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Photochemistry of Cytosine Derivatives. 1. Photochemistry of Thymidylyl-(3′→5′)-deoxycytidine†

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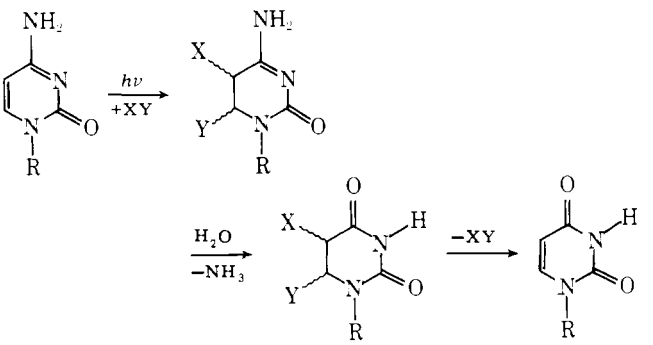
ABSTRACT: The photochemistry of thymidylyl-(3′→5′)-deoxycytidine (dTpdC) was studied as a model system of adjacent thymine and cytosine bases in DNA. Acetophenone-sensitized irradiation causes the cytosine moiety in dTpdC to react with the thymine moiety intramolecularly. Three unstable photoproducts are formed initially which are converted into three isomeric dinucleoside phosphates of thymine-uracil cyclobutane photodimer in a ratio of 4.2:2.2:1. Under the same irradiation condition thymidylyl-(3′→5′)-thymidine (dTpdT) yields two products in a ratio of 6:1. The structures of these products are established by chemical and spectroscopic methods. The major product in these reactions has been identified as the stereoisomer which has the same anti,anti relationship between the pyrimidine rings and the deoxyribose group as in the parent dinucleoside phosphates. The efficiency of the intramolecular dimerization of dTpdC is about one-third that of dTpdT. The results suggest that the cytosine base in DNA may be converted to a uracil base via photodimerization with an adjacent pyrimidine base, hydrolysis, and photoreactivation.

The objective of this research is to correlate the photochemistry and photophysics of nucleic acid derivatives with the photobiology of microorganisms in order to provide a molecular basis for mutagenesis induced by ultraviolet light (for a recent review of the photochemistry and photobiology of nucleic acid derivatives, see Wang, 1976). Photophysically, cytidylic acid has the lowest singlet excited state among all nucleotide units in DNA (Guéron et al., 1967; Lamola, 1973). After photoexcitation, singlet excited energy may localize at the cytosine residue in DNA. Photochemically, the reaction of cytosine and its derivatives may result in the saturation of their 5,6-double bond (Guéron et al., 1974). Consequently, the 4-amino group in the primary photoproduct is no more stabilized by the resonance existing in cytosine, and hydrolytic deamination of the primary product will lead to the formation of a dihydrouracil derivative which may undergo further chemical or photochemical transformation to give a uracil derivative (Scheme I). If such a sequence of events will occur in vivo, the formation of dU from C may lead to the miscoding of C as dU or T. Photobiologically, results from in vivo studies on T4 and S13 phages indicate that the major portion of UV-induced mutation corresponds to the C to T base transition (Drake, 1963, 1966a,b; Howard & Tessman, 1964). We have previously reported the photochemical conversion of cytosine

SCHEME I



to uracil derivatives in the presence of mercaptans including cysteine (Yang et al., 1974) and wish to explore additional pathways for such conversions.

Photodimerization of pyrimidines is the most thoroughly studied photoreaction of nucleic acid derivatives (for reviews, see Burr, 1968; Setlow, 1968; Fahr, 1969; Varghese, 1972; Fisher & Johns, 1976). The formation of pyrimidine dimers has been correlated with many of the biological effects of UV irradiation (Setlow, 1966; Meistrich, 1972; Meistrich & Drake, 1972). Thymine and its derivatives undergo photodimerization readily under a variety of experimental conditions, but the principal photochemical process of cytosine and its monomeric derivatives is the photohydration (Fisher & Johns, 1976; Yang & Liu, 1977). Although there are only a few examples of photodimerization involving cytosine reported in the literature, cytosine-containing dimers such as Thy[]Cyt and Cyt[]Cyt have been detected in UV-irradiated DNA and characterized as Thy[]Ura and Ura[]Ura, respectively (Setlow & Carrier, 1966; Setlow et al., 1965). Cytosine-containing dimers may be formed in amounts comparable to the thymine

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dimer and become more abu nt as the G-C content of DNA increases (Setlow & Carrier 966).

One of the important properties of pyrimidine cyclobutane photodimers is their reversion to monomers by short-wavelength UV light (Herbert et al., 1969) or by visible light in the presence of a photoreactivating enzyme system (for a review, see Harm, 1976). If the deamination of cytosine-containing dimers occurs before photoreactivation, the overall change will be the conversion of C to U and, consequently, a change in genetic code. Such a process may be responsible for the mutagenic action of UV light. Since cytosine and its monomeric derivatives do not normally undergo cross dimerization with thymine, the purpose of this work is to study the photochemistry of a model system of adjacent thymine and cytosine bases in DNA, a dinucleoside phosphate, thymidylyl-(3′→5′)-deoxycytidine (dTpdC,[1] **1**).

Photochemistry of several dinucleoside phosphates has been studied. The direct irradiation of dTpdT yields four products; two of them are internal cyclobutane photodimers (Johns et al., 1964; Pearson et al., 1965). The UV irradiation of UpU and CpC yields both internal cyclobutane photodimers and photohydrates (Helleiner et al., 1963; Brown et al., 1966; Freeman et al., 1965; Hariharan & Johns, 1968). The photochemistry of dTpdC has also been studied previously; however, the structures of photoproducts have not been established (Haug, 1964). In this study, both the direct and sensitized irradiations of dTpdC were investigated, particularly with respect to its conversion to the intramolecular photodimer dTp[]pdC, the deamination of these products to dTp[]pdU and the photoconversion of dTp[]pdU to dTpdU. Results from these reactions were compared with a parallel investigation on the sensitized irradiation of dTpdT. The possible implication of this investigation on the photobiology of microorganisms will be discussed.

Experimental Section

Proton Magnetic Resonance. Proton magnetic resonance spectra were recorded on a Bruker HS-270 (270 MHz) spectrometer. Proton FT-NMR spectra were acquired on the same spectrometer operating in the pulsed Fourier-transform mode. Field stabilization was obtained from the deuterium resonance of the solvent (D_2O). Free-induction decay data were accumulated and transformed with an 8K Nicolet computer. Computer simulations of NMR spectra were performed on the same computer. Programs NTCFT and ITRCAL were used for FT-NMR and computer simulation, respectively. All NMR spectra were run at 20 ± 1 °C. For spectra taken in D_2O, samples were lyophilized from 99.8% D_2O in order to exchange the exchangeable protons to deuterium.

Thin-Layer and Preparative-Layer Chromatography. Analytical thin-layer chromatography (TLC) was carried out using Eastman silica gel chromatogram (6060) or Brinkman cellulose plate with fluorescence indicator (CE 300 UV_{254}). Preparative layer chromatography (PLC) was performed on glass plates (20 × 20 cm) precoated with a 2-mm thickness of silica gel (E. Merck F-254). Visualization was accomplished with 254-nm light or a reagent system developed by Habermann (1960), chlorine vapor followed by Toluidine Blue/KI/HOAc. The solvent systems used were: solvent I, 1-butanol-acetic acid-water (5:2:3); solvent II, isobutyric acid-concentrated ammonia-water (66:1:33); solvent III, 2-propanol-concentrated ammonia-water (7:1:2).

Irradiation. All photochemical reactions were performed with a Hanovia 450-W medium-pressure Hg lamp in a chilled water cooled quartz immersion well and a light filter as noted. For irradiation of fluid solutions, the immersion well was placed in a Pyrex jacket equipped with a reflux condenser and nitrogen dispersion inlet tube. The reactants were placed in the jacket and purged with nitrogen for at least 30 min before the lamp was activated. A gentle stream of nitrogen was admitted for the duration of irradiation. The reaction temperature was maintained at approximately 10 °C. For irradiation of frozen aqueous solutions, the solution was frozen in a Petri dish and placed on a dry ice bath, and the above-mentioned lamp in the quartz well was placed at 2 cm above the Petri dish. The progress of these photoreactions was monitored with UV spectroscopy.

Thymidylyl-(3′→5′)-deoxycytidine (**1**, *dTpdC*). Compound **1** was prepared as reported in the literature (Gilham & Khorana, 1958) with minor modifications. N^4,3′-*O*-Diacetyldeoxycytidine 5′-phosphate was reacted with 5′-*O*-tritylthymidine in pyridine in the presence of 2,4,6-tri-2-propylbenzenesulfonyl chloride (TPS). After the reaction and the removal of protecting groups, dTpdC was separated from the crude reaction mixture by PLC on 20 cm × 20 cm × 2 mm silica gel plate with 2-propanol-ammonia-water (17:1:2) in 70% yield. The product exhibits identical R_f values and UV and ir spectra with those of an authentic sample (P-L Biochemicals) and is hydrolyzed to thymine and cytosine with 1 N HCl at 100 °C for 4 h.

