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The Photochemistry of Thymidylyl-(3′-5′)-5-methyl-2′-deoxycytidine in Aqueous Solution¶

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ABSTRACT

The photochemistry of the dinucleoside monophosphate thymidylyl-(3′-5′)-5-methyl-2′-deoxycytidine (Tpm^{5}dC) has been studied in aqueous solution using both 254 nm and UV-B radiation. A variety of dinucleotide photoproducts containing 5-methylcytosine (m^{5}C) have been isolated and characterized. These include two cyclobutane dimers (CBD) (the *cis-syn* [*c,s*]and *trans-syn* forms), a (6-4) adduct and its related Dewar isomer, and two isomers of a product in which the m^{5}C moiety was converted into an acrylamidine. Small amounts of thymidylyl-(3′-5′)-thymidine (TpT) were also formed, presumably as a secondary photoreaction product. In addition, a photoproduct was characterized in which the m^{5}C moiety was lost, thus generating 3′-thymidylic acid esterified with 2′-deoxyribose at the 5-hydroxyl on the sugar moiety. The *c,s* CBD of Tpm^{5}dC readily undergoes deamination to form the corresponding CBD of TpT. The kinetics of this deamination process has been studied; the corresponding enthalpy and entropy of activation for the reaction have been evaluated at pH 7.4 as being, respectively, 73.4 kJ/mol and −103.5 J/K mol. Deamination was not observed for the other characterized photoproducts of Tpm^{5}dC.

INTRODUCTION

The photochemistry of 5-methylcytosine (m^{5}C) has been a topic of a significant amount of interest during the last 15 years. This particular nucleobase is a significant constituent of mammalian and plant DNA. For example, methylated cytosines (Cyt) account for about 5% of the total Cyt residues in calf thymus DNA (see table 1.2, pp. 6–7 in Ref. 1); a similar level of methylation of Cyt is observed in human DNA as well (2). In plant DNA, the level of methylation of Cyt is considerably higher. For example, about 31% of the Cyt are in the form of m^{5}C in wheat DNA (1).

The presence of m^{5}C in DNA has potential for increasing the amount of light absorbed by that DNA in the UV-B region of the spectrum. This nucleobase has an absorption spectral profile that is shifted toward longer wavelengths, as compared with Cyt itself. For example, the absorption spectrum of 5-methyl-2′-deoxycytidine (m^{5}dC) displays an absorption maximum at 278 nm, as compared with 271 nm for 2′-deoxycytidine (3). This implies that m^{5}C residues are more likely to capture light in the UV-B region of the spectrum than Cyt residues. This, in turn, indicates that substituting Cyt in DNA with m^{5}C will increase the UV-B absorbance of the DNA, as compared with DNA not containing m^{5}C.

In the past few years, it has become clear that m^{5}C residues within DNA play an important biological role (for an overview, see Ref. 4). Methylated Cyt appear to be involved in the regulation of chromatin structure and gene expression (5), whereas changes in normal patterns of Cyt methylation seem to be involved in tumorigenesis (Ref. 6 and references therein), ageing (Refs. 7,8 and references therein) and a variety of human syndromes involving abnormal gene expression (9). On the photobiological level, it appears that m^{5}C photoproducts, in particular *cis-syn* (*c,s*) cyclobutane dimers (CBD), are likely to be involved in mutagenic events. A variety of studies have demonstrated that mutagenic hot spots in UV-B–irradiated DNA are associated with 5′-pyrimidine-m^{5}C-guanine sequences (see for example Refs. 10–13 and references therein). Such sequences in native DNA should be susceptible to formation of *c,s* CBD. Deamination of, say, the *c,s* thymine (Thy)–m^{5}C CBD would yield the corresponding *c,s* Thy-Thy CBD, which could then be fixed as a mutated site.

The photochemistry of m^{5}C itself has become increasingly well explored during the past 15 years. This compound and its 2′-deoxyribonucleoside undergo light-induced rearrangement reactions (through putative Dewar intermediates) in acetonitrile to form ureidoacrylonitriles (14). The m^{5}C moiety reacts with alkyl amines to form ring-opened products, namely akylcarbamoyl-3-aminoacrylamidines (15) and with alcohols to form carboalkoxy-3-aminoacrylamidines (16). Both m^{5}C and its deoxyribonucleoside react with water to form acrylamidines (17). Other studies of the reactions of m^{5}C in water have suggested that photodeamination, photooxidation and photo-induced demethylation occur to form Thy, 5-hydroxymethylcytosine and Cyt, respectively (18).

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Abbreviations: CBD, cyclobutane dimer; *c,s, cis-syn*; Cyt, cytosine; DAD, diode array detector; dCpdC, 2′-deoxycytidylyl-(3′-5′)-2′-deoxycytidine; dCpT, 2′-deoxycytidylyl-(3′-5′)-thymidine; ESI, electrospray ionization; HPLC, high-performance liquid chromatography; MALDI, matrix-assisted laser desorption ionization; m^{5}C, 5-methylcytosine; m^{5}dC, 5-methyl-2′-deoxycytidine; m^{5}dCpdC, 5-methyl-2′-deoxycytidylyl-(3′-5′)-2′-deoxycytidine; m^{5}dCpT, 5-methyl-2′-deoxycytidylyl-(3′-5′)-thymidine; MeOH, methanol; NMR, nuclear magnetic resonance; Thd, thymidine; Thy, thymine; TpdA, thymidylyl-(3′-5′)-2′-deoxyadenosine; TpdC, thymidylyl-(3′-5′)-cytosine; Tpm^{5}dC, thymidylyl-(3′-5′)-5-methyl-2′-deoxycytidine; TpT, thymidylyl-(3′-5′)-thymidine; *t,s, trans-syn*; TSP, 3-(trimethylsilyl)propionic-2,2,3,3-d$_4$ acid, sodium salt.

The photochemical reactions of m^5C to form CBD, (6-4) adducts and Dewar adducts have also received some attention. Early studies suggested that there was no evidence for formation of CBD when m^5dC was irradiated at 254 nm in the frozen state (19). A second study from the same laboratory investigated the photochemistry of m^5C at 254 nm when incorporated into a viral DNA in which all Cyt residues were replaced with m^5C (20). Once again, no evidence was found for formation of CBD. However, a later study suggested that a CBD containing m^5C, as well as a monomeric m^5C-containing product, were formed when *de novo* methylated DNA was irradiated at either 254 or 302 nm (21); however, these products were not structurally characterized. The photoreactions of m^5C in the context of the duplex poly-2′-deoxyguanosine:poly-2′-deoxy-5-methylcytidine have also been studied. Applying radioimmunoassay methods, it was found that CBD and (6-4) type adducts of m^5C were both present in this duplex of homopolymers after it was irradiated with either UV-B or UV-C light (22). Recently, it has been shown that m^5C, when irradiated in the frozen state with light predominately in the UV-B region, formed three m^5C cyclobutane homodimers (23) that could be structurally characterized. Similar irradiation of equimolar mixtures of m^5C and Thy produced three m^5C-Thy cyclobutane heterodimers (23), including the biologically relevant *c,s* and *trans-syn* (*t,s*) dimers.

The photoreactions of dinucleoside monophosphates have received considerable attention during the past 40 years. One reason is that the reactions involving the nucleobase components contained in these dinucleotides (or in oligonucleotides as well) are considered to be more reflective of those occurring in DNA or RNA than those reactions occurring when the same moieties are irradiated as free nucleobases, nucleosides or nucleotides in fluid solution. For example, the Dewar adduct of Thy was discovered only when the structure of the last uncharacterized photoproduct formed in the thymidylyl-(3′-5′)-thymidine (TpT) system was finally determined (24,25). The Dewar adduct of Thy in the TpT system is not a primary photoproduct, but is a secondary product produced from the corresponding (6-4) adduct by absorption of UV-B light (24). Isolation of the Dewar product of either the nucleobase Thy or the nucleoside thymidine (Thd), after the irradiation of the (6-4) adducts of these compounds, has not been reported. The presence of structural constraints, found in the context of the dinucleotide environment, may be conducive to light-induced formation of the Dewar adduct of Thy from its precursor (6-4) adduct in TpT.

A significant body of work exists on the photochemistry of dinucleoside monophosphates. Most experimental attention has been directed toward those dinucleotides containing two pyrimidines, although studies on the photochemistry of thymidylyl-(3′-5′)-2′-deoxyadenosine (TpdA) and 2-deoxyadenosylyl-(3′-5′)-2′-deoxyadenosine have been fruitful. The remaining dinucleoside monophosphates containing purine nucleobases appear to be unreactive toward formation of specific photoproducts (26) (for a review of the photochemistry of the purine containing dinucleoside phosphates, see Ref. 27; more recent information on the structure of the TpdA photoproduct is given in Ref. 28). Along with studies of the photochemistry of TpT, the photochemical reactions of three dinucleoside monophosphates of particular interest in DNA have been investigated, namely 2′-deoxycytidylyl-(3′-5′)-2′-deoxycytidine (dCpdC), thymidylyl-(3′-5′)-2′-deoxycytidine (TpdC), and 2′-deoxycytidylyl-(3′-5′)-thymidine (dCpT). CBD, (6-4) products, Dewar adducts and hydrates of Cyt are among the products arising from these reactions (Refs. 29–32 and references therein). Mass spectral methods have been developed recently for detection and quantitation of the four *c,s* CBD, the four (6-4) adducts and the four Dewar adducts of TpT, dCpdT, TpdC and dCpdC in photoreacted DNA (Ref. 33 and references therein). This method involves digestion of the irradiated DNA with an enzymatic cocktail that reduces it to dinucleoside monophosphates containing these photoproducts and nucleosides of unreacted bases. (The literature concerning dinucleotide photochemistry up to 1989 is surveyed in Ref. 34, whereas a more recent review is given in Ref. 35; leading references to more recent work are given in Refs. 33,36.)

