td_1 were unsuccessful. Mild acid hydrolysis of Td_1 gave one major product (R_F 0.29 in solvent F) which appeared as a fluorescent band under an ultraviolet lamp, the eluent of the band showing an ultraviolet absorption maximum at 316 nm. The product, after purification by Dowex column chromatography, was crystallized from hot water. The infrared (Varghese and Wang, 1968b) and ultraviolet spectra (Figure 2b) were found to be identical with those of 5-hydroxy-6-4′-[methylpyrimidin-2′-one]dihydrothymine (T–T adduct; IV) isolated from thymine. By refluxing T–T adduct in 0.5 N HCl for 90 min 6-4′-[5′-methylpyrimidin-2′-one]thymine (V), the dehydration product, was obtained. Td_1 is therefore most probably the deoxyribonucleoside of the T–T adduct.

Isolation and Characterization of Td_3. Pure Td_3 was obtained after rechromatography in a number of solvent systems. The ultraviolet spectra of Td_3 in aqueous solution at different pH are shown in Figure 3a. In aqueous solution Td_3 is almost as resistant as thymidine to ultraviolet irradiation (λ 254 nm); attempts to crystallize it were unsuccessful. Paper chromatograms of the trifluoroacetic acid hydrolysate of Td_3 developed in solvent F showed only one detectable product (R_F 0.37) which appeared as a dark band when the chromatogram was viewed under an ultraviolet lamp. After elution from the chromatograms, the hydrolyzed product was purified by Dowex column chromatography and crystallized from hot water. The ultraviolet (Figure 3b) and infrared spectra (A. J. Varghese, submitted for publication) of the crystalline material were found to be identical with those of TDHT (VI) (Varghese, 1970) isolated from DNA, indicating that Td_3 is most probably the deoxyribonucleoside of TDHT. TDHT is not affected to any significant extent by boiling in 1 N NaOH for 30 min and it appears that it is almost as stable as cis-syn thymine dimer toward acid and alkali.