For the relative quantum efficiency study, dTpdC was further purified by anion exchange chromatography on a DEAE-cellulose (Bio-Rad Cellex-D) column with ammonium bicarbonate as the eluent.

Thymidylyl-(3′→5′)-thymidine (**6**, *dTpdT*). Compound **6** was prepared in the same manner as for dTpdC by reacting 3′-*O*-acetylthymidine 5′-phosphate with 5′-*O*-tritylthymidine in pyridine in the presence of TPS in 72% yield. For the relative quantum efficiency study, dTpdT was further purified on a cellulose column with ethanol-water (9:1) as the eluent.

Thymidylyl-(3′→5′)-deoxyuridine (**4**, *dTpdU*). The method of converting cytosine to uracil with sodium bisulfite (Shapiro et al., 1970; Hayatsu et al., 1970) was applied to the preparation of dTpdU from dTpdC. A solution of 10 mg (0.018 mmol) of dTpdC in 0.2 mL of distilled water containing 2.5 M $NaHSO_3$ was incubated at 50 °C for 13 h. The mixture was diluted with several volumes of water and 315 mg (1 mmol) of $Ba(OH)_2 \cdot 8H_2O$ was added. The resulting precipitate was removed by centrifugation and washed twice with water. The combined supernatants were treated with a suspension of Bio-Rad AG 50W-X8 to give an acidic solution. The resin was removed by filtration and washed several times with water. The filtrate was evaporated to dryness under reduced pressure to give a chromatographically homogeneous sample (8 mg, 80% yield). R_f (silica gel) solvent I 0.37, solvent II 0.48, solvent III 0.58; IR (KBr) 1700 cm^{-1} (C=O) UV λ_{max}^{pH7} 263 nm (15 000); NMR (D_2O) δ_{TSP} 1.87 (s, 3, C_5-CH_3 (T)), 2.39 and 2.54 (2 m, 4, $H_{2'}$ (T + U)), 3.81 (m, 2, $H_{5'}$ (T)), 4.15 (m, 4, $H_{5'}$ (U), $H_{4'}$ (T) and $H_{4'}$ (U)), 4.57 (m, 1, $H_{3'}$ (U)), 4.79 (m, 1, $H_{3'}$

[1] Abbreviations for photodimers of pyrimidines used in this manuscript are according to those formulated by Cohn et al. (1974). Abbreviations are: dTpdC, thymydylyl-(3′→5′)-deoxycytidine; dTpdU, thymydylyl-(3′→5′)-deoxyuridine; dTpdT, thymydylyl-(3′→5′)-thymidine; Sym[]Sym, cyclobutane photodimers of pyrimidines; their stereochemistry may be indicated by a suffix, such as Thy[]Thy(*c,s*) for the cis,syn photodimer of thymine where *c*, *t*, *s*, and *a* stand for cis, trans, syn, and anti, respectively; X[]Y, photodimers of pyrimidine nucleosides, such as dT[]dT for the photodimer of thymidine; Xp[]pY, internal photodimers of dinucleoside phosphates where the suffix will indicate the stereochemistry of the cyclobutane and the prefix will indicate the orientation between the riboside and the pyrimidine ring, such as *anti*-dTp[]pdC(*c,s*) for the anti isomer of the cis,syn internal photodimer of thymydylyl-(3′→5′)-deoxycytidine.

(T)), 5.87 (d, 1, J = 7.8 Hz, H_5 (U)), 6.27 (m, 2, $H_{1'}$ (T), $H_{1'}$ (U)), 7.60 (s, 1, H_6 (T)), and 7.82 ppm (d, 1, J = 7.8 Hz, H_6 (U)).

A portion of the product was heated with 1 N hydrochloric acid at 100 °C. After 4 h, the material was degraded completely to thymine and uracil as identified by TLC in various solvent systems.

cis-syn-Thymine-Uracil Cyclobutane Photodimer (Thy[]Ura(c,s), **5a**). The compound was separated from a mixture of Thy[]Thy, Thy[]Ura, and Ura[]Ura obtained from the irradiation of an equimolar mixture of thymine and uracil in frozen aqueous solution by cation-exchange chromatography on a column of AG 50W-X8 (ammonium formate) according to the procedure of Weinblum (1967). The IR spectrum of this compound is identical with the one reported.

cis-syn-Thymine Cyclobutane Photodimer (Thy[]Thy(c,s), **8a**). The compound was prepared by irradiating thymine in frozen aqueous solution. Recrystallization of the crude product from water gave a crystalline material which exhibits an IR spectrum identical in all respects to that reported in the literature (Weinblum & Johns, 1966; Weinblum, 1967).

*Dimethyluracil Cyclobutane Photodimers, (c,s)-***11a**, *(t,s)-***11b**, *(c,a)-***11c**, *and (t,a)-***11d**. All four cyclobutane photodimers of dimethyluracil were prepared from the irradiation of 1,3-dimethyluracil in dioxane (Elad et al., 1971). NMR spectra (270 MHz) of **11a–d** exhibit two singlets for the N_1 and N_3 methyl groups and two groups of peaks for the cyclobutane protons. The hyperfine splitting patterns of the cyclobutane protons of **11c** (two triplets) and **11d** (two doublets of doublets) were analyzed by first-order analyses. Those for **11a** and **11b** were analyzed as AA′XX′ systems (Günther, 1972) and checked by computer simulations. The coupling constants thus resolved and chemical shifts are listed in Table V. The 270-MHz NMR spectra and computer simulated spectra of **11a** and **11b** are shown in Figure 6.

Direct Irradiation of dTpdC. When a solution of thymidylyl-(3′→5′)-deoxycytidine (dTpdC, **1**, 33 mg, 0.07 mmol) in 450 mL of distilled water was irradiated under a nitrogen atmosphere through a Vycor filter sleeve, 70% of the starting material was consumed after 2 h as indicated by UV spectroscopy. Concomitant with the decrease in the absorbance at 267 nm were the appearances of an absorption at longer wavelength (>300 nm) and a maximum at 245 nm. After a sample of the irradiated solution was warmed at 68 °C, the absorbance at 267 nm was restored to 70% of the original value of the solution before irradiation. Presumably, the unstable photohydrate was the major photoproduct which was reverted to dTpdC. Three stable products were detected on TLC in addition to the starting material as reported by Haug (1964). One of the photoproducts has the same R_f values in different solvent systems very close to those of dTpdU. Another product has an absorption maximum above 300 nm and may be a dinucleoside phosphate of a thymine-cytosine adduct (Wang & Varghese, 1967). None of the products are cyclobutane-type photodimers.

Sensitized Irradiation of dTpdC. A solution of dTpdC (**1**, 57 mg, 0.1 mmol) in 450 mL of distilled water containing 680 mg of acetophenone (5.7 mmol) was irradiated through a Pyrex filter sleeve under an atmosphere of nitrogen at 10 °C. After 13 h, 90% of the starting material was consumed as indicated by UV spectroscopy at 267 nm. When a sample of the irradiated solution was heated to 50 °C, there was no increase in absorbance at 267 nm but a decrease in absorbance from 220 to 290 nm, indicating the absence of photohydrates but a typical dihydrocytosine to dihydrouracil conversion. The solvent was removed by lyophilization. NMR spectrum (270 MHz) of the crude reaction mixture shows the presence of five methyl singlets at δ 1.68, 1.57, 1.51, 1.42, and 1.33 ppm from the internal TSP standard. Upon heating to 50 °C, peaks at δ 1.51 and 1.33 gradually grew at the expense of peaks at δ 1.57 and 1.42, respectively, while the peak at δ 1.68 became sharper. Thermally unstable products were also detected on TLC sheets which were found to undergo conversion to stable products. Finally after 8 h, only three peaks at δ 1.68, 1.51, and 1.33 remained with an integration ratio of 4.2:2.4:1, respectively. TLC on silica gel or cellulose sheets indicated the presence of only three products and the starting material. In order to simplify the work-up, the lyophilized product mixture was dissolved in a small amount of water and warmed to 50 °C until unstable products were no longer detectable by TLC. The average half-life of isomers of dTp[]pdC with respect to their conversion to dTp[]pdU was about 1 h.

The solution was then applied to two silica gel plates (20 cm × 20 cm × 2 mm) and developed with solvent system II for 24 h. One intense band and three weak bands were visualized under UV light. Bands were scrapped off and washed with anhydrous ether to remove the residual solvent and the compounds were extracted with 95% ethanol. Solids collected after removing the solvent were dissolved in distilled water and passed through a bed of celite in a small fritted disc funnel and the filtrates and washings lyophilized. A small amount of starting material (dTpdC, 6 mg) was recovered and three products were isolated: **3a,** 27 mg (53%), R_f (silica gel), solvent I 0.22, solvent II 0.21, solvent III 0.50; **3b,** 17.2 mg (34%), R_f (silica gel), solvent I 0.27, solvent II 0.33, solvent III 0.51; **3c,** 6.6 mg (12%), R_f (silica gel), solvent I 0.32, solvent II 0.42, solvent III 0.55.