Of particular interest here is the photochemistry of dinucleoside monophosphates containing m^5C. Only one study of the photochemistry of such compounds has been published, namely that of Douki and Cadet (29); these workers described their work on three compounds, namely 5-methyl-2′-deoxycytidylyl-(3′-5′)-thymidine (m^5dCpT), 5-methyl-2′-deoxycytidylyl-(3′-5′)-2′-deoxycytidine (m^5dCpdC), and thymidylyl-(3′-5′)-5-methyl-2′-deoxycytidine (Tpm^5dC). Of these three compounds, the photochemistry of m^5dCpT was the most amenable to study. Two compounds that contained m^5C were isolated and characterized by proton nuclear magnetic resonance (NMR) and UV spectroscopy and by mass spectrometry; one was identified as the *t,s* m^5C-Thy CBD and the other as the m^5C-Thy (6-4) adduct. Mass spectral and photochemical evidence indicated that the *c,s* m^5C-Thy CBD and the m^5C-Thy Dewar adduct were present in a fraction that could not be separated into pure components. Additional evidence, supporting the assignment of the (6-4) adduct of m^5dCpT as a compound containing an undeaminated m^5C residue, was provided by a study that showed that the fluorescence properties of this compound were different from those of the (6-4) adduct of TpT (37). In addition to the products containing m^5C, two products were formed that were also produced when TpT was irradiated; these were the *c,s* Thy-Thy CBD and the (6-4) adduct of TpT. Douki and Cadet (29) suggested that these particular products may have arisen from photoreactions of excited m^5CpdT, presumably via deamination of the m^5C component of the dinucleotide in its excited state.

In the case of the Tpm^5dC and m^5dCpdC systems, m^5C-containing photoproducts were not isolated (29), although the authors indicated that they could not rule out that certain products eluting with very small high-performance liquid chromatography (HPLC) peak areas could be such compounds. However, it was found that the predominant photoreactions of Tpm^5dC led to the same *c,s* CBD and (6-4) adduct that were formed in the UV-irradiated TpT system. Irradiation of the m^5dCpdC system produced two products that were characterized; these were the (6-4) product and the corresponding Dewar adduct of TpdC (29).

In this article, we present the results of a study of the photochemistry of Tpm^5dC in aqueous solution. This dinucleoside monophosphate is one of the compounds whose photoreactions were studied previously by Douki and Cadet (29), but for which these workers were unsuccessful in isolating m^5C-containing products for characterization. The study of the photoreactivity of Tpm^5dC has proven more tractable under the conditions used in our experiments, and therefore, we have been able to isolate and characterize a number of photoproducts containing m^5C. This dinucleotide sequence is of particular photobiological interest because it is one that occurs naturally in mammalian DNA in the trinucleotide sequence thymidylyl-(5′-3′)-5-methyl-2′-deoxycytidylyl-(5′-3′)-2′-deoxyguanosine.

MATERIALS AND METHODS

General aspects. HPLC solvents were from Fisher (Fair Lawn, NJ); NMR solvents were from Aldrich (Milwaukee, WI). The dinucleotides TpT and TpdA were purchased from Sigma (St. Louis, MO). A Rainin binary gradient pumping system (Emeryville, CA) coupled to a Hewlett-Packard 1040A diode array detector (DAD) (Palo Alto, CA) was used for HPLC. Analytical and preparative separations were carried out on a Shiseido Capcell UG120 RP18 4.5 × 150 mm reverse phase column (5 μm particle size; Yokohama, Japan); hereafter, this column is termed the Capcell column. Before injection of HPLC samples, they were subjected to spin filtration, using Costar Spin-X micro-centrifuge filter tubes containing a 0.2 μm nylon filter (Corning Incorporated, Corning, NY).

UV spectra were run on a Hewlett-Packard 8452A diode array spectrometer. One-dimensional NMR spectra were run either at 600 MHz on a Varian INOVA NMR spectrometer (Palo Alto, CA) or at 500 MHz on a Bruker DRX 500 MHz NMR spectrometer (Rheinstetten, Germany). Most assignments of resonances corresponding to particular protons were made by comparison with those seen in the NMR spectra of similar well-studied compounds.

Matrix-assisted laser desorption ionization (MALDI) mass spectrometry was carried out on an Applied Biosystems 4700 mass spectrometer (Applied Biosystems, Foster City, CA) operating in the reflector mode and using external calibration. The matrix used was α-cyano-4-hydroxycinnamic acid. Electrospray ionization (ESI) mass spectrometry was done on either a Sciex API300 triple quadrupole electrospray instrument (Toronto, Canada) or on a Waters Micromass ZQ 4000 instrument (Beverly, MA).

Irradiation methods. Exploratory runs were done in stoppered black self-masking quartz UV cuvettes holding a volume of 0.7 mL (Type 30, Precision Cells, Farmingdale, NY); the sample compartment in these cells is 2 mm wide and 1.0 cm deep. Preparative irradiations were done on 3.5 mL volumes contained in stoppered quartz spectrophotometer cuvettes (Type 110, Hellma Cells, Plainview, NY), which are constructed to hold this total volume; the sample compartment in these cells is 1.0 cm deep. Most irradiations for preparative purposes were done with 254 nm light provided by unfiltered Spectronics BLE 1T155 lamps housed in a Spectroline XX-15A dual lamp holder (Spectronics, Westbury, NY). Irradiations for preparative purposes were done in a cold room maintained at 4°C. A cuvette containing the sample to be irradiated was placed on its side on an aluminum block (9 cm long, 7.5 cm wide and 5 cm high) such that it was situated directly underneath and parallel to one of the lamp tubes; the lamp housing itself was supported on blocks 7.5 cm high. The distance between the bottom of the cylindrical lamp tube and the top surface of the cuvette was 2.3 cm. The irradiance at the surface of the cuvette was about 16 J m^{-2} s^{-1}, as measured using a Spectronics DM-254N Ultraviolet Meter. Some irradiations were done with light centered at 312 nm; in these situations, the light was provided by Spectronics BLE 1T158 lamps housed in the same holder and the sample was situated as for the 254 nm irradiations.

Preparation of Tpm^{5}dC. The synthesis of Tpm^{5}dC was performed using the phosphotriester approach (38,39). 5′-*O*-Dimethoxytritylthymidine was phosphorylated with 2-chlorophenylphosphorodi-(1,2,4-triazolide) to give, after hydrolysis, the triethylammonium salt of the 3′-(2-chlorophenyl)-phosphate of 5′-*O*-dimethoxytritylthymidine. The latter compound was allowed to react with 4-*N*-benzoyl-5-methyl-2′-deoxycytidine in the presence of 1-(mesitylenesulphonyl)-3-nitro-1,2,4-triazole in pyridine as a condensing agent. The product of the reaction, protected Tpm^{5}dC, was purified by silica gel column chromatography (silica gel 70–230 mesh 60 A, Aldrich) using chloroform–methanol (MeOH) (50:1 vol/vol) as eluent (yield 75%, 230 mg). Deblocking of the protected Tpm^{5}dC was accomplished via a two-step procedure (40). In the first step, the acid labile 5′-*O*-dimethoxytrityl group was removed by treatment of the protected Tpm^{5}dC with a 2% solution of benzenesulfonic acid in a chloroform–MeOH solution (7:3 vol/vol) at 0°C for 10 min. The partially protected Tpm^{5}dC was then purified by silica gel column chromatography (silica gel 70–230 mesh 60 A, Aldrich) using chloroform–MeOH (25:1 vol/vol) as eluent. The base labile groups 4-*N*-benzoyl and 2-chlorophenyl were removed by treatment with concentrated ammonia at 50°C for 5 h. Finally, the unprotected Tpm^{5}dC was purified by HPLC on a Shiseido Capcell Pak C_{18} UG 120 reverse phase column (10 × 250 mm, 5 μm particle size), using 25 m*M* ammonium formate–MeOH (84/16) flowing at a rate of 5 mL/min. The appropriate fraction containing pure Tpm^{5}dC from each HPLC run was collected. The collected eluent was then distributed into a number of 50 mL plastic tubes and lyophilized to remove excess ammonium formate. The tubes containing the resultant freeze-dried material were kept in a freezer at –20°C until required for use. The resultant material was characterized by mass spectrometry and by proton and ^{13}C NMR spectroscopy. Greater experimental detail concerning this synthesis of Tpm^{5}dC and its spectroscopic characterization, as well as similar information for m^{5}dCpT, will be reported elsewhere (L. Celewicz, in preparation).

RESULTS AND DISCUSSION

Results of exploratory studies on the effects of irradiation of the Tpm^{5}dC system

Preliminary studies were done to optimize irradiation times and separation conditions and to explore the general nature of the photoproducts produced when Tpm^{5}dC (**I**) is irradiated. In a typical run, the content of one tube of lyophilized Tpm^{5}dC (as the ammonium salt; see above) was dissolved in distilled water to make a stock solution. A portion of this stock solution was then diluted 300-fold and the UV spectrum measured. Using an estimated ε_{max} value of 18 150 M^{-1} cm^{-1} at 271 nm (obtained by adding the ε_{max} values for Thd and m^{5}dC [9650 M^{-1} cm^{-1} Ref. 41 and 8500 M^{-1} cm^{-1} Ref. 42, respectively]), the concentration of the diluted solution was obtained. From this value and the dilution factor, the concentration of the stock solution was calculated. A portion of the stock solution was then diluted with doubly distilled water to 0.5 m*M* and the remainder of the stock was frozen until needed for further use.