Isolation and Properties of Td_4. Td_4 is the least stable photo-

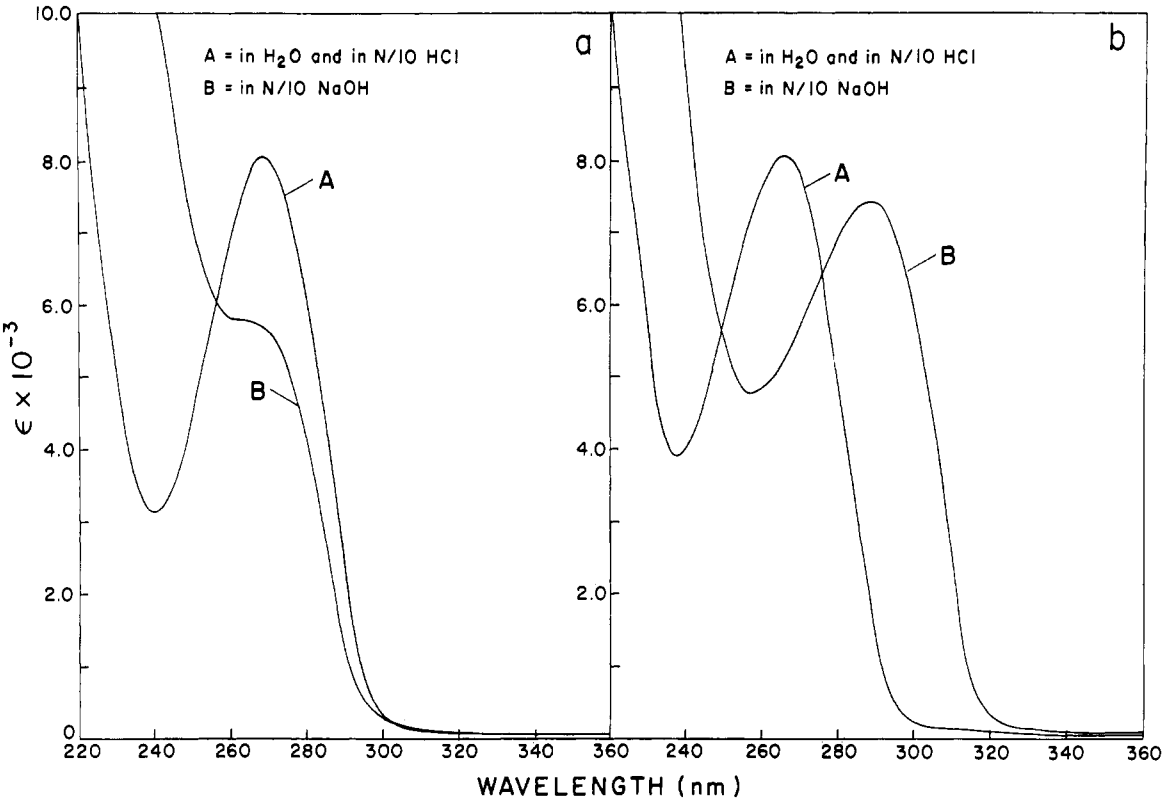


FIGURE 3: Ultraviolet absorption spectrum of (a) Td_3 and (b) TDHT isolated from Td_3 after acid hydrolysis.

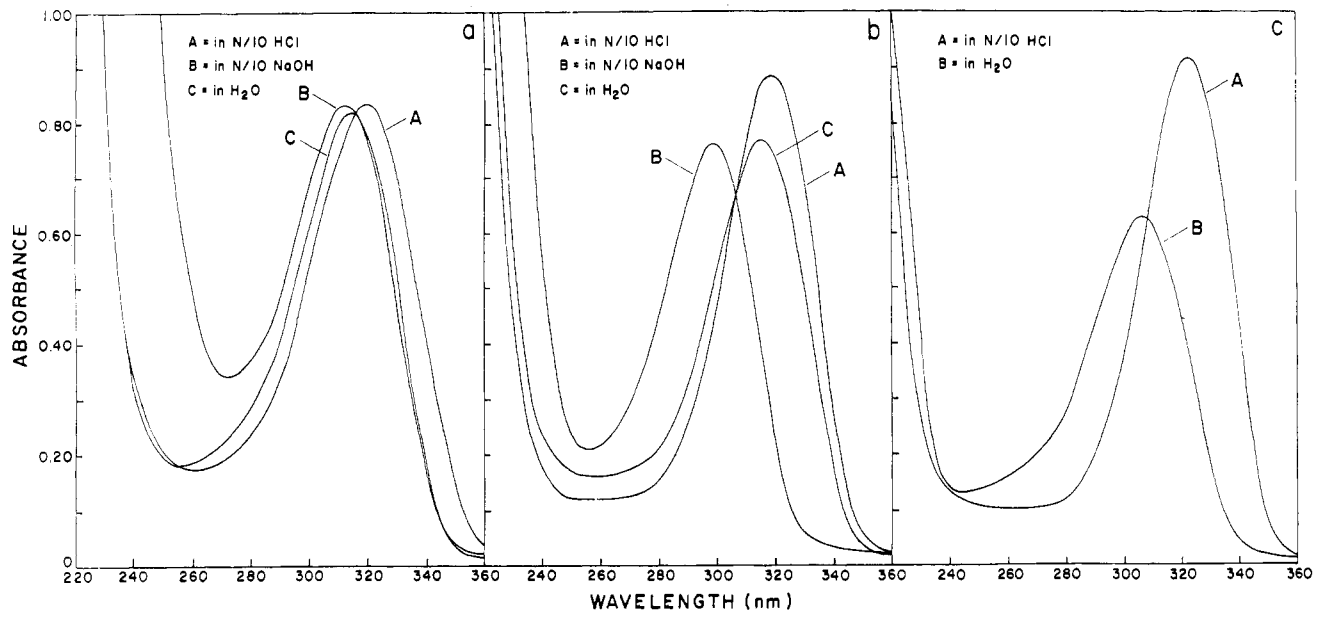


FIGURE 4: Ultraviolet absorption spectra of (a) Td_4, (b) major product of acid hydrolysis, and (c) product obtained after heating Td_4 in 0.1 N NH_4OH.