Acetone-sensitized irradiation of **1** also yielded compounds **3a–c** in about the same ratio as in the acetophenone-sensitized irradiation. In addition, another unidentified product with R_f value between that of **1** and **3c** was detected. The presence of additional methyl signals in the NMR indicates that the unidentified product may be an adduct of acetone to dTpdC. Photochemical addition of acetone to the 5,6-double bond of pyrimidines to form oxetane type of products has been reported previously (Varghese, 1972).

Preliminary spectroscopic analyses of **3a, 3b,** and **3c** indicate that they may be isomeric internal photodimers of dTpdU, dTp[]pdU. All compounds exhibit only end absorption in their UV spectra. NMR spectra (270 MHz) of these compounds exhibit methyl singlets at higher field than that of dTpdC. They also exhibit, instead of signals for olefinic protons in dTpdC, signals for three protons in ABX patterns with chemical shifts similar to those found for cyclobutane protons in pyrimidine photodimers (see Results and Discussions).

Acetophenone-Sensitized Irradiation of dTpdT. A solution of dTpdT (90 mg, 0.16 mmol) in 400 mL of distilled water containing acetophenone (637 mg, 5.3 mmol) was irradiated under the same condition as for the irradiation of dTpdC. After 3.5 h, UV absorbance at 267 nm decreased to 10% of the original value. Solvent was removed by lyophilization and the residue was dissolved in a small amount of water and applied to two silica gel plates (20 cm × 20 cm × 2 mm) and developed in solvent system II. Three bands were visualized under UV light and the products were recovered by the same procedure as described before: **7a,** 64.8 mg (80%); R_f (silica gel), solvent I 0.24, solvent II 0.21; **7b,** 10.8 mg (14%); R_f (silica gel), solvent I 0.32, solvent II 0.30.

Photoconversion of Internal Photodimers of Dinucleoside Phosphates to Dinucleoside Phosphates. Solutions of the photoproducts of dTpdC and dTpdT were spotted on silica gel and cellulose TLC sheets and the compounds were exposed to

a germicidal lamp (λ_{em} 254 nm) until dark spots were revealed on the sheets. dTpdU or dTpdT was spotted on the sheet as the reference after the UV irradiation, and the TLC sheets were then developed in the solvent system I–III. Compounds **3a–c** were shown to be converted to dTpdU and compounds **7a** and **7b** were shown to be converted to dTpdT by this method.

Conversion of Internal Photodimers of Dinucleoside Phosphates to Pyrimidine Dimers. Samples of photoproducts (5 mg) were treated with 1 mL of 6 N HCl at 100 °C for 1 h. The solvent was removed under reduced pressure and the residual HCl was removed by repeated coevaporation with water. Crude products were recrystallized from distilled water to yield crystalline compounds which were identified by comparison with authentic samples of **5a** and **8a.** Compounds **3a** and **7a** yielded products which were identical in all respects to **5a** and **8a,** respectively.

Relative Reaction Rate Study. A conventional "merry-go-round" apparatus was used (Murov, 1973). The 313-nm line of a 450-W Hanovia medium pressure mercury arc was isolated by two filter systems: (i) a precooled solution, 3.5 mm in depth, of 0.6 g/L potassium chromate and 0.17 g/L sodium hydroxide circulating about the arc and (ii) Corning 7-54 filters. The irradiation tubes were made from a single length of Pyrex glass tubing of uniform o.d. and 6 mm i.d. The tubes were sealed to Ace Teflon right-angle valves. The volume of samples used was 1 mL.

The samples to be irradiated were phosphate buffer (5×10^{-3} M, pH 7.4) solutions containing 5×10^{-4} M in dTpdC or dTpdT and 10^{-3} M in acetophenone. The relative concentration of acetophenone to the dinucleoside phosphate was chosen such that the sensitizer absorbs almost all of the light. All samples were degassed by three freeze-pump-thaw cycles. The progress of the reaction was monitored by UV absorbance at 270 nm (1-mm quartz cell) with a solution of acetophenone at the same concentration as the reference. The absorbance of irradiated samples of dTpdC was rechecked after heating the sample at 50 °C for 1 h. No increase in absorbance was observed indicating that photohydration did not contribute to the observed decrease of absorbance.

Results and Discussions

A. *Direct Irradiation of dTpdC* (**1**). Mixed photodimer Thy[]Cyt has been detected in the UV-irradiated DNA and isolated as Thy[]Ura (Setlow & Carrier, 1966; Setlow et al., 1965). The formation of Thy[]Cyt may be substantial compared with the formation of Thy[]Thy. Since cytosine has the lowest singlet excited level among all bases in nucleic acids (Guéron et al., 1967; Lamola, 1973), singlet excitation energy will be mostly localized in cytosine groups upon direct excitation of polynucleotides or nucleic acids. We synthesized dTpdC as a model for adjacent TC bases in DNA. The irradiation of this dinucleoside phosphate had been previously investigated by Haug (1964). Irradiation of dTpdC in water resulted in the decrease in the absorption at 267 nm with a concomitant appearance of a new maximum at 245 nm. After a sample of the irradiated solution was warmed to 68 °C, a major portion of the decrease (70%) at 267 nm was restored. The result indicated that the major photochemical conversion was the photohydration of the cytosine group in the dinucleotide. In addition, three heat stable photoproducts were detected by TLC as reported by Haug (1964). One of them was tentatively identified as dTpdU according to its R_f values which might be formed via the photohydrate, hydrolytic deamination, and dehydration. No intramolecular photodimer was detected in the reaction mixture. Apparently, the singlet excited cytosine group in dTpdC undergoes bimolecular interaction with water (Greenstock & Johns, 1968) in the medium faster than the intramolecular photodimerization with the thymine group. Therefore, dTpdC serves only as a poor model for the direct irradiation of native DNA. In native DNA, cytosine and thymine groups are in the core of DNA where little or no water may come in contact with cytosine. Hydration of cytosine becomes relatively unimportant (Lamola, 1973), and pyrimidine dimer formation may thus be the primary photochemical process.

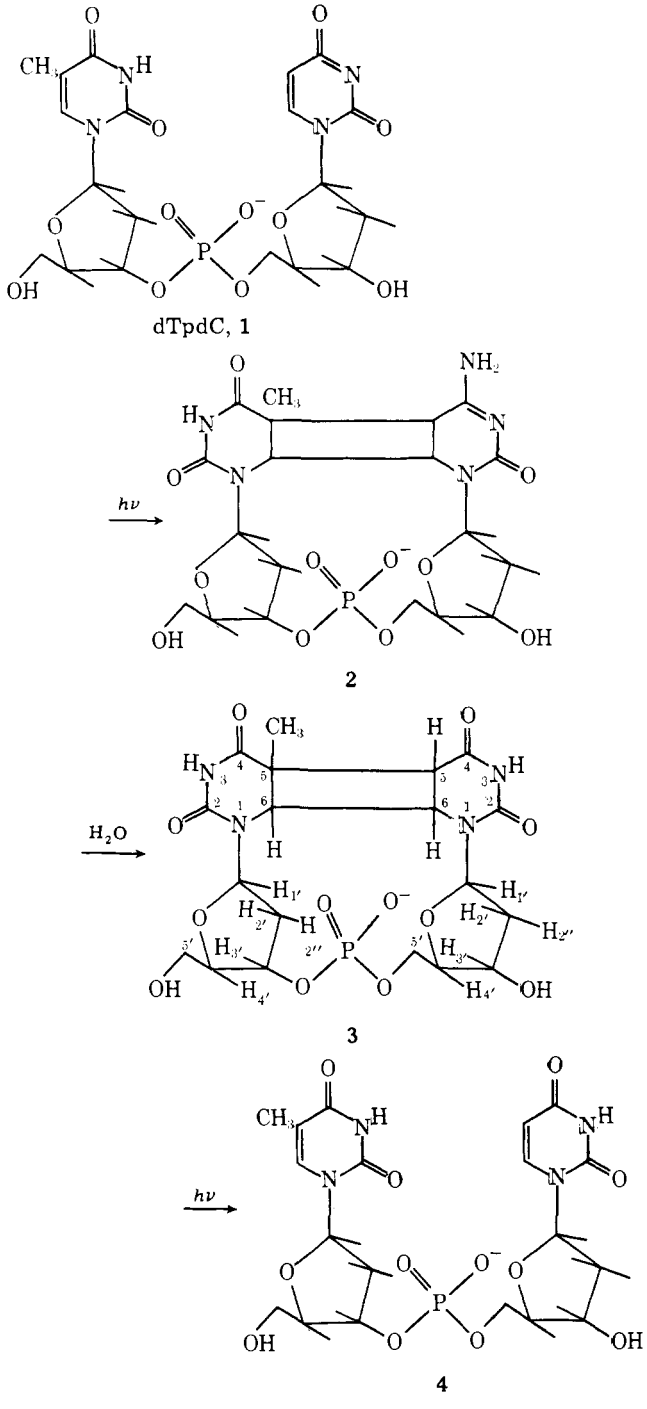
B. *Sensitized Irradiation of dTpdC* (**1**). On the bases that thymine has the lowest triplet energy level of all bases in nucleic acids and acetophenone has a triplet energy level similar to that of thymine, Lamola developed the experimental method to introduce triplet excitation energy selectively into the thymine groups in DNA via acetophenone sensitization (Guéron et al., 1967; Lamola, 1973). The chemical consequence of such a sensitized irradiation of native DNA is Thy[]Thy formation (Lamola & Yamane, 1967; Lamola, 1973). When this technique was applied to dTpdC, we found that the dinucleotide was consumed as indicated by both TLC and UV spectroscopy. Upon heating, not only the decrease in absorption at 267 nm was not restored indicating the absence of photohydrates, but also there was a further decrease in absorbance from 220–290 nm suggesting a dihydrocytosine to dihydrouracil conversion. Similar results were obtained when acetone was used as the sensitizer.