A sample (0.7 mL) of the 0.5 m*M* solution of Tpm^{5}dC was placed in a Type 30 cuvette and irradiated at about 4°C with 254 nm light for varying lengths of time (see above); the pH of the solution at this temperature was about 4.6. After each irradiation time, 20 μL of the contents of the cuvette was removed and injected on the Capcell column (see above). The following gradient (Gradient A) was used: t = 0 min: % MeOH = 0; 4 min, 0; 6 min, 25; 7 min, 25; 7.25, 0; 10 min, 0. The flow rate was 3 mL/min and the aqueous eluent was 25 m*M* ammonium formate, pH 7. After 128 min (a dose of 123 kJ m^{-2}) about 26% of the parent compound had reacted, whereas after 300 min (288 kJ m^{-2}), about 50% had disappeared. After 16 h irradiation under the same conditions, most of the parent compound had disappeared; however, the HPLC chromatogram indicated that the set of reaction products was very complex after this extended period of irradiation. A second set of exploratory experiments was done in which a similar Tpm^{5}dC sample was irradiated at pH 4.6 with light centered at 312 nm from unfiltered BLE 1T158 lamps. After 128 min, about 20% of the parent had disappeared. To get an idea of how far the reaction could be pushed with this longer wavelength radiation, we irradiated for 15 h and found that around 48% of the Tpm^{5}dC had reacted.

A typical HPLC chromatogram, resulting from injection of 200 μL of freshly irradiated Tpm^{5}dC irradiated for 128 min at 254 nm at pH 4.6 and 4°C with an unfiltered 1T155 lamp is shown in Panel b of Fig. 1. We found nine major product peaks to be of interest for this study; these are designated 1–9 in Fig. 1b. Four peaks, corresponding to minor products, were present. Three of these, labeled with small letters (v, w and x) were not studied in detail; however, one minor product peak was identified, namely that labeled TpT (6-4), which elutes at about 4.6 min and corresponds to the (6-4) photoadduct of TPT. This identification was based on the identity of its elution time with that of an authentic sample of this compound and the close correspondence of its absorption spectrum profile, as obtained with the spectral capture capability of

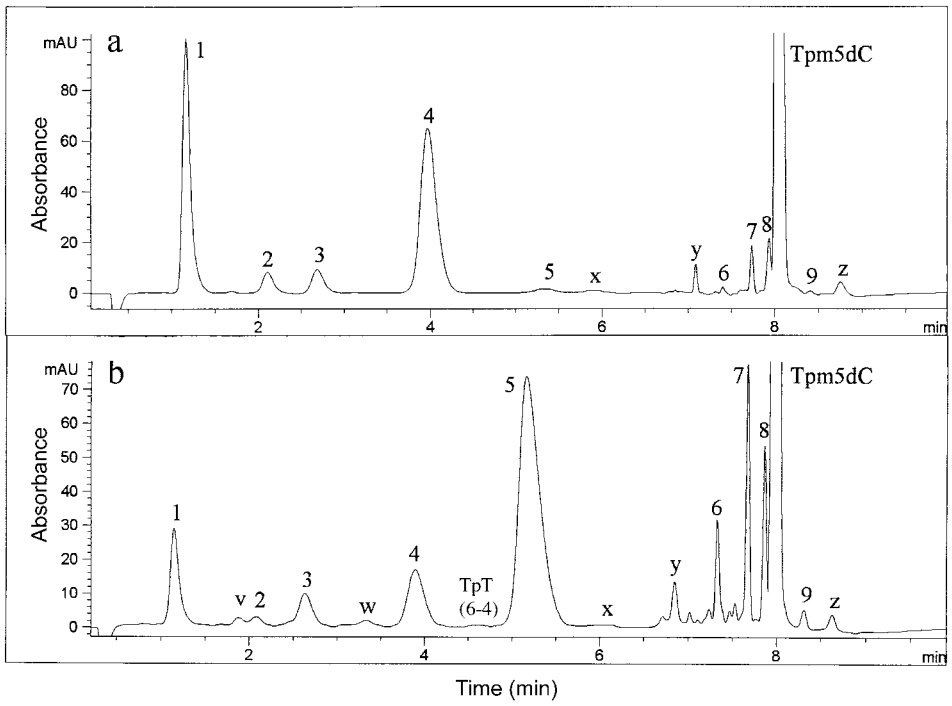


Figure 1. Panel a displays the HPLC chromatogram of a freshly irradiated reaction mixture of Tpm[5]dC photoproducts, obtained via irradiation of 0.5 m*M* Tpm[5]dC for 128 min with an unfiltered Spectroline BLE-1T158 lamp (UV-B output centered at 312 nm). Panel b shows the HPLC chromatogram of a second freshly photolyzed Tpm[5]dC sample (0.5 m*M*) that had been irradiated for 128 min with a Spectroline BLE-1T155 lamp with output mainly at 254 nm. Both irradiated solutions were at pH 4.6 and at 4°C. The injection volume in each case was 200 μL and the wavelength of detection was 230 nm. The irradiation conditions, the column used and the HPLC gradient used are as described in Materials and Methods.

the DAD, with that of the authentic TpT (6-4) adduct. The peaks labeled y and z are minor impurities in the parent compound. Panel a in Fig. 1 shows the HPLC chromatogram obtained after Tpm[5]dC at pH 4.6 and 4°C was similarly irradiated with light centered at 312 nm. The biggest difference between the chromatograms displayed in Fig. 1a,b is that Peak 5 eluting at 5.2 min in Panel 1b is barely detectable in Panel 1a. We did not find any evidence for formation of the TpT (6-4) product in this irradiated solution.

The UV spectra for the compounds corresponding to a number of these peaks, obtained using the ''on the fly'' spectral capture capabilities of the DAD, are shown in Fig. 2. These spectra will be discussed in the course of the discussion below.

We noted variation in the pH of solutions prepared from different lyophilized tubes of Tpm[5]dC (see the last section of Materials and Methods) after dissolution of the contents of such tubes in doubly distilled water, followed by dilution to 0.5 m*M* in Tpm[5]dC. The measured pH ranged from 4.6 to 7.5, with the contents of most tubes yielding solutions with pH values above 7. The reason for this variability is probably because of the tubes containing differing amounts of residual ammonium formate after lyophilization of the parent Tpm[5]dC, which was isolated using ammonium formate eluent at pH 7; larger amounts of residual ammonium formate would give solutions with pH values near 7. The qualitative and quantitative aspects of the observed photochemistry are similar at both pH 4.6 and pH 7–7.5. However, the pH does affect the stability of the *c,s* Tpm[5]dC CBD corresponding to Peak 1 (see below for details relevant to the identification of this compound); this compound readily decomposes, particularly at acid pH, to form the corresponding TpT CBD, which elutes as Peak 2 in Fig. 1. Although most of our studies were done with samples at pH 7 or above, the run made to prepare Fig. 1 was done with a sample irradiated at pH 4.6, so as to show the relationship of elution time of the TpT *c,s* CBD to that of other photoproducts. HPLC chromatograms similar to those shown in Fig. 1, obtained via analysis of material freshly irradiated at pH 7 or above, do not show a significant presence of Peak 2.

Preparative isolation of the Tpm[5]dC photoproducts

In a typical preparative run, 3.5 mL portions of Tpm[5]dC (0.5 m*M*, pH 7.5) were placed in Type 21 quartz cuvettes (see above) and irradiated for 5 h at 254 nm in the cold as described above. (A total of about 45 mL of 0.5 m*M* Tpm[5]dC was irradiated to ensure isolation of a sufficient amount of each photoproduct for NMR and mass spectral study.) The resulting irradiated solution was partitioned directly on the Capcell column, using an isocratic flow of 3 mL/min. The eluent used was 84% (25 m*M* ammonium formate)–16% MeOH. Each run required 7 min under these conditions. Seven fractions were collected: F1 (0.25–0.5 min), F2 (0.5–0.8 min), F3 (1–1.2 min), F4 (1.6–2.1 min), F5 (2.6–3.1 min), F6 (3.1–4.1 min) and F7 (5.6–6.3 min). Rechromatography of F1 using Gradient A on the Capcell column showed that F1 contained the material corresponding to Peaks 1, 2, 3 and 4 in Fig. 1, along with some material corresponding to Peak 5. The UV spectrum of the peak corresponding to F2 showed that this fraction mainly corresponded to Peak 5, whereas the spectra of the peaks collected in F3, F4 and F5 showed that these fractions contained material corresponding to Peaks 6, 7 and 8, respectively. Fraction F6 contained unreacted Tpm[5]dC, whereas the spectrum of the peak corresponding to F7 showed that it corresponds to Peak 9 in Fig. 1.