product of thymidine and was purified by repeated chromatography in solvent A followed by Bio-Gel P-2 column chromatography. Ultraviolet absorption spectra of Td_4 in aqueous solution at different pH values are shown in Figure 4a. Irradiation of an aqueous solution of Td_4 with ultraviolet light ($\lambda > 300$ nm) forms a product or products with no ultraviolet absorption maxima. Acid hydrolysis (HCl or trifluoroacetic acid) of Td_4 produces thymine and a number of other products. Analysis of paper chromatograms of the hydrolysates developed in solvent F showed a major product

TABLE II: λ_{max} and ϵ_{max}[a] of Thymidine Photoproducts at Different pH Values.

Photoproducts	pH 2		pH 6		pH 12	
	λ_{max} (nm)	ϵ_{max}	λ_{max} (nm)	ϵ_{max}	λ_{max} (nm)	ϵ_{max}
Td_1	316	4.3×10^3	316	4.8×10^3	312	4.8×10^3
Td_3	267	8.2×10^3	267	8.2×10^3	267	5.8×10^3
Td_4	320	4.4×10^3	316	4.3×10^3	312	4.4×10^3
TDHT	265	8.0×10^3	265	8.0×10^3	290	7.5×10^3
T–T adduct	316	4.9×10^3	316	4.9×10^3	306	5.6×10^3
Hydrolysis product of Td_4	322		314		308	

[a] Calculated on the assumption that Td_1, Td_3, and Td_4 contain two thymidine residues.

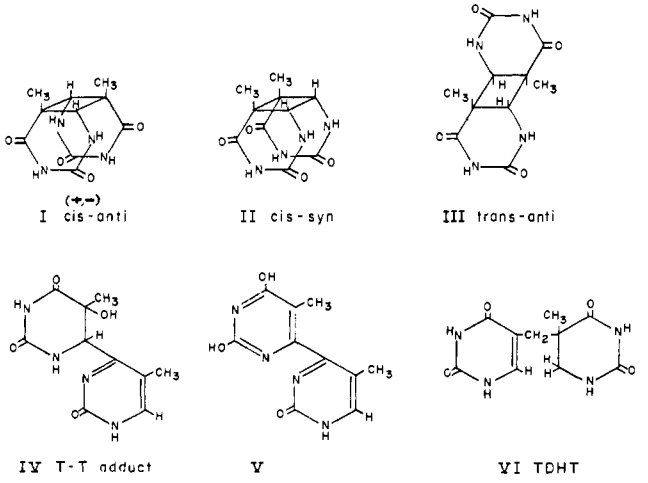
(R_F 0.65) which appeared as a fluorescent band under an ultraviolet lamp. Ultraviolet absorption spectra of this product at different pH values are shown in Figure 4b. Ultraviolet absorption characteristics of Td_4 and its major hydrolysis product suggest that Td_4 is an unstable isomer of Td_1.

By heating at 80° in 0.1 N NH_4OH for 30 min Td_4 is converted into thymidine and another product. On a paper chromatogram developed in solvent G, thymidine appears as a dark band (R_F 0.43) and the second product as a fluorescent band (R_F 0.62) under an ultraviolet lamp. The ultraviolet absorption spectrum of the eluent of the fluorescent band (Figure 4c) is identical with that of 5-methylpyrimidin-2-one deoxyribonucleoside (Laland and Serck-Hanssen, 1964). The conversion of Td_4 into these two products also suggests that Td_4 is probably derived from two thymidine residues. Because of the inherent instability of Td_4 further elucidation of the structure was not possible.