When we carried out the irradiation at 12 °C and worked up the reaction mixture carefully to avoid any unnecessary decomposition of heat sensitive products, we found that the products were already a mixture of dTp[]pdC and dTp[]pdU isomers. This result clearly indicates that the hydrolysis of dTp[]pdC to dTp[]pdU will occur under mild conditions and characterization of photoproducts such as dTp[]pdC will be extremely difficult. Qualitatively, we found that the average half-life of dTp[]pdC isomers with respect to their deamination to dTp[]pdU at 50 °C was less than 1 h. Biologically, this result implies that the photochemical lessions of dT[]dC dimers in DNA may also be facily converted to dT[]dU dimers.

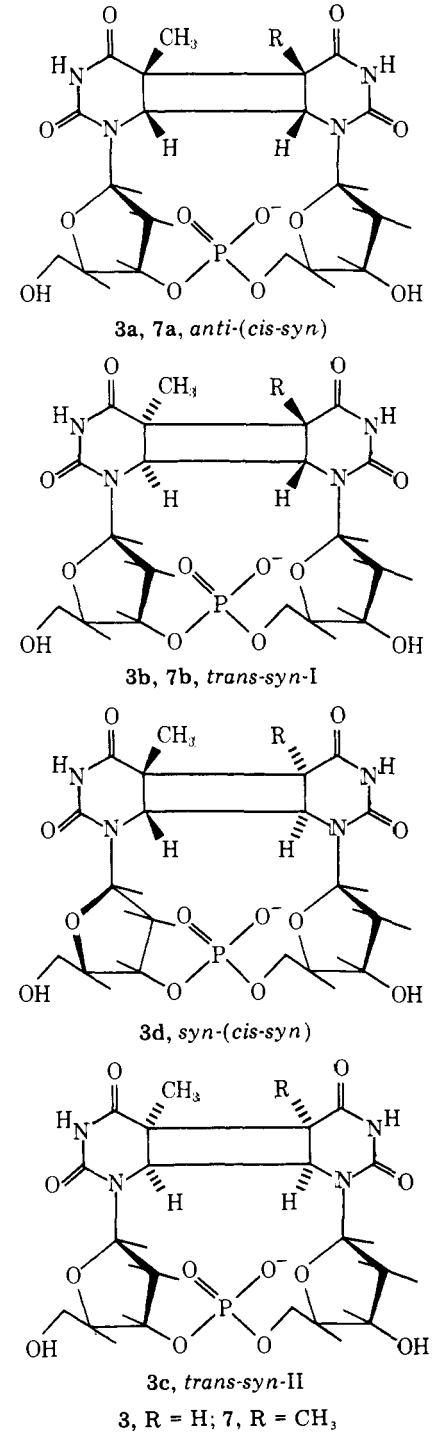
Since three isomeric dTp[]pdC (**2a–c**) were formed in this sensitized irradiation, the resulting system was a mixture of seven components including starting dTpdC, three isomeric dTp[]pdC and three dTp[]pdU. In order to simplify the workup, the dTp[]pdC were hydrolyzed to the corresponding dTp[]pdU isomers (**3a–c**) which were then separated by chromatography and characterized (Scheme II).

C. *Structural Determination of Photoproducts from the Sensitized Irradiation of dTpdC.* Preliminary spectroscopic analysis of **3a, 3b,** and **3c** indicates that they are isomeric internal photodimers of dTpdU, dTp[]pdU. All compounds exhibit only end absorption in their UV spectra. However, after the compounds have been irradiated with UV light at 254 nm, absorption maxima at 260 nm reappear in their UV spectra, a characteristic of pyrimidine photodimer. NMR spectra of these compounds exhibit, instead of signals for olefinic protons in dTpdC, signals for cyclobutane like protons at higher fields. There are two possible head-to-head mixed photodimers of thymine and uracil, Thy[]Ura(*c,s*) (**5a**) and Thy[]Ura(*t,s*) (**5b**). There are two possible isomeric nucleotides derivable from each mixed photodimer Thy[]Ura due to the difference in the arrangement of nucleotide phosphate linkage relative to the pyrimidine dimer group, *anti*-(*c,s*)-**3a** and *syn*-(*c,s*)-**3d** from **5a,** and (*t,s*)-I (**3b**) and (*t,s*)-II (**3c**) from **5b** (Scheme III). The head-to-tail isomers are excluded from consideration due to the geometrical constraint in this type of configuration. The structures were established by the following chemical degradations and high resolution NMR spectroscopy.

SCHEME II



SCHEME III



C-1. *Photoreversion.* Compounds **3a–c** were all converted to dTpdU by irradiation with UV light at 254 nm indicating that they are internal photodimers of dTpdU (**4**).

C-2. *Acid Hydrolysis.* Dinucleoside phosphates may be converted to their constituent bases by heating in 6 N HCl at 100 °C (Scheme IV). When **3a** was treated under this condition for 1 h, an acid stable product **5a** was formed, which was identified to be a heterodimer of thymine and uracil by comparison with an authentic sample prepared from the irradiation of thymine and uracil in frozen aqueous solution (Weinblum, 1967). The IR spectrum of this compound was reported to be identical with the Thy[]Ura isolated from UV-irradiated DNA (Weinblum, 1967), which is most probably the cis-syn isomer. The 270-MHz NMR spectrum of **5a** in 1 N NaOD exhibits a three-proton singlet at δ 1.37, which is attributable to the methyl group of the thymine moiety, and three peaks in an ABX pattern at δ 2.88 (doublet), 3.83 (doublet or doublets), and 3.68 (doublet) which may be assigned to the H_5 and H_6 protons of the uracil moiety and the H_6 proton of the thymine moiety, respectively. The coupling constant between the H_6 protons ($J_{6,6*}$)[2] was found to be 4.8 Hz. The $J_{6,6*}$ coupling constants for Thy[]Thy and Ura[]Ura in the cis-syn configuration have been reported to be 4.8–5.2 Hz from the C-13 satellite peaks of the respective H_6 signals (Anet, 1965; Blackburn & Davis, 1966; Hollis & Wang, 1967; Fahr et al., 1972). The structures of *cis-syn*-Thy[]Thy and Ura[]Ura have been elucidated unambiguously by X-ray diffraction analysis

[2] For simplicity, the H_6 proton of the thymine moiety in the photodimers of thymine and uridine derivatives is determined as H_{6*}. The coupling constant between the H_6(U) and H_{6*}(T) protons is $J_{6,6*}$.

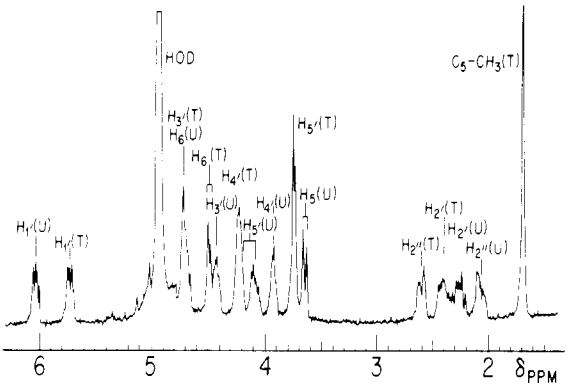


FIGURE 1: NMR (270 MHz) spectrum of internal photodimer of dTpdU (**3a**) in D_2O.

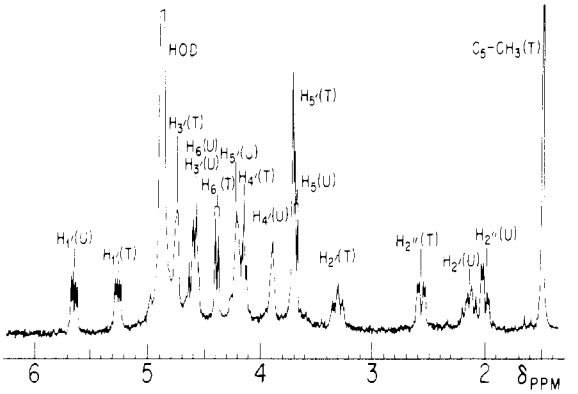
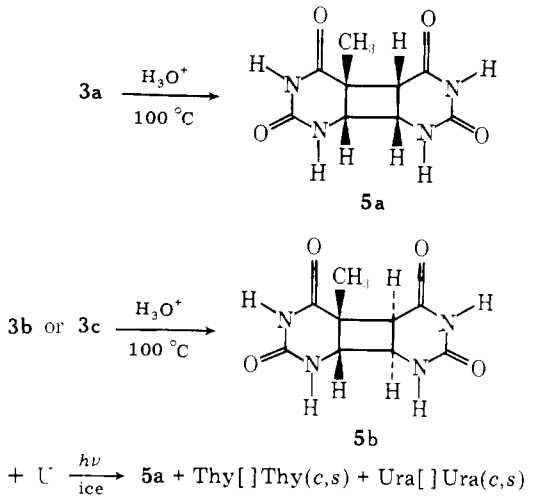


FIGURE 2: NMR (270 MHz) spectrum of internal photodimer of dTpdU (**3b**) in D_2O.