Fraction 1 was chromatographed preparatively using Gradient A and the individual compounds corresponding to Peaks 1, 2, 3 and 4 were collected (F1-1, F1-2, F1-3 and F1-4); each of these fractions contained one major product. Each major product was then repurified for NMR and mass spectrometric study, using the same chromatographic conditions used for their isolation. Between 0.3 and 1.0 mg of each product was obtained using this procedure. In the subsequent text, we will use the following designations for the various products, which correspond to individual fractions in

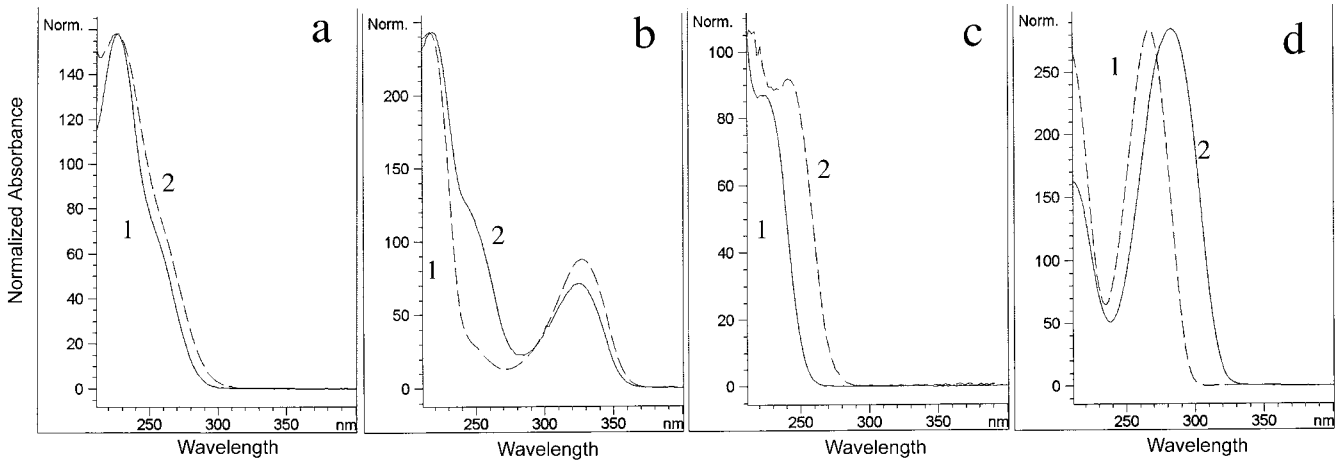


Figure 2. Normalized overlaid UV spectra of various photoproducts produced in the Tpm^{5}dC system, along with those of related compounds. Panel **a** displays the normalized spectral absorbance of the *c,s* and *t,s* CBD, namely **II** (C1) (2a.2) and **V** (C4) (2a.1) as a function of wavelength in nanometers. Panel **b** presents the spectra corresponding to the (6-4) products arising from irradiation of Tpm^{5}dC (C5, **VI**) (2b.1) and m^{5}dCpT (2b.2). Panel **c** shows the spectra of the Dewar adducts produced upon irradiation of Tpm^{5}dC (C3, **VII**) (2c.1) and m^{5}dCpT (2c.2). Finally, Panel **d** displays the spectra of acrylamidine product C7 (**VIII**) (2d.2) and the depyrimidation product C6 (**IX**) (2d.1) produced upon irradiating Tpm^{5}dC. The spectra were obtained by using the ''on the fly'' spectrometric capabilities of a Hewlett-Packard 1040A DAD. The spectrum of each purified compound of interest was captured as its corresponding peak eluted from the Capcell column; the eluent used was 25 m*M* ammonium formate flowing at a rate of 2 mL/min.

which they are found: C(ompound)1: F1-1; C2 (F1-2); C3 (F1-3); C4 (F1-4); C5 (F2); C6 (F3); C7 (F4); C8 (F5); C9 (F7). (Table 1 summarizes the relationship between the product[s] in each fraction [*e.g.* F2 contains C5], as well as to the peak[s] in Fig. 1 that correlate to each fraction [*e.g.* Peak 5] and the structure of the corresponding product[s] in that fraction [*e.g.* **VI**], as given in Scheme 1.)

Characterization of the photoproducts

Products C1 and C4 have UV spectra similar to those of m^{5}C-Thy mixed dimers (23). (The UV spectra of C1 and C4 are shown in Panel a of Fig. 2). This suggests that one of these products is the *c,s* CBD and the other the *t,s* CBD of Tpm^{5}dC. (Steric constraints make it unlikely that *cis,anti* and *trans,anti* CBD can be formed in dinucleotides, such as Tpm^{5}dC.) Irradiation of either of these two compounds with 254 nm light results in its complete conversion to Tpm^{5}dC, as evidenced by production of a single compound with an absorption spectrum and HPLC behavior identical to that of Tpm^{5}dC.

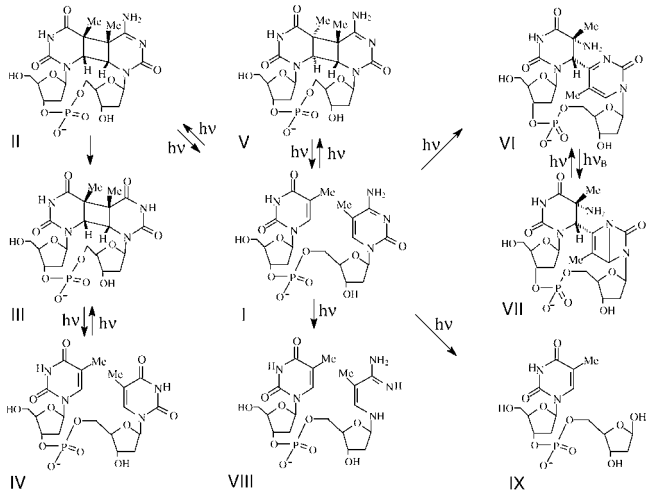
When C1 is allowed to stand, it converts into C2. Irradiation of C2 with 254 nm light produces TpT (**IV**). Irradiation of TpT itself at 254 nm, using the conditions described above, produces as a major product a compound with a retention time and UV spectrum identical to that of C2. On the basis of the published chromatographic behavior of the *c,s* TpT dimer (see, for example, fig. 5 in Ref. 29), C2 can be identified as this compound (**III** in Scheme 1). Therefore, C1 can be identified as the *c,s* CBD of Tpm^{5}dC (**II**), which undergoes thermal deamination to form **III** as shown in Scheme 1. This implies that C4 is the Tpm^{5}dC *t,s* CBD (**V**), shown in Scheme 1. This latter compound is thermally stable upon standing at 4°C and at room temperature.

Consistent with the identification of C1 as **II** are mass spectral data. In particular, the ESI mass spectrum in the negative ion mode gives the expected value for [M − H] of 544. Under conditions required to prepare a pure NMR sample, **II** decomposed to a mixture of **II** and **III**, the *c,s* TpT CBD. However, the mass spectral data and the UV absorption spectral profile, along with the observations that this compound decomposes to form the corresponding **III** and that it reverts upon irradiation with 254 nm light to form parent Tpm^{5}dC, provides conclusive evidence that C1 is indeed described by the structure given by **II**.

The mass spectral and proton NMR data are consistent with the assignment of C4 as the *t,s* CBD of Tpm^{5}dC (**V**). Electrospray mass spectrometry in the negative ion mode indicates that C4 has the expected value for [M − H] of 544. The assignable NMR data are as follows: (D_2O, 600 MHz, 3-(trimethylsilyl)propionic-2,2,3,3-d_4 acid, sodium salt [TSP]) Tp: 5.26 (1H, H1′(dd)), 3.70 (1H, H2′(m)), 2.59 (1H, H2″(dd)), 4.78 (1H, H3′(m)), 4.22 (1H, H4′(dd)), 3.69 (2H, (H5′ and H5″)(m)), pm^{5}dC: 5.75 (1H, H1′(dd)), 2.14 (1H, H2′(m)), 1.93 (1H, H2″(dd)), 4.52 (1H, H3′(overlapping dd)), 4.00 (1H, H4′ (broadened doublet)), 4.11 (2H, (H5′ and H5″)(m)). Two singlet peaks, each corresponding to three protons, occur at 1.54 and 1.45 ppm and can be assigned to the two methyl groups. Two broad singlets occur at 4.17 and 4.12 ppm and probably correspond to the protons at Position 6 on the two pyrimidine rings. These assignments are consistent with the extensive sets of NMR data provided by Liu and Yang (31) and by Kan *et al.* (43) for the TpT *t,s* CBD, as well as the data given by Douki and Cadet (29) for the m^{5}dCpT *t,s*

Table 1. Correlation between fraction number (F), product designation (C), peak number (as labeled in Fig. 1) and the corresponding structure in Scheme 1

Fraction	Peak label in Fig. 1	C(ompound)	Structure or identity
F1	1, 2, 3, 4	C1, C2, C3, C4	**II**, **III**, **V**, **VII**
F1-1	1	C1	**II**
F1-2	2	C2	**III**
F1-3	3	C3	**VII**
F1-4	4	C4	**V**
F2	5	C5	**VI**
F3	6	C6	**IX**
F4	7	C7	**VIII**
F5	8	C8	**VIII**
F6	Tpm^{5}dC	Tpm^{5}dC	**I**
F7	9	C9	**IV**



Scheme 1. The photoreactions of Tpm^5dC in aqueous solution. Also shown are the thermal deamination reaction of the *c,s* CBD of Tpm^5dC (**II**) to form **III**, the corresponding CBD of TpT, and the photoinduced reaction of **III** to form TpT (**IV**). The notation $h\nu_B$ indicates that this reaction is most significant when UV-B light is used to induce photoreaction.

CBD. (For comparative purposes, the chemical shift data for C4 (**V**), as well as that for C3 and C5, are tabulated in Table 2, along with the corresponding data given by Douki and Cadet (29) for the *t,s* CBD and the (6-4) adduct of TpT and by Taylor *et al.* (25) for the TpT Dewar adduct.)

Product C5 has an absorption spectrum characteristic of a (6-4) product, with a λ_{max} of 327 nm and λ_{min} at 271 nm (Fig. 2b.1). The molecular mass [M − H] of C5, obtained using ESI mass spectrometry in the negative ion mode, is 544; this corresponds to the expected molecular mass of the structure given by **VI**. When the ESI spectrum was run in the positive ion mode, the predominant peak was at 568, which corresponds to the sodium adduct of **VI** in which the phosphate is protonated. Irradiation of C5 with 312 nm light for 16 min converts it completely into a compound with the same elution properties and absorption spectrum as C3. The absorption spectrum of C3 (Fig. 2c.1) has λ_{max} = 223 nm and a shallow minimum at 221 nm. Conversely, C3, when irradiated with 254 nm light, is converted quantitatively into a compound with elution properties and a UV spectrum identical to those of C5. The molecular mass [M − H] of C3 is also 544, as expected for an adduct containing both the Thy and m^5C moiety. These observations indicate that C5 should be identified as the (6-4) adduct of Tpm^5dC (**VI**), whereas C3 is the corresponding Dewar adduct of Tpm^5dC (**VII**).