Discussion

Ultraviolet irradiation of thymidine in frozen aqueous solution produces three main types of product. The cyclobutane-type thymidine dimers comprise about 70% of the total products. They have low ultraviolet absorption above 250 nm and are converted into thymidine on irradiation in aqueous solution with 254-nm light. The second class of product is the adduct type. One characteristic of this class is an ultraviolet absorption peak above 300 nm. It is acid and alkali labile and fluoresces under ultraviolet light. The adduct-type product constitutes about 16% of the total products. The third type of thymidine product (14% of total products) has an ultraviolet absorption maximum at 267 nm and is stable to acid and alkali. The spectral properties of Td_1, Td_3, and Td_4 are summarized in Table II. The structures of these products are given in Chart I.

CHART I



Considerable variation in the amounts of thymidine photoproducts was observed when the irradiation was carried out in 0.1 N hydrochloric acid, 0.1 N sodium hydroxide, and 1 M sodium chloride solutions. In all cases Td_2 is the major product. The amounts of the various products were estimated on the basis of their molar extinction coefficients and the results are presented in Table III. In hydrochloric acid, a product (R_F 0.45 in solvent A) having an ultraviolet absorption maximum at 275 nm was also formed. The identity of this product is not known.

Ultraviolet-induced formation of these three different types of product appears to be a general phenomenon for thymine derivatives. The same types of product are formed in different proportions when thymidine is irradiated as a thin solid film with ultraviolet light. Johns *et al.* (1964) and Pearson *et al.* (1965) have reported that ultraviolet irradiation of thymidylylthymidine (TpT) in aqueous solution yields, in addition to the cyclobutane-type dimers, two products, referred to as

TABLE III: Relative Yields of Thymidine Photoproducts under Different Conditions.

Photoproducts	% Yields			
	0.1 N HCl	0.1 N NaOH	1.0 M NaCl	H_2O
Td_1		18	17	6.0
Td_2	50	58	57	70
Td_3	4	22	21	14
Td_4		2	5	10

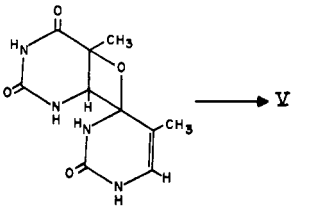
TpT_3 and TpT_4, having ultraviolet absorbance maxima at about 320 nm. It is probable that TpT_3 and TpT_4 are structurally related to Td_1 and Td_4. These investigators have reported that TpT_3 and TpT_4 are photoreversible. Td_1 cannot be converted into Td_4 under any irradiation condition nor can it be easily photolyzed at 313 nm, as in Td_4.

The photochemical behavior of thymine, however, is quite different from that of its nucleoside; of the four isomers possible, only the cis-syn isomer is produced by ultraviolet irradiation of thymine in frozen aqueous solution. Furthermore, a trimeric product such as the one isolated from thymine (Varghese and Wang, 1968a) has not been detected in irradiated thymidine. Thymidine and TpT produce two adduct-type compounds, while thymine gives rise to only one T–T adduct.

Of the four isomers of the cyclobutane-type thymine dimer, only the cis-syn isomer has been detected in irradiated native DNA. Small amounts of the trans-syn isomer (Ben-Hur and Ben-Ishai, 1968) have been reported to be formed in denatured DNA. Reports from several laboratories (Smith, 1963; Haug, 1964; Varghese and Wang, 1967a) indicate that products with ultraviolet absorbance maxima at 320 nm are formed in DNA as a result of ultraviolet irradiation. No derivative of either Td_1 or Td_4 has yet been isolated from DNA although the deamination product of a cytosine–thymine adduct (Wang and Varghese, 1967) having an absorbance maximum at 316 nm has been isolated as minor photoproduct from DNA. Failure to detect these compounds might be due to their extensive degradation during acid hydrolysis. It has been shown by a number of investigators that ultraviolet irradiation of bacterial spores (Donnellan and Setlow, 1965), frozen bacteria (Smith, 1967), dry DNA (Smith and Yoshikawa, 1966), and frozen DNA (Rahn and Hosszu, 1968) produce a thymine-derived product which is not a cyclobutane dimer. This product, generally referred to as spore product, SP, has recently been isolated from DNA irradiated at low temperature and characterized as TDHT. The biological importance of SP has been studied in detail by Donnellan and his associates (Donnellan and Stafford, 1968; Stafford and Donnellan, 1968) who have also shown that SP is derived from two adjacent thymine residues in DNA.