SCHEME IV



$$T + U \xrightarrow[\text{ice}]{h\nu} \mathbf{5a} + \text{Thy[]Thy}(c,s) + \text{Ura[]Ura}(c,s)$$

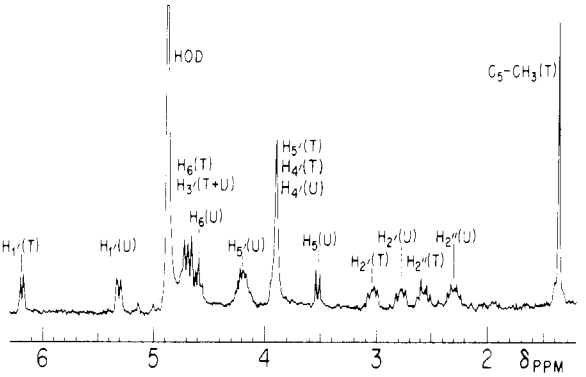


FIGURE 3: NMR (270 MHz) spectrum of internal photodimer of dTpdU (**3c**) in D_2O.

(for a review, see Karle, 1976). The consistency in the coupling constants can be considered as an additional support for the structure of **5a** to be Thy[]Ura(*c,s*). Compounds **3b** and **3c** were similarly hydrolyzed to yield **5b,** identified as a Thy[]Ura based on its photoreversion to thymine and uracil, but it is different from **5a.** FT-NMR spectrum (270 MHz) of **5b** in D_2O exhibits a three-proton singlet at δ 1.42 which is assigned to the methyl group of the thymine moiety and three peaks in an ABX pattern at δ 3.61 (doublet), 4.22 (doublet of doublets), and 3.98 (doublet) which are assigned to the H_5 and H_6 protons of uracil moiety and the H_6 proton of the thymine moiety, respectively. Since the H_5 proton of the uracil moiety is a doublet, it indicates that **5b** is also a head-to-head dimer and it must be trans-syn by inference. Compound **5b** is more polar than **5a** in its chromatographic behaviors which is consistent with the observed polarity trend for other isomeric pyrimidine photodimers (Weinblum & Johns, 1966; Elad et al., 1971; Weinblum et al., 1968); Thy[]Ura(*t,s*) is thus characterized for the first time. The coupling constant $J_{6,6*}$ of **5b** is 7.0 Hz; however, there is no reliable report in the literature on the value of $J_{6,6*}$ of trans-syn photodimers of pyrimidines for comparison.

Therefore, the Thy[]Ura moiety of *3a* has the cis-syn stereochemistry, while those in **3b** and **3c** have the trans-syn stereochemistry. Detailed analysis of the NMR spectra of these compounds verify this assignment (vide infra).

C-3. *The 270-MHz NMR Spectra of Compound 3a-c.* NMR spectra (270 MHz) for **3a** (Figure 1), **3b** (Figure 2), and **3c** (Figure 3) were obtained in D_2O. Most protons exhibit resolved signals. The proton assignments were made on the basis of chemical shift data of some reference compounds, and verified by spin decoupling experiments. Values for chemical shifts and coupling constants of **3a-c** together with reference compounds are summarized in Tables I, II, and III. The numbering of protons is indicated in the structural formula of **3.** The structural assignments based on the NMR spectra are discussed in two parts: (a) the cyclobutane ring protons and (b) the deoxyribose ring protons.

(a) *The Cyclobutane Ring Protons.* The cyclobutane protons for **3a-c** appear as ABX systems at δ 3.5-3.7 for the X proton and 4.4-4.7 for the A and B protons, respectively. The doublet at δ 3.5-3.7 is assigned to the H_5 proton of the uracil moiety, and the signals (2 H) in the δ 4.4-4.7 region are assigned to the H_6 protons of thymine (H_{6*})[2] and uracil (H_6) moieties. Since the H_{6*} proton is only coupled with the H_6 proton, while the H_6 proton is coupled with both the H_{6*} and the H_5 protons, the H_{6*} and H_6 protons will appear as a doublet and a doublet of doublets, respectively. In dinucleoside phosphates, the $H_{3'}$ protons of the deoxyribose moieties also appear in the same region as the H_6 protons and thus may interfere with the assignment. It was found that $H_{3'}$ signal interferes with the H_{6*} signal of **3c** and with the H_6 signal of **3a** and **3b.** However, the assignments were resolved unambiguously by spin decoupling experiments, i.e., H_5 is coupled only with H_6, while H_6 is coupled with both H_5 and H_{6*}. The results are summarized in Table I.

The coupling constants $J_{6,6*}$ in the cyclobutane rings were found to be 5.2 Hz for **3a** and 7.5 Hz for **3b** and **3c.** Since these values are in good agreement with the corresponding ones for **5a** and **5b,** the result indicates that the conformations of cy-

TABLE I: Chemical Shift (ppm from TSP Standard)[a] and Coupling Constant (Hz) Data of the Cyclobutane Protons of **3**, **5**, and **7** (D_2O, 20 ± 1 °C).

	5a[b]	**5b**	**3a**	**3b**	**3c**	**7a**	**7b**
C_5-CH_3(T)[c]	1.37	1.42	1.68	1.51	1.33	1.54 1.49	1.51 1.45
H_5(5′ or U)[c]	2.88	3.61	3.63	3.70	3.51		
$J_{5,6}$	J = 7.5	J = 9.9	J = 8.3		J = 8.7		
H_6(5′ or U)[c]	3.83	4.22	4.71	4.59	4.58	4.36	4.27
H_6(3′ or T)[c]	3.68	3.98	4.49	4.40	4.67	4.27	4.27
$J_{6,6*}$	J = 4.8	J = 7.0	J = 5.2	J = 7.5	J = 7.5	J = 5.4	J = 7.3

[a] *tert*-Butyl alcohol was used as internal reference which has methyl singlet at 1.25 ppm from TSP. [b] Solvent D_2O–1 N NaOD. [c] T and U in the parentheses refer to the thymine and uracil moiety of the pyrimidine photodimers, respectively, and 5′ and 3′ refer to 5′-nucleotidyl and 3′-nucleotidyl moiety of the dinucleoside phosphates, respectively.

TABLE II: Chemical Shift Data of the Deoxyribose Protons of **3**, **7**, and Reference Compounds (D_2O, 20 ± 1 °C, ppm from TSP).[2,a]

		$H_{1'}$	$H_{2'}$	$H_{2''}$	$H_{3'}$	$H_{4'}$	$H_{5'}$
dTpdC	(5′)[b]	6.22	2.31	2.31	4.57	4.15	4.12
	(3′)	6.22	2.31	2.48	4.77	4.18	3.79
dTpdT	(5′)	6.31	2.38	2.38	4.61	4.15	4.15
	(3′)	6.21	2.38	2.58	4.81	4.21	3.83
3a	(5′)	6.03	2.27	2.07	4.43	3.92	4.09 4.22
	(3′)	5.73	2.38	2.61	4.71	4.22	3.73
3b	(5′)	5.66	2.17	2.02	4.59	3.90	4.22
	(3′)	5.27	3.32	2.59	4.74	4.16	3.70
3c	(5′)	5.31	2.77	2.29	4.65	3.88	4.19
	(3′)	6.19	3.01	2.55	4.65	3.88	3.88
7a	(5′)	5.96	2.32	2.11	4.36	3.94	4.02 4.11
	(3′)	5.64	2.38	2.63	4.67	4.17	3.72
7b	(5′)	5.71	2.22	2.03	4.57	3.92	4.27
	(3′)	5.28	3.34	2.62	4.77	4.16	3.73
pdT	(5′)[c]	6.33	2.41	2.32	4.57	4.16	3.99
pdC	(5′)	6.27	2.39	2.23	4.52	4.17	4.04
dTp	(3′)	6.27	2.36	2.49	4.76	4.15	3.79
dTH_2[d]		6.27	2.23	2.23	4.37	3.86	3.70

[a] *tert*-Butyl alcohol was used as internal reference which has a methyl singlet at 1.25 ppm from TSP. [b] The numbers 5′ and 3′ in the parentheses refer to the 5′-nucleotidyl and 3′-nucleotidyl moiety of the dinucleoside phosphates, respectively. [c] Davies & Danyluk (1974). [d] Kondo & Witkop (1968).

clobutane rings are not changed appreciably from the pyrimidine photodimers to the internal photodimers of dinucleoside phosphates.