Analysis of the proton NMR spectra of these two compounds yields data that are consistent with these identifications. In the case of C5, the following proton NMR assignments can be made: (D_2O, 600 MHz, TSP) Tp: 6.18 (1H, H1′(dd)), 1.31 (1H, H2′(ddd)), 2.13 (1H, H2″(ddd)), 3.86 (1H, H3′(m)); 3.70 (1H, H4′(m)), 3.96 (1H, H5′(dd), 3.86 (1H, H5″(m)), 1.68 (3H, Me(s)), 5.01 (1H, H6(s)), pm^5dC: 6.54 (1H, H1′(dd)), 3.02 (1H, H2′(ddd)), 2.58 (1H, H2″(m)), 4.83 (1H, H3′(m)), 4.15 (1H, H4′(m)), 3.80 (1H, H5′(dd)), 3.70 (1H, H5″(m)), 2.35 (3H, Me (s)), 8.03 (1H, H6,(s)). For C3, similar assignments can be made as follows: (D_2O, 500 MHz, TSP) Tp: 6.31 (1H, H1′(d)), 2.52 (1H, H2′(m)), 2.38 (1H, H2″(m)), 4.54 (1H, H3′(m)); 1.55 (3H, Me, (s)), 4.76 (1H, H6(s)), pm^5dC: 5.65 (1H, H1′(dd)), 2.28 (1H, H2′(m)), 2.28(1H, H2″(m)), 4.51 (1H, H3′(m)), 2.15 (3H, Me(s)), 5.36 (1H, H6(s)). The H4′, H5′ and H5″ protons for the Tp and pm^5dC ribose rings appear in a region containing overlapping multiplets lying between 3.92 and 4.10 ppm. The preceding assignments are consistent with the previous assignments for the (6-4) and Dewar adducts of TpT given by Rycyna and Alderfer (44), Kan *et al.* (43), Taylor *et al.* (25) and Douki and Cadet (29), as well as the data given for the (6-4) product of m^5dCpdT given in Ref. 29.

Table 2. Proton NMR chemical shifts (in ppm with respect to TSP as an internal standard) of selected photoproducts of Tpm^5dC. All spectra were run in D_2O. For comparative purposes, the chemical shift data for the *t,s* CBD and (6-4) product of TpT, given by Douki and Cadet (29), as well as the corresponding data for the TpT Dewar adduct of Taylor *et al.* (25) are also tabulated. The parentheses in the second column of the table denote the nucleoside component of the dinucleoside monophosphate the NMR data in that row refers to. Information about the multiplet structure of individual resonances is given in the discussion of the structural assignment for each species

Compound		H1′	H2′	H2″	H3′	H4′	Me	H6
TpT *t,s* CBD	(T)pT	5.36	3.39	2.68	4.84	4.21	1.58	4.22
	Tp(T)	5.80	2.27	2.07	4.63	3.98	1.52	4.33
V (C4)	(T)pm^5dC	5.26	3.70	2.59	4.78	4.22	1.54	4.12
	Tp(m^5dC)	5.75	2.14	1.93	4.52	4.00	1.45	4.17
TpT (6-4)	(T)pT	6.27	1.55	2.25	4.08	3.76	1.83	5.17
adduct	Tp(T)	6.60	3.15	2.67	4.82	4.23	2.40	8.09
VI (C5)	(T)pm^5dC	6.18	1.31	2.13	3.86	3.70	1.68	5.01
	Tp(m^5dC)	6.54	3.02	2.58	4.83	4.15	2.35	8.03
TpT Dewar	(T)pT	6.30	2.52	2.36	4.56	3.89	1.57	4.73
adduct	Tp(T)	5.64	2.28	2.27	4.49	3.89	2.10	5.33
VII (C3)	(T)pm^5dC	6.31	2.52	2.38	4.54	*	1.55	4.76
	Tp(m^5dC)	5.65	2.28	2.28	4.51	*	2.15	5.36

* These chemical shifts cannot be determined with certainty because of extensive overlap in the regions where the corresponding resonances occur.

The UV spectra of C7 and C8, obtained using the spectral capture capabilities of the DAD, suggest that they contain moieties that are unsaturated; in addition, the two spectra are almost superimposable, differing only slightly in the values of λ_{max}; C7 has an absorption maximum at 283 nm, whereas the λ_{max} for C8 is at 281 nm. The λ_{min} for both compounds is at 239 nm. (The UV spectrum of C7 is shown in Fig. 2d.2.) The compounds C7 and C8 rapidly interconvert; HPLC analysis of either freshly ''purified'' product indicates that both compounds are present. Because these compounds cannot be individually maintained in a pure state, the mass spectrum of a mixture containing both C7 and C8 was determined using ESI in the positive ion mode. The ($M + H^+$) peak for these compounds occurs at 520.2, whereas the ($M + Na^+$) peak occurs at 542.2; an M value of 519.2 corresponds to the mass that would be expected for the acrylamidine product given by structure **VIII**. In this compound, a CO fragment has been lost, but two hydrogens have been gained for a net loss of 26 from the mass (545) of the parent Tpm^5dC. This type of compound has been previously characterized by Celewicz and Shetlar (17) as the nature of photoproduct arising when either m^5C or m^5dC are irradiated in water. It was shown in this study that the acrylamidine type products exists as a mixture of two isomeric compounds that differ only slightly in their UV spectra. It was suggested that the difference between these two products is that one corresponds to a compound in which the acrylamidine moiety takes the E conformation and the other the Z conformation; the acrylamidine moiety shown in **VIII** is in Z conformation. Further evidence that C7 and C8 are acrylamidines is provided by the results

of a chemical study. Treatment of a mixture of these two compounds with 1,1′-carbonyldiimidazole, as described in Ref. 17, produced Tpm^5dC in good yield; this was proven by the correspondence of the HPLC behavior and UV spectrum of the product with that of Tpm^5dC.

The proton NMR spectrum of freshly isolated C7 is quite complex because of a number of overlapping multiplets that arise because of the presence of both C7 and C8 (see above); such overlap is particularly evident in the resonances corresponding to the acrylamidine moiety and its attached sugar. However, the following NMR features can be assigned with some degree of certainty. The relative spectral intensities in several cases show that one isomer of **VIII** predominates in amount; the chemical shifts corresponding to the dominant form (when evident) are italicized. (D_2O, 600 MHz, TSP, AA = aminoacrylamidine) Tp: 6.33 (1H, H1′(t)) and *6.29* (1H, H1′(dd)), 2.26 (2H, H2′, H2″(m)), 3.98 (1H, H3′(m)), 3.80 (1H, (H5′or H5″)(dd)), 3.84 (1H, (H5′ or H5″)(dd)), *1.898* and 1.905 (3H, Me(s)), *7.65* (1H, H6(s)) and 7.66 (1H, H6(s)); pAA: *5.44* (1H, H1′(t)) and 5.39 (1H, H1′(t)), 2.43 and 2.63 (1H, H2′, H2″(both complex multiplets)), 4.78 (1H, H3′(overlapped by the H_2O peak), *1.70* and 1.72 (3H, Me(s)), 7.35 and *7.36* (1H, 3H(s)). Complex peaks centered at 3.98 and 4.20 probably contain resonances corresponding to the Tp 4′ resonance and the pAA 4′, 5′ and 5″ resonances. On the basis of the ratio of the areas of the AA methyl peaks, it can be calculated that the two isomers are present in about a 2:1 ratio at the ambient temperature of the NMR experiment.

Product C9 has an absorption spectrum and HPLC retention time identical to that of authentic TpT (**IV**). Additional support for this assignment is provided by identity of the chemical shift values of the various resonances in the proton NMR spectrum, run at 600 MHz in D_2O with TSP as standard, with those published by Rycyna and Alderfer (44).