Mechanism of Formation. α,β-Unsaturated ketones are known to form photodimers of the cyclobutane type quite readily. These reactions have been shown to involve the attack of an excited molecule on an unexcited one (Eaton, 1962). The process, involving $n-\pi^*$ excitation, is considered to involve singlet–triplet crossover producing metastable excited molecule. In the case of pyrimidine bases in dilute solution it has been shown that a molecule in its lowest excited triplet state combines with another molecule in its ground state (Brown and Johns, 1968; Charlier *et al.*, 1969). In concentrated or frozen solution, excited singlet-state molecules are also involved. The orientation and stereochemistry of the photodimerization of α,β-unsaturated ketones have been investigated by Corey *et al.* (1964). In the 2-cyclohexenone system, a biradical intermediate has been proposed to explain the stereochemistry of the reaction. It was also shown that substitution in the 2 or 4 position considerably alters the nature and amounts of the photoproducts. Therefore, if photodimerization of pyrimidine bases follow the pattern of α,β-unsaturated ketones, the photoproducts of substituted pyrimidine bases might be different from those of simple pyrimidine bases. This also might explain the variation in the photoproducts of uracil, thymine, and thymidine.

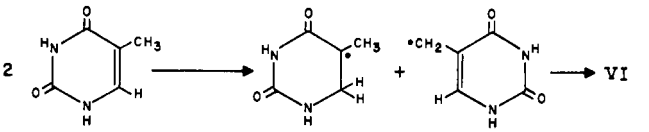
For Td_1, and Td_4, an oxetane derivative (VII), similar to that proposed for the formation of T–T adduct (Varghese and Wang, 1968b) from thymine, may be the precursor. Such an intermediate may be stable only at low temperature and



VII

rearranges to the adduct on warming. The observation of Rahn and Hosszu (1969) that fluorescence due to the adduct does not appear directly on irradiation of thymine at −196°, but comes about upon annealing at elevated temperatures, supports the idea of such a precursor for T–T adduct. Many carbonyl compounds are known to form oxetanes on irradiation in the presence of an olefin. Earlier studies by Buchi and coworkers (1954) have shown that the oxetane would arise from the most stable biradical produced by the addition of the triplet carbonyl component to the olefin. Reaction mixtures are shown often to contain all possible stereoisomers of the oxetane, in addition to products from other reactions. Oxetanes are known to form various rearranged products by acid catalysis.

Ultraviolet excitation of pyrimidines in frozen solution gives not only triplet-state molecules (after intersystem crossing) but also radicals (Pershan *et al.*, 1964; Lacroix and Van de Vorst, 1968). From electron spin resonance studies, many investigators (Pershan *et al.*, 1964; Pruden *et al.*, 1965) have reported the formation of a thymyl radical (VIII) in DNA and thymidine as a result of ultraviolet irradiation. The thymyl radical is formed by the addition of a hydrogen atom at the C_6 position of a thymine moiety. In situations such as may exist in dry or frozen DNA or thymidine, the methyl group of an adjacent thymine residue may serve as the hydrogen donor. The resulting thyminyl radical (IX) adds on to the thymyl radical, forming the stable TDHT derivative. Ultraviolet-induced formation of the thyminyl radical has also been reported (Alcantara and Wang, 1965).



VIII IX

Isolation of these different photoproducts of thymidine emphasizes the fact that measurement of photoproduct production by analysis of variation of optical density with dose may be a hazardous undertaking. Moreover, identification of specific products based only on chromatographic mobilities of labeled samples may also be misleading.

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