Chemical shifts for the C_5 methyl group of the thymine moiety are δ 1.68, 1.51, and 1.33 for **3a**, **3b**, and **3c**, respectively. An anisotropic diamagnetic shielding to higher field is predicted for protons situated above the plane of a carbonyl group (Jackman & Sternhell, 1969a) and such shieldings have been observed previously (Chapman & Smith, 1961; Werbin & Strom, 1968). Examination of molecular models indicates that the methyl group in a trans-syn isomer **3b** or **3c** should be more shielded by the C_4 carbonyl on the opposite pyrimidine ring than that in the cis-syn isomer **3a**. We have also found that the methyl protons in the unstable precursor **2b** and **2c** are at δ 1.57 and 1.42, respectively, which are less shielded than those in **3b** and **3c**, while the methyl protons in **3a** and its precursor **2a** have very close chemical shifts. The methyl protons in the trans-syn configuration, where they are situated above the opposite pyrimidine rings, will be more effected by the change in the diamagnetic anisotropic shielding from the conversion of an enamine function in cytosine to a carbonyl group in uracil, i.e., 2 → 3.

These results together verify that **3a** is in the cis-syn configuration, while **3b** and **3c** are in the trans-syn configuration.

(b) *The Deoxyribose Ring Protons.* The next problem in the structural assignments for **3a–c** is to determine the stereochemical relationship between the cyclobutane ring and the sugar phosphate. These assignments may be made by the comparison of chemical shifts of their deoxyriboside protons with reference compounds.

Two isomeric dTp[]pdU dinucleoside phosphates may be derived from a given heterophotodimer of dT[]dC due to the difference in the position of the sugar–phosphate linkage rel-

TABLE III: Coupling Constants between the Deoxyribose Protons of **3**, **7**, and Reference Compounds (Hz).

		$J_{1',2'}$	$J_{1',2''}$	$J_{2',2''}$	$J_{2',3'}$	$J_{2'',3'}$
TMP-5′[a]		7.6	6.2	−14.0	6.6	2.6
dCMP-5′[a]		7.0	6.3	−14.1	6.0	4.0
dTpdT	(5′)	6.5	6.5	*b*	*b*	*b*
	(3′)	6.6	6.6	−14.7	*b*	3.3
3a	(5′)	9.0	6.0	−13.5	8.4	3.3
	(3′)	9.6	4.5	−15.0	5.1	*c*
3b	(5′)	10.5	4.7	−13.5	9.0	*c*
	(3′)	10.5	5.0	−14.3	4.5	*c*
3c	(5′)	9.0	*c*	−13.5	7.2	8.1
	(3′)	9.6	*c*	−15.0	9.6	9.0
7a	(5′)	8.5	5.6	−13.5	6.6	3.2
	(3′)	5.1	8.2	−13.8	3.0	5.0
7b	(5′)	10.2	4.5	−13.8	*b*	*b*
	(3′)	10.2	4.5	−15.0	*b*	*b*

[a] Davies & Danyluk (1974). [b] Unable to discern. [c] Small value (<2 Hz).

ative to the juncture of the cyclobutane ring. Therefore, (*c,s*)-**5a** may form dTp[]pdU **3a** and **3d.** Compound **3a** contains the sugar–phosphate linkages orienting away from the C_2 carbonyl of the pyrimidines or has the anti-(cis-syn) conformation,[2] while **3d** contains the sugar–phosphate linkage in the opposite direction or has the syn-(cis-syn) conformation. The heterophotodimer (*t,s*)-**5b** may form two dTp[]pdU dinucleoside phosphates, **3b** containing the deoxyribosyl-3′-phosphate linkage syn to the C_2 carbonyl group of thymine and the deoxyribosyl-5′-phosphate linkage anti to the C_2 carbonyl of uracil, while **3c** contains both sugar phosphate linkages in the syn configuration.

By examining the molecular models of internal photodimers **3a–c** and dTpdC, it is clear that the $H_{5'}$ protons of the dTp moiety are comparatively isolated from the rest of these molecules. The chemical shift of these $H_{5'}$ protons thus will not vary appreciably from thymidyl 3′-phosphate to dTpdC and the internal photodimers **3a–c.** By assigning the two-proton peak at δ 3.8 of these compounds to the $H_{5'}$ protons of the dTp moiety, the chemical shifts for individual deoxyriboside protons in both sugar units may then be established by extensive spin decoupling experiments. The deoxyriboside protons in **3a–c** appear in three groups of signals and are listed in Table II:

(i) Two sets of doublets of doublets at δ 5.2–6.2 region for the $H_{1'}$ protons. Unlike the two $H_{1'}$ protons in dTpdC which have overlapping chemical shifts, these two sets of peaks are separated by more than 0.3 ppm and vary appreciably in chemical shifts for different isomers.

(ii) Four separate one-proton signals in the δ 2.0–3.3 region for the $H_{2'}$ protons ($H_{2'}$ and $H_{2''}$). They exhibit both the geminal coupling and vicinal couplings with the $H_{1'}$ and $H_{3'}$ protons, and may thus be broad doublet, doublet of doublets, triplet of doublets, octet, or multiplet depending on the size of the vicinal coupling constants. Compared with the $H_{2'}$ protons in dTpdC, these signals are more widely separated and are shifted to lower field. This implies more interaction between the $H_{2'}$ protons and other protons and functional groups in **3a–c.**

The assignments of $H_{2'}$ and $H_{2''}$ protons (see structural formula **3** for the numbering) were made with the aid of molecular models. Basically, $H_{2''}$ of pdU moiety of the dinucleoside phosphate is at higher field than $H_{2'}$ of the same deoxyriboside. This is due to the anisotropic shielding effect of the hydroxyl group at the $C_{3'}$ position which is cis to the $H_{2''}$ proton (Davies & Danyluk, 1974; T'so et al., 1969; Schweizer et al., 1968). On the other hand, $H_{2''}$ of the dTp moiety is at lower field than the $H_{2'}$ proton due to the field effect of the phosphate group (Davies & Danyluk, 1975; Fang et al., 1971).

The coupling constants $J_{1',2'}$, $J_{2',2''}$, $J_{2',3'}$, and $J_{2'',3'}$ are resolved by spin decoupling experiments and are listed in Table III.

(iii) Groups of peaks in the δ 3.7–4.7 region for the $H_{3'}$, $H_{4'}$, and $H_{5'}$ protons. The chemical shifts of these protons are of the same order as those in dTpdC. For **3a** and **3b,** two $H_{3'}$ signals are separated and they may be differentiated by their relationship with the $H_{5'}$ protons of the dTp moiety at δ 3.8 through spin decoupling via the $H_{4'}$ signal.

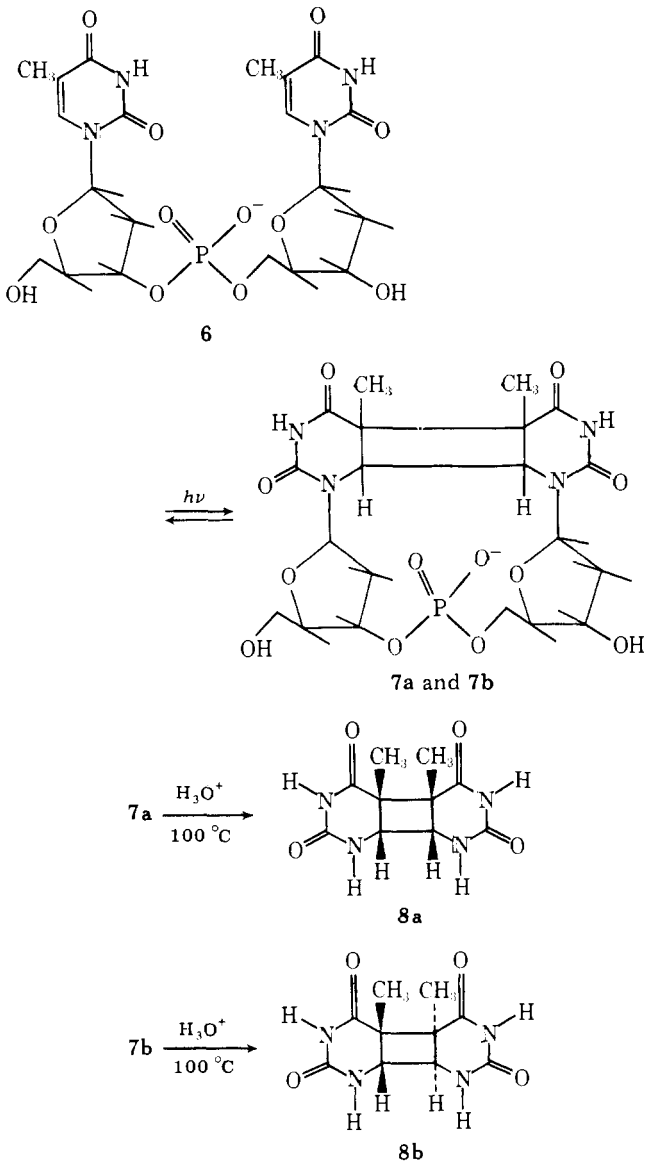
(iv) *anti*-dTp[]pdU(*c,s*), **3a.** The effects of the position of C_2 carbonyl on the shielding and deshielding of various riboside protons in nucleosides and their derivatives have been reported previously. For example, the NMR of 6-methylcytidine and 6-methyluridine show the downfield shifts of 0.5–0.6 ppm for $H_{2'}$, 0.15–0.20 ppm for $H_{3'}$, and the upfield shift of 0.16–0.25 ppm for $H_{1'}$ relative to the 5-methyl derivatives (Schweizer et al., 1971). The results indicate that the 6-methyl nucleosides are in the syn conformation rather than the usual anti conformation (T'so, 1974) for pyrimidine nucleosides. Apparently, the stereointeraction between the 6-methyl group and the ribose ring causes the syn conformation to be more favorable. Similar shifts have been observed also for orotidine and ribosyl-β-cyanuric acid with respect to uridine (Hruska, 1971; Dugas et al., 1971). Therefore, the chemical shifts of $H_{1'}$, $H_{2'}$, and $H_{3'}$ may be diagnostic for the structural assignments (Table II).