When the absorption spectra of C6 and C9 are compared in normalized form, they are almost superimposable; C6 displays a λ_{max} at 267 nm and λ_{min} at 235 nm; see Fig. 2.d.1. However, the molecular mass of C6 corresponds to that expected for TpT that has lost one Thy (or Tpm^5dC that has lost a m^5C). After replacement of the m^5C moiety with OH, the net loss in molecular mass for Tpm^5dC is 107; this corresponds to an expected molecular mass of 438 for a species, which is protonated on the phosphate moiety. The molecular mass [M − H] determined in the negative ion mode by ESI is 436.8, whereas the molecular mass of the $[M + Na^+]$ peak determined by MALDI mass spectrometry is 460.98. These two masses indeed correspond to the molecular mass expected for a species of structure **IX**, assuming that the phosphate is protonated in both the ESI and the MALDI mass spectra. The structure displayed as **IX** can be regarded as the 5-ester of 3′-thymidylic acid with 2-deoxy-β-*erythro*-pentofuranose with the free 2′-deoxyribose taking the β anomeric form. Aspects of the proton NMR data are consistent with the assignment of C6 as having this general type of structure; in particular, there is only one methyl resonance and one resonance corresponding to H6. However, the sugar regions of the proton NMR spectrum are very complex with many resonances being significantly broadened, as compared with the corresponding resonances in the compounds studied above. This is probably because of the fact that the free deoxyribose moiety can exist in two ring forms that are anomeric at C1 in that sugar moiety, as well as in an open chain conformation; these multiple forms probably equilibrate with one another reasonably rapidly, accounting for the fact that we see only one HPLC peak. Thus the structure depicted by **IX** should be considered as a symbol representing these multiple forms, rather than a single structure. Although it is difficult to make a definitive assignment of the resonances in this spectrum, we were able to make several assignments, as follows: (D_2O, 600 MHz, TSP, AP = apyrimidinic) Tp: 6.33 (1H, H1′(broad triplet)), 1.91 (3H, Me(s)), 7.67 (1H, H6(s)); pAP: 5.62 (∼0.5 H, H1′(broadened t or overlapping dd)) and 5.59(∼0.5H,H1′(broadened d)). (The presence of two peaks for H1′ suggests that both the α and β anomeric forms of the 2′-deoxyribose moiety are indeed contributing to the NMR spectrum.) There are two complex peaks centered at 2.44 and 2.55; these probably correspond to the Tp and pAP 2′ and 2″ protons. Two very complex regions of overlapping resonances lie between 3.77 and 3.89 ppm and between 3.89 and 4.07 ppm. These regions probably contain the Tp and the pAP 4′, 5′ and 5″ resonances. In addition, there are four broadened resonances between 4.20 and 4.50 in which splitting and shoulders can be detected. A couple of these have integrations corresponding to half a proton, suggesting that they are representative of different anomeric forms of the AP deoxyribose moiety. These peaks, in total, probably correspond to resonances due to H3′ for the Tp and pAP moieties. Between 2.10 and 2.60 ppm are three complex resonances corresponding to a total of four protons; these likely correspond to the H2′ and H2″ protons of the Tp and pAP groupings.

To confirm that our assignment of C6 as **IX** is correct, we prepared an authentic sample of **IX**. We depurinated the dinucleoside monophosphate TpdA by letting it stand in 0.1 *M* HCl at 37°C for either 5 h or 15 h; after the former time, small amounts of TpdA remained, whereas incubation for 15 h produced quantitative depurination. The protocol used is similar to that described by Weinfeld *et al*. (45) for production of **IX** from TpdA. Analysis of the reaction mixture by HPLC using Gradient A on the Capcell column, after neutralization with 1 *M* ammonium formate, showed that only two products were present in significant amounts, namely adenine and the desired depurinated product. Compound **IX** was purified using the Capcell column with 90% ammonium formate–10% MeOH flowing at 3 mL/min as eluent; under these conditions, the adenine peak eluted as a tailing peak at 0.7 min and the desired **IX** at 1.99 min. The UV spectral properties of the purified **IX**, as measured by the DAD (which measures spectral intensities at 2 nm intervals) were very similar to those in the literature (46) (λ_{max} = 267, λ_{min} = 235, A_{260}/A_{280} = 1.6).

The HPLC chromatogram of purified **IX** using Gradient A on the Capcell column yielded a single peak that had the same retention time as C6 in a freshly irradiated solution of Tpm^5dC (0.5 m*M*, irradiated in the cold at 254 nm for 2 h). Overlay of the UV absorption spectra of C6 and **IX** indicated that the spectrum of C6 was identical to that of **IX**. Coinjection of **IX** with the irradiated Tpm^5dC solution resulted in significant enhancement of the height of C6; there was no indication of peak broadening. Because **IX** and C6 coelute and have identical UV absorption spectra, we conclude that C6 is indeed **IX**.

Compounds II, V, VI and VIII are primary products in the photoreaction of Tpm^5dC

In one set of experiments, we followed the course of the photochemical reaction of Tpm^5dC as a function of irradiation time. For these studies, samples of Tpm^5dC (0.5 m*M*, pH 7.4) were irradiated at 254 nm and 4°C in self-masking 0.7 mL cuvettes as described in Materials and Methods. Irradiation times of 8, 16, 32,

64, 96 and 128 min were used and the irradiance was 16 J m^{-2} s^{-1} at the upper surface (area = 0.7 cm^2) of the irradiation cell (see Materials and Methods). Calculation shows that after 8 min the dose, on the basis of surface area was 7.68 kJ m^{-2}, which corresponds to an amount of energy of 0.54 J being received at the surface of the cell. Thus, a corresponding average dose of about 0.77 J cm^{-3}, on the basis of volume, was received by the contents of the cell after 8 min irradiation. Doses at other times can be readily obtained by multiplication by an appropriate time scaling factor. The irradiated solutions (20 μL injections) were analyzed by HPLC using Gradient A on the Capcell column. Each of the products could be detected using 230 nm radiation. However, peak areas corresponding to **VI** and the two forms of **VIII** were considerably larger when detected at 327 and 280 nm, respectively. The presence of five compounds (**II**, **V**, **VI** and the two forms of **VIII**) was evident after 8 min, as evidenced by the appropriate retention time and absorption spectrum for the each peak. The peaks areas for **VI** and the two forms of **VIII** increased steadily throughout the irradiation period. The peak areas of **II** and **V** both increased in area between 8 and 64 min and then remained essentially constant over the next two irradiation intervals. The photochemical behavior observed for the peaks corresponding to **II**, **V**, **VI** and **VIII** is that expected for primary photoproducts. The behavior exhibited by **II** and **V** is that expected of a compound that reaches a photoequilibrium in which photoreversal of product to parent compound occurs at the same rate as it is formed.

The *c,s* TpT CBD (**III**) and the Tpm^5dC Dewar adduct (**VII**) could be first detected after 64 min of irradiation and then increased in amount over the next two irradiation intervals. This is the behavior expected of secondary products; in these two cases, **III** is putatively produced by thermal deamination of **II** and **VII** by photoisomerization of the initially produced **VI**. In these time-course studies, we did not detect a peak eluting at a retention time corresponding to that of the TpT (6-4) adduct (see Fig. 1, Panel b).

The question of whether IX is a primary photoproduct

In the studies described above, we were unable to determine if **IX** was a primary product. Under the conditions of the gradient used for HPLC analysis in those studies, the peak for this particular compound was poorly resolved from a peak corresponding to an impurity in the parent Tpm^5dC. Only after 64 min irradiation could we determine that **IX** was definitely present in the reaction mixture. We therefore developed HPLC conditions that achieved better separation of **IX** from this impurity peak. For our studies of **IX**, 0.7 mL samples of Tpm5dC (0.45 m*M*, 10 m*M* in sodium phosphate, pH 7) were irradiated at 254 nm and 4°C as described in Materials and Methods. Irradiation times of 4, 8, 16 and 24 min were used. The samples were analyzed on the Capcell column using 86% ammonium formate–14% MeOH, flowing at a rate of 3 mL/min. We focused on the appearance of a peak at 1.2 min, which corresponded in elution time to **IX**; under these conditions, the two isomers of **VIII** elute at 1.8 and 2.6 min, whereas parent Tpm^5dC elutes at 3.5 min. The peaks corresponding to **II**, **V**, **VII** and **VII** elute as a set of poorly resolved peaks nearer to the beginning of the chromatogram.

Even using these improved conditions, we found examination of the formation of **IX** to be problematic. It is produced in a considerably smaller amount than the photoproducts whose study was described in the previous section. An impurity, present in a small amount in unirradiated samples, elutes at the same retention time as **IX**. This impurity has a very similar absorption spectrum to that of **IX** and, indeed, may be **IX**. However, using a second impurity with a significantly larger peak area (eluting at 1.05 min and with an area that remained essentially constant with increasing irradiation time) as an internal standard, it was found that the difference in the normalized area of the peak eluting at 1.2 min after a given irradiation time and the normalized area corresponding to the impurity peak in the unirradiated sample steadily increased. Between irradiation times of 4 and 24 min, this area increased about five-fold. At the same time, the area corresponding to Tpm^5dC decreased to about 90% of its original area. Although these results tend to support the idea that **IX** is a primary photoproduct, rather than a secondary photoproduct, they must be at this point be regarded as only suggestive. More extensive work will be required to prove this point beyond doubt.

Effect of sample deoxygenation on the photoreactions of Tpm^5dC

We did a study to determine whether the qualitative photochemisty of Tpm^5dC was altered by removal of oxygen from the reaction mixture, as compared with the photochemistry occurring when the irradiated samples were equilibrated with air. These comparative studies were done on two samples (0.45 m*M*, 10 m*M* in sodium phosphate buffer, pH 7.0) contained in quartz NMR tubes from Wilmad (Buena, NJ) (sample size, 500 μL). One sample was fitted with a rubber septum designed for NMR tubes (Wilmad) and the other with a normal plastic top. The tube with the septum was deoxygenated for 20 min by bubbling with 99.997% nitrogen flowing through a long syringe needle (Wilmad) that reached to the bottom of the NMR tube; a second small narrow-gauge needle piercing the septum allowed egress of the nitrogen. The two tubes were taped in equivalent positions to the block described in Materials and Methods and irradiated for 1 h at 22°C. The resultant reaction mixtures were analyzed via HPLC using the Capcell column.

For studies designed to examine the effect of deoxygenation on production of the two isomers of **VIII** and **IX**, the eluent was 86% ammonium formate–18% MeOH flowing at a rate of 3 mL/min. These conditions nicely resolve **IX** from any possible Thd contamination (which elutes at 1.47 min). The resultant chromatograms for the two systems indicated no qualitative differences between the aerated and deoxygenated system in the region where **IX** and the two isomers of **VIII** elute, although there were slight quantitative differences in peak areas for the peaks corresponding to these three compounds in the chromatograms for the two irradiated systems.

For the sake of completeness, a second set of HPLC runs was made on the same two samples to study the effects of sample deoxygenation on formation of **II**, **V**, **VI** and **VII**. Therefore, Gradient A (see Materials and Methods) was used on the Capcell column. Again, there were not large differences in the peak areas for those peaks corresponding to these compounds in the chromatogram corresponding to the deoxygenated solution, as compared with the aerated solution.

The results outlined above, taken as a whole, suggest that oxygen is not an effective quencher for those excited states participating in formation of the various photoproducts of Tpm^5dC discussed in this paper and also indicate that oxygen is not required as a reactant on the pathway leading to loss of m^5C by Tpm^5dC to form **IX**.

Effects of irradiation wavelength on the distribution of products in the Tpm^{5}dC system

Figure 1a,b indicate that there are significant differences in the distribution of products formed when Tpm^{5}dC is irradiated for 128 min with 254 nm light and with UV-B light. As pointed out previously, the (6-4) product **VI** (corresponding to Peak 5 in Fig. 1) is present in only small amounts in this system when irradiated with radiation that is predominately in the UV-B, whereas it is the dominant product when irradiation occurs at 254 nm. Another significant difference between the product distributions at the two wavelengths is the considerably greater amounts of the dimer products **II** and **V** that are present in the Tpm^{5}dC system when irradiation is done with long wavelength light. An enhanced feeling for the differences in the two profiles in Fig. 1 can be obtained by comparison of the areas corresponding to the various classes of products at each irradiation wavelength. When the irradiation was done using 254 nm light (Fig. 1b), the integrated areas corresponding to Peaks 1 and 4 were 194 and 232, respectively, whereas the areas of Peaks 3 and 5 were 120 and 1242, respectively. However, when the long wavelength light was used (Fig. 1a), the corresponding areas for Peaks 1 and 4 were 660 and 873 and the areas of Peaks 3 and 5 were 97 and 35. The relatively small areas corresponding to **II** and **V** in the system irradiated at 254 nm, as compared with the area corresponding to **VI**, is to be expected. These two compounds have very significant absorbance values at 254 nm (see Fig. 2a) and, thus, continuous destruction of these dimers by absorption of light of this wavelength would occur. As described above, a study of the peak area for each of the various products formed as a function of irradiation time, using 254 nm radiation with time points of 8, 16, 32, 64, 96 and 128 min and using the irradiation conditions described in Materials and Methods, showed that the areas corresponding to **II** and **V** had reached a steady state at 64 min, whereas the peak area corresponding to **VI** and the sum of the peak areas of the two isomers corresponding to **VIII** continued to increase with time of irradiation. These results indicate that if one desires to isolate CBD **II** and **V**, then irradiations should be done with lamps emitting primarily long wavelength radiation. On the other hand, irradiation at 254 nm is the best route to take if a relatively high yield of the (6-4) product **VI** is needed. (It should be noted that direct comparisons of peak areas for various products in Fig. 1a,b are not valid because the amount of light absorbed by the two irradiated parent systems is different. Thus, it is not feasible to compare peak areas to evaluate the relative reactivity of Tpm^{5}dC to form a given individual product under the two sets of irradiation conditions.)

A study was made of the effect of filtering the 254 nm lamps with Vycor shields on the amounts of **IX** and the two isomers of **VIII** formed during 2 h of irradiation under the conditions described in Materials and Methods. It was found that the amount of **IX** decreased by a factor of about 3.3, as compared with the situation where the lamp was unfiltered, whereas the summed areas corresponding to C7 and C8 decreased by a factor of about 1.1. Thus, it appears that short-wavelength components of the unfiltered germicidal lamp enhance the rate of the depyrimidation process while having little effect on acrylamidine formation.

Acetophenone photosensitizes formation of both the *c,s* and *t,s* Tpm^{5}dC CBD

In one of their studies on the photochemistry of the m^{5}dCpT system, Douki and Cadet examined the results of using acetophenone to sensitize the triplet state reactions of m^{5}dCpT. They found evidence for four products; the two main products were the *c,s* TpT CBD and a second product, eluting with the same retention time as the *c,s* m^{5}dCpT CBD that slowly decomposed to form the *c,s* dimer of TpT. Minor products with the same retention time as the *t,s* CBD of TpT and m^{5}dCpT were also observed.

We carried out a similar experiment with Tpm^{5}dC. A solution (0.7 mL, pH 7.4) that was 0.5 m*M* in Tpm^{5}dC and 14 m*M* in acetophenone was irradiated in the cold as described in Materials and Methods, using the long-wavelength lamp emitting mainly at 312 nm. The reaction was followed as a function of time, using Gradient A and the Capcell column. After 128 min, about 45% of the parent had reacted and three products were present, namely **II**, **V** and **III**, the *c,s* TpT CBD. The ratio of the integrated peak areas for the two Tpm^{5}dC dimers (**II/V**), measured at 230 nm, was 2.2, whereas the ratio of the areas corresponding to **II** and **III** was about 7.9. There were no peaks corresponding to the Tpm^{5}dC (6-4) product or the corresponding Dewar adduct. Similarly, peaks suggesting the formation of **VI**, **VIII** and **IX** were absent. These results indicate that the triplet state of Tpm^{5}dC can react to form the two CBD of this dinucleotide monophosphate and, at the same time, supports (but does not prove) the hypothesis that the reactions to form **VI**, **VIII** and **IX** occur from the singlet state of Tpm^{5}dC.

Thermal stability of the *c,s* CBD of Tpm^{5}dC near neutral pH

As mentioned in the Introduction, deamination of *c,s* Tpm^{5}dC CBD in the context of DNA is thought to play a role in fixing mutations at sites in which a Thy residue has replaced a m^{5}C moiety. To gain quantitative information about the kinetics of the thermal deamination of the *c,s* CBD of Tpm^{5}dC (**II**) to form **III**, we followed the kinetics of decomposition of **II** as a function of temperature using a sample obtained by irradiating under the following conditions: 0.5 m*M* Tpm^{5}dC, pH 7.4, irradiation time of 128 min with long-wavelength radiation. (Although external buffers [*e.g.* phosphate] were not added to this solution, it is likely the pH of 7.4 is because of the presence of residual ammonium formate in the solution that remained after lyophilization; for example, 25 m*M* ammonium formate that has stood in air for several days has a pH of around 7.2.) We analyzed the solution immediately after irradiation, using Gradient A on the Capcell column; there was a barely detectable peak corresponding to **III** in the freshly irradiated solution. For each run, 70 μL portions of a freshly irradiated solution of Tpm^{5}dC were placed in five 1.5 mL screw-top plastic vials (Sarstedt, Newton, NC). These vials were placed in covered water-filled beakers situated in water baths maintained at a constant temperature. The remaining irradiated solution was stored on ice. After 6 min, to allow the vials to come to equilibrium with the surrounding water, the clock was started and, after set time intervals, individual vials were removed and plunged into an ice bath. After agitation of each vial on a vortex mixer, followed by centrifugation, 20 μL was injected on the Capcell column and chromatographed using Gradient A as described in Materials and Methods; samples for each time point were run in duplicate. The integrated area, measured at 230 nm, corresponding to Peak 1 (see Fig. 1) for each incubation time was averaged. The averaged integrated area corresponding to the Tpm^{5}dC *t,s* CBD (Peak 4), which is stable over the heating periods used and which can thus serve as an internal standard, was also evaluated for each time point. A normalization factor for each time point, to be applied to corresponding averaged peak areas for Peak

1, was calculated by dividing the averaged area for Peak 4 associated with the first time point by the averaged area for Peak 4 corresponding to the time point to be corrected. The experimental peak area for Peak 1, at each time point, was then multiplied by its corresponding normalization factor. This normalization eliminates the effects of injection volume variation.

The natural log of the normalized peak area for Peak 1 at each time, measured at 230 nm, was plotted as a function of time of incubation using Graph III for the Macintosh (Computer Associates, Islandia, NY); the slope of each line was then evaluated using a least squares fitting procedure included in this program. Runs were made at four temperatures; the values for k, the pseudo first-order rate constants (in h^{-1}), obtained from these four runs are as follows: 54°C, 0.164; 59°C, 0.297; 65°C, 0.431; 71°C, 0.684. All correlation coefficients were 0.995 or higher.

The above kinetic data were plotted appropriately to estimate the enthalpy and entropy of activation for the deamination reaction of Tpm^{5}dC *c,s* dimer. According to the Eyring transition state theory of chemical kinetics, the temperature dependence of the rate constant for a reaction in solution can be mathematically described as shown in the equation given immediately below (see, for example, Ref. 47, pp. 476–480), in which the transmission coefficient has been set equal to one:

$$k = (\mathrm{RT/Nh})e^{\Delta S^{\ddagger}/R}e^{-\Delta H^{\ddagger}/RT}$$

This can be rewritten in the form of $\ln(k/T) = \ln(R/Nh) + \Delta S^{\ddagger}/R - \Delta H^{\ddagger}/RT$. According to the latter equation, the value of $\Delta H^{\ddagger}$ can be evaluated from the slope of a graph of $\ln(k/T)$ *versus* 1/T, and the y intercept of such a graph yields a quantity from which $\Delta S^{\ddagger}$ can be calculated. We analyzed the above data using this equation; the resulting plot yields values of $\Delta H^{\ddagger} = 73.4$ kJ/mol and $\Delta S^{\ddagger} = -103.5$ J/K mol for the deamination reaction of the *c,s* CBD of Tpm^{5}dC. The correlation coefficient describing the linear fit of the rate constant data for the Eyring plot was 0.992. Note that, because the rate constants listed in the previous paragraph are in h^{-1}, the corresponding value of R/Nh must also be in h^{-1} K^{-1}; therefore we used a value for R/Nh of 7.48×10^{13} h^{-1} K^{-1} in the evaluation of $\Delta S^{\ddagger}$.