Examination of molecular models indicates that in *syn*-dTp[]pdU(*c,s*), **3d,** the C_2 carbonyl groups of pyrimidines are in the proximity of the $H_{2'}$ and $H_{3'}$ protons of both deoxyribose rings, while, in **3a,** they are in the proximity of the $H_{1'}$ protons. One would expect a large shift of the $H_{2'}$ proton peaks in **3d.** Since the chemical shift of $H_{2'}$ protons in **3a** is not appreciably different from those in dTpdC and dTpdT and other nucleotides which are known to be in the anti conformation, and the peaks for $H_{1'}$ protons are shifted to the lower field, the anti-(*c,s*) conformation is assigned to **3a.**

(v) Compounds **3b** and **3c.** There are two possible isomers for dTpdU derived from dT[]dU(*t,s*), *trans-syn*-I, and *trans-syn*-II (Scheme III). NMR spectrum of **3b** shows a dramatic shift of one of the $H_{2'}$ protons of the dTp moiety ($H_{2'}$(T)) to downfield (ca. 1 ppm to **3a**). Examination of space-filling models of both isomers indicates that in *trans-syn*-I the $H_{2'}$(T) is "surrounded" by the C_2 carbonyl of the Tp moiety and the phosphate oxygen. This downfield shift may thus be accounted for, and *trans-syn*-I configuration is assigned to **3b.** Supporting this assignment is the upfield shift of the $H_{1'}$ proton of the Tp moiety of **3b** which is less deshielded by the C_2 carbonyl group of the thymine moiety than that of **3a.** The remaining *trans-syn*-II structure is assigned to **3c** by inference. The proton assignments of **3c** are only tentative due to the overlap of the signals for the $H_{3'}$ and $H_{4'}$ protons. However, downfield shifts of both $H_{2'}$ protons are consistent with the structure *trans-syn*-II.

D. *Structural Determination of Photoproducts from dTpdT.* Two products, **7a** (80%) and **7b** (14%), were isolated from the acetophenone sensitized irradiation of dTpdT (**6**), while four products were formed in the direct irradiation of **6** (Johns et al., 1964). Compounds **7a** and **7b** were both converted

SCHEME V



into dTpdT by irradiation with short wavelength light and hydrolyzed by acid to the known Thy[]Thy(*c,s*) and Thy[]Thy(*t,s*) (**8a** and **8b**), respectively. These results indicate that they are internal cyclobutane photodimers of dTpdT (Scheme V).

The NMR spectra of **7a** and **7b** (Figures 4 and 5) are very similar to those of **3a** and **3b.** Chemical shifts and coupling constants are listed in Tables I, II, and III. By comparing the NMR spectra of **3** and **7,** we find that the cyclobutane protons are more shielded in **7.** Theoretical consideration suggested that a proton cis to a methyl group on a cyclobutane ring should be more shielded (Jackman & Sternhell, 1969b), which may account for the higher shielding of methyl protons in **7a** relative to those in **3a.** Therefore, anti-(*c,s*) structure was assigned to **7a** and *trans-syn*-I was assigned to **7b.** It is to be noted that the coupling constants between two H_6 protons ($J_{6,6*}$) for **7a** and **7b** are 4.8 and 7.3 Hz, respectively, and are in good agreement with those of **3a** and **3b.**

E. *The Relationship between the Conformation of Dinucleoside Phosphate and the Structure of Photoproducts.* Dinucleoside phosphates have been reported to exist mainly in the "anti,anti,right-handed" conformation, in which both nucleosidyl units have the anti conformation with respect to the sugar-base torsion angle (T'so et al., 1969; Fang et al., 1971; Chan & Nelson, 1969). It is of interest to note that the major photoproducts from dTpdC (53% of total products) and, especially, from dTpdT (80%) are in the anti,anti configuration with respect to the sugar-base torsion angle. Pyrimidine bases in dinucleoside phosphates are associated in the ground state by a π-stacking interaction (Brown et al., 1968) and also in the excited state by the formation of exciplex (Eisinger & Lamola, 1971). Therefore, the formation of cyclobutane photodimers from dinucleoside phosphates is expected to favor the retention of the conformation of dinucleoside phosphates in the ground state. Our results not only offer additional support for the conformation of dinucleoside phosphates but also suggest that dTpdC has relatively less π-stacking interaction than dTpdT. It may also be noted that the cyclobutane photodimer in the syn-(*c,s*) configuration such as **3d,** which has both nucleosidyl units in the syn configuration, is not found among the photoproducts.

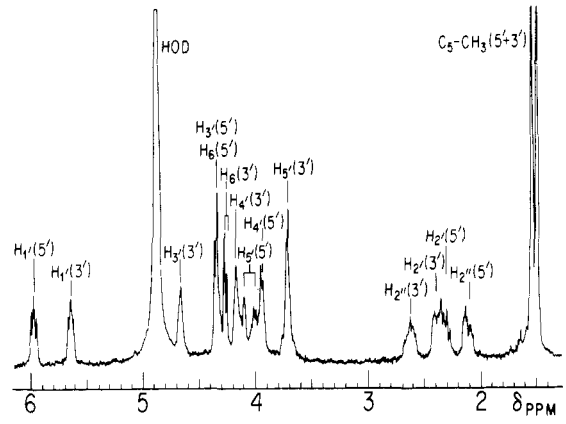


FIGURE 4: NMR (270 MHz) spectrum of internal photodimer of dTpdT (**7a**) in D_2O.

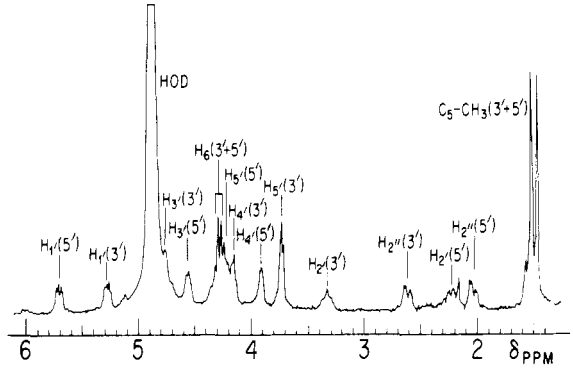
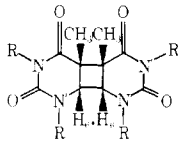


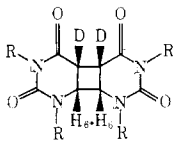
FIGURE 5: FT-NMR (270 MHz) spectrum of internal photodimer of dTpdT (**7b**) in D_2O, 16 scans.

F. *Coupling Constants between Cyclobutane Protons in Pyrimidine Photodimers.* Since we have observed in this study several coupling constants between the cyclobutane protons in pyrimidine photodimers, it will be of interest to compare these values with those reported in the literature to see whether coupling constants are diagnostic for configuration assignments. The coupling constants are listed in Table IV. The $J_{6,6*}$ for all of the cis-syn photodimers studied fall in the range of 4.8–5.2 Hz. The only reported value for $J_{6,6*}$ of a trans,syn pyrimidine photodimer is that of the dimethyluracil photodimer **11b** which is not in agreement with our data. Fahr & coworkers (1972) have analyzed the spin-coupling patterns of cyclobutane protons in the 100-MHz NMR spectrum of four dimethyluracil photodimers as AA'BB' systems with the aid

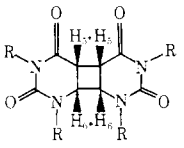
TABLE IV: Coupling Constants in Cyclobutane Pyrimidine Photodimers (Hz).



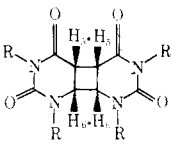
9a, R = CH_3
b, R = H



10, R = CH_3



11a, R = CH

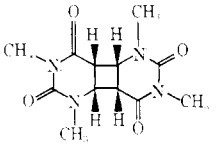


11b, R = CH_3

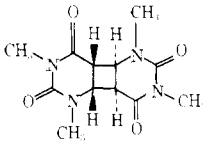
	cis-syn photodimers							trans-syn photodimers				
	9a	**9b**	**10**	**3a**	**5a**	**7a**	**11a**	**3b**	**3c**	**5b**	**7b**	**11b**
$J_{5,5*}$							9.27					7.31
$J_{5,6}$				8.3	7.5		8.13	*a*	8.7	9.9		9.82
$J_{6,6*}$	4.8	5.2	5	5.2	4.8	5.4	4.19	7.5	7.5	7.0	7.3	3.15
ref	*b*	*c*	*d*	*e*	*e*	*e*	*f*	*e*	*e*	*e*	*e*	*f*

[a] Unable to discern. [b] Anet (1965). [c] Hollis & Wang (1967). [d] Blackburn & Davies (1966). [e] This work. [f] Fahr et al. (1972).

TABLE V: Chemical Shifts and Coupling Constants of Cyclobutane Protons of **11a–d.**



11c



11d

	$J_{5,5}$	$J_{5,6}$	$J_{5,6*}$	$J_{6,6*}$	H_5 (Hz)	H_6 (Hz)
11a	9.31	7.92	1.70	4.21	1031.4	1108.5
11b	2.81	9.79	−1.63	7.01	987.1	1064.4
11c		8.25	8.25		1029	1119
11d		9.20	5.00		966	1117.5

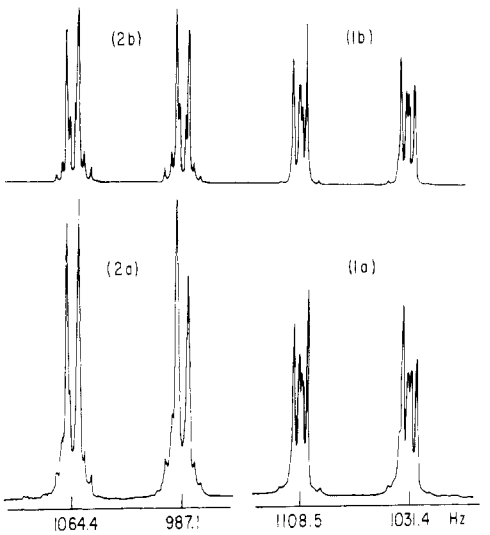


FIGURE 6: NMR spectra (270 MHz) of the cyclobutane protons of **11a** (1a) and **11b** (2a) and computer simulated spectra of **11a** (1b) and **11b** (2b).

of computer simulation. They have obtained a set of coupling constants for each isomer. Those for cis-syn and trans-syn isomers are included in Table IV. Although $J_{6,6*}$ for the cis-syn isomer (4.19 Hz) is in reasonable agreement with $J_{6,6*}$ of other photodimers, the one for trans-syn isomer (3.15 Hz) deviates appreciably from what we have observed for trans-syn photodimers. In the computing and matching procedures for obtaining the coupling constants, Fahr & co-workers had to estimate the values of $J_{5,5*}$, $J_{6,6*}$, $J_{5,6*}$, and $J_{6,5*}$ from the vicinal and long-range coupling constants between cyclobutane protons of a number of compounds reported in the literature. The vicinal coupling constants in NMR are known to be sensitive to the change in the conformation of cyclobutane ring and the electronegativity of substituents (Karplus, 1963). The Karplus equation of the dependence of vicinal coupling constant on the dihedral angle may break down if one of the carbon atoms carries an electronegative substituent. Since C_6 and $C_{6'}$ of these photodimers are attached to the 1 and 1′ nitrogen, the assignment by Fahr & co-workers may be hazardous unless the coupling constants from closely related compounds are known. We have also analyzed the coupling patterns of cyclobutane protons of photodimers of dimethyluracil as AA′XX′ systems. An AA′XX′ spectrum may be treated by an analytical approach in which the sums and differences of coupling constants are obtained directly from the spacing of lines in the spectrum (Günther, 1972). The coupling constants resolved in this manner may be checked with the computer simulation technique (Figure 6). Through this approach two sets of coupling constants were obtained for the cis-syn and trans-syn isomers of dimethyluracil photodimers which are very close to those reported by Fahr (Table V). Due to the symmetrical nature of these spectra, the coupling constants $J_{A,A'}$ and $J_{X,X'}$ obtained may be assigned to $J_{5,5*}$ and $J_{6,6*}$, respectively, or vice versa. For the trans-syn photodimer of dimethyluracil, two sets of coupling constants are obtained, 7.31 Hz and 3.15 Hz. Since 7.31 Hz is much closer to our observed values for $J_{6,6*}$ of other photodimers in the trans-syn configuration, we believe that the assignment of Fahr will have to be reversed. In the cyclobutane photodimers we have studied, the vicinal coupling constant J_{cis} is smaller than J_{trans}, although J_{cis} is generally found to be larger than J_{trans} for simple cyclobutane derivatives (Fleming & Williams, 1967). Therefore, the assignment of stereochemistry of cyclobutane compounds on the basis of vicinal coupling constants may be hazardous unless a comparison may be made between compounds of closely related structures. It may be noted, however, that $J_{6,6*}$ of 7.5 and 5.2 Hz have been assigned to the cis-syn and trans-syn internal photodimers of "abbreviated" dinucleoside respectively (Logue & Leonard, 1972). This variation of the vicinal coupling constants from our values may be due to the change of the conformation of cyclobutane ring.

G. *Comparison of the Rates of Internal Photodimerization of dTpdC and dTpdT.* Since the relative reactivity of triplet excited thymine with adjacent pyrimidine bases will determine the relative population of Thy[]Cyt and Thy[]Thy dimers formed in the sensitized irradiation of DNA, dTpdC and

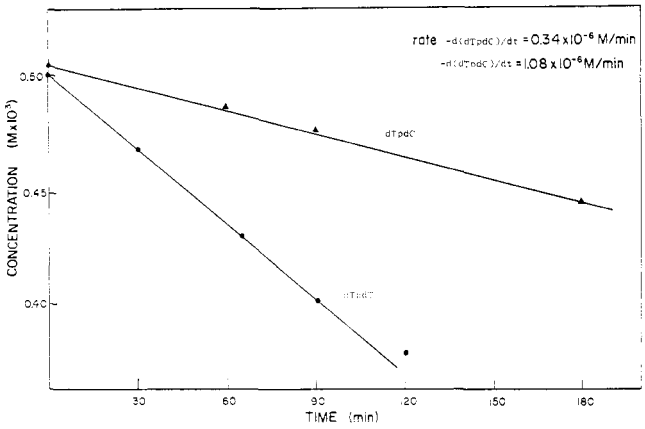


FIGURE 7: Kinetics of acetophenone-sensitized intramolecular photodimerization of dTpdC and dTpdT.

dTpdT were irradiated both simultaneously in the same solution and parallelly in two different solutions. dTpdT was found to dimerize 5–6 times faster than dTpdC when they were irradiated simultaneously and about 3 times faster when irradiated in parallel (Figure 7). Acetophenone has a triplet energy comparable to that of thymine but lower than those of other bases (Lamola et al., 1967). Acetophenone may thus transfer its triplet energy selectively to thymine but not necessarily at a diffusion controlled rate. Therefore, dTpdT may be excited by acetophenone sensitization more efficiently than dTpdC on a statistical basis. The diversity in the rate of dimerization of two dinucleoside monophosphates also suggests that cytosine may have a lower reactivity than thymine with thymine triplet. Photosensitization has been found to yield pyrimidine dimers in solution (for a review, see Fisher & Johns, 1976), and this technique has been used to introduce mainly thymine photodimers into DNA (Lamola, 1969; Meistrich & Lamola, 1972). However, the relative yield of Thy[]Cyt to Thy[]Thy (0.03:1.00) in the acetophenone sensitized irradiation of DNA (Lamola, 1973) is much less than what would be expected from our result of the relative efficiency of the acetophenone sensitized dimerization of model compounds of dTpdC and dTpdT. This discrepancy may be due to the higher restraint of cytosine group in DNA for the internal Thy[]Cyt formation exerted by three H bonds in G:C pairs than that of thymine group for the internal Thy[]Thy formation exerted by two H bonds in A:T pairs. Since cytosine is less reactive than thymine with triplet thymine in the dinucleoside phosphates to begin with, the steric restriction may have a more pronounced effect on the reactivity of cytosine than that of thymine in photodimer formation via triplet sensitization in DNA. It is probable that the difference in relative yields of Thy[]Cyt to Thy[]Thy formation via sensitization will be much less in single strand DNA or locally denatured DNA than in native DNA.

Conclusion

Our experimental results on the sensitized irradiation of dinucleoside phosphates, dTpdC and dTpdT, together with the reports in the literature on the direct irradiation of DNA (Setlow & Carrier, 1966; Setlow et al., 1965) demonstrated that the Thy[]Cyt mixed dimer may be formed photochemically in appreciable yield relative to the Thy[]Thy dimer. We have shown that Thy[]Cyt will undergo deamination under relatively mild experimental conditions. In Scheme I, the first step may be either a direct or sensitized photoreaction of DNA and the last step may be effected by photoreactivation in the presence of photoreactivating enzymes. If the deamination of the intermediate Thy[]Cyt has occurred before the photoreactivation in DNA, these transformations may result in the conversion of cytosine to uracil. Our study on the acetophenone-sensitized irradiation of dTpdC, the conversion of dTp[]pdC to dTp[]pdU and the reversion of dTp[]pdU to dTpdU may thus provide a molecular basis of UV-induced mutagenesis in bacteriophage systems.

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