Using the values of $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$ evaluated above, estimates can be made of the half-lives of the *c,s* Tpm^{5}dC CBD at various temperatures; we evaluated these at four different temperatures of interest: 37°C, 17.8 h; 25°C, 58.3 h; 4°C, 592.3 h = 24.7 days and 0°C, 959.8 h = 39.9 days. The value for the calculated half-life at 37°C is comparable with that experimentally measured at 37°C for the *c,s* m^{5}C-Thy mixed CBD, namely 21.6 h (23).

A second, less extensive run was made in which the pH was buffered at pH 7.55 by making the solution 5 m*M* in sodium phosphate. Three values of k (in h^{-1}) were determined: 54°C, 0.188; 59°C, 0.271 and 65°C, 0.477; all correlation coefficients were 0.997 or better. Plotting and analysis of these data, as described above, led to values of $\Delta H^{\ddagger} = 75.3$ kJ and $\Delta S^{\ddagger} = -97.3$ J/K, which is in reasonable agreement with the studies made directly on the unbuffered irradiated solution at pH 7.4. The correlation coefficient for the linear fit of the rate constant data for the Eyring plot was 0.997.

It is interesting to compare the calculated half-lives of the *c,s* Tpm^{5}dC CBD with the half-lives that have been measured for corresponding CBD formed when dinucleotides containing Cyt and Thy or two Cyt are irradiated. Douki and Cadet (30) measured the rate constant for deamination of the *c,s* CBD of dCpT at pH 7 in 10 m*M* phosphate buffer at room temperature; from this rate constant, they calculated the half-life to be 6.8 h. In another study, Lemaire and Ruzsicska (48) measured the rate constant for deamination of the thymidylyl-(3′-5′)-2′-deoxycytidine *c,s* CBD as 1.5×10^{-3} min^{-1} in 0.05 *M* phosphate buffer at 25°C and pH 7; from this value, the half-life can be evaluated as 7.7 h. In yet another study, Hariharan and Johns (49) examined the stability of the cytidine cyclobutane homodimers formed when cytidylyl-(3′-5′)-cytidine was irradiated and found that the originally formed dimer had a half life of 7.2 h at 9°C and pH 6.8. The product from this reaction, a heterodimer containing both uracil and Cyt residues, underwent deamination with a half-life of about 5 h.

Comparison of the appropriate half-lives, given above for several Cyt-containing *c,s* CBD, with those of **II** indicates that methylation of Cyt at the 5-position results in significant stabilization of this moiety toward thermal deamination. At 25°C, for example, the calculated half-life of **II** at pH 7.4 was about 58 h, as compared with 7.7 h for the analogous dimer of TpdC (see above).

The *c,s* dimer of Tpm^{5}dC is quite stable to deamination under the conditions of our experiments, particularly at low temperatures. However, it is not as stable as the corresponding *c,s* CBD of m^{5}dCpT, for which the half life was measured at pH 7 at 25°C in 0.01 *M* phosphate buffer. For this deamination reaction, the rate constant was measured as 10^{-5} min^{-1} (=6×10^{-4} h^{-1}) (29). The corresponding half-life is 1155 h = 48.1 days.

The effect of pH on deamination of the Tpm^{5}dC *c,s* CBD

Figure 1 shows HPLC chromatograms obtained after HPLC analysis of a system in which the pH of the irradiated Tpm^{5}dC solution was 4.6. In these chromatograms, the presence of the *c,s* TpT CBD (Peak 2) is evident. Indeed, this particular chromatogram was chosen to display the elution behavior of this compound. When the irradiation was done with the long-wavelength lamp (Fig. 1a), the area of Peak 2 was comparable with that corresponding to the Dewar adduct **VII**. The HPLC analysis was done immediately after irradiation, which implies that **III** was formed during the course of the irradiation. However, after carrying out analogous irradiations at pH 7.5 accompanied by immediate HPLC analysis of the photolyzed solution, we found that TpT *c,s* CBD was present in, at most, trace amounts in the reaction mixtures. To establish that this was an effect of pH on deamination of the Tpm^{5}dC *c,s* dimer, rather than some other factor present in the system at pH 4.6, we adjusted the pH of 0.7 mL of the solution, originally at a pH of 4.6, to 7.4 by making it 10 m*M* in phosphate. Irradiation of this sample for 90 min in the cold at long wavelengths, followed immediately by HPLC analysis, produced a chromatogram in which there was no evidence of formation of **III** during the course of the irradiation. (We found no evidence for deamination of **V**, **VI** and **VII** in any of our experiments.)

In a qualitative experiment designed to test the effect of standing at pH 4.6 on **II**, as compared with standing at near neutral pH, we irradiated a 0.7 mL sample at pH 4.6 for 90 min in the cold with long wavelength radiation and then placed 70 μL portions of the irradiated sample into two plastic 1.5 mL Sarstedt tubes with screw caps. To the contents of one tube, we added 7 μL of 100 m*M* sodium phosphate (pH 7.4). We then allowed the two tubes to stand for 64 h in a refrigerator and analyzed the two solutions via HPLC as described previously. The HPLC chromatogram of the sample at pH 4.6 displayed only a trace of Tpm^{5}dC *c,s* CBD, whereas the other sample, adjusted in pH with phosphate buffer, retained around 85% of the original **II**.

To obtain an idea of how much faster **II** underwent deamination at pH 4.6 than at pH 7.4, we studied the kinetics of deamination of **II** at 54°C, as described in the previous section. The pseudo first-order rate constant determined for the deamination process was $k = 3.4\ h^{-1}$, which compares with the corresponding value of $k = 0.169\ h^{-1}$ obtained at pH 7.4. Thus, the deamination process at pH 4.6 is about 20-fold faster than the same process at pH 7.4, at least at 54°C. The rate of deamination has also been found to be dependent on pH for *c,s* CBD of dinucleoside monophosphates containing Cyt, rather than m^5C. For example, detailed studies by Lemaire and Ruzsicska indicated that the deamination rate of the *c,s* CBD of TpdC and dCpT are dependent on both pH and buffer concentration; both have maximal rates of deamination at a pH of around 3.8.

The above experiments suggest that careful control of pH is required to maximize the stability of the Tpm^5dC *c,s* CBD. Although it does slowly deaminate at physiological pH when stored at temperatures near 0°C, the rate of its deamination is accelerated markedly in acid solution (*e.g.* pH 4.6). Further studies will be required to elucidate the detailed dependence of the rate of deamination upon pH and its mechanistic implications.

Effects of temperature of irradiation on deamination

Almost all of our irradiations were carried out at 4°C. However, we did an experiment to qualitatively compare the photochemistry occurring at ambient temperature with that taking place at 4°C. We irradiated two 0.7 mL samples of 0.5 m*M* Tpm^5dC (pH 4.6) at 254 nm for 120 min under almost identical conditions, the only difference being that one sample was maintained at 4°C and the other was maintained at room temperature (about 22°C). In both cases, the irradiation setup was that described in Materials and Methods; in each case, the sample being irradiated rested on an aluminum block (9 cm long, 7.5 cm wide and 5 cm high), so as to maintain the temperature constant during the period of photoreaction. Both samples were placed on ice immediately after photolysis and successively analyzed by HPLC. The significant differences between the two systems, in terms of the HPLC profile, were that the peak area for C6 (**IX**) is increased about three-fold, as compared with the irradiation done at 4°C, whereas the peak area of TpT (**IV**) was enhanced about seven-fold at 22°C. The amounts of the TpT *c,s* CBD (**III**) found in both systems were comparable in amount. The integrated peak area corresponding to **II**, measured at 230 nm, was about 15% larger in the system irradiated at 4°C, as compared with that irradiated at 22°C. One additional interesting observation was made. The leading edge of the peak corresponding to **II** for the sample irradiated at room temperature contains a substance that displays an absorption maximum at about 285 nm. The elution time and the spectrum of this new compound are very similar to that of the Z isomer of the acrylamidine formed by reaction of m^5C with water (17). A trace of this material was also present in the leading edge of the peak corresponding to **II** in the system irradiated at 4°C. (Indeed, the UV spectrum of the material eluting in Peak 1 in Panel b in Fig. 1 indicates some contamination of this peak with putative acrylamidine; this was not true of Peak 1 in Panel a.) The greater amount of this acrylamidine product in the system irradiated at 22°C may be because of the larger amounts of m^5C released in this system by formation of **IX**. This enhanced amount of m^5C could then react in a secondary photoprocess to form the larger amount of the acrylamidine observed in the system irradiated at 22°C.

Evidence that acrylamidine products analogous to C7 and C8 are formed in the m^5dCpT system

In view of our observation that products C7 and C8, the two isomeric forms of acrylamidine product **VIII**, are formed in the Tpm^5dC system, we did a study seeking evidence for the formation of analogous compounds in the m^5dCpT system. The parent compound (0.5 m*M*), which was prepared using essentially the method described in Ref. 29, was irradiated with 254 nm radiation under the same conditions described in Materials and Methods. The HPLC of 20 μL of this reaction mixture, run using Gradient A on the Capcell column, showed two closely eluting peaks at 6.75 and 6.82 min with absorption spectra very similar to those of C7 and C8; parent m^5dCpT eluted at 7.62 min. Using the spectral capture capability of the DAD, it was found that the peak eluting first had a λ_{max} at 277 nm, whereas the peak eluting at 6.82 min had $\lambda_{max} = 283$ nm; both had λ_{min} at 240 nm. Using the same gradient on Column A, but with an injection size of 500 μL of irradiated material, the material eluting between 6 and 6.4 min, corresponding to the unresolved peaks of the putative acrylamidines, was collected and submitted for ESI mass spectral analysis in the positive ion mode. The $[M + H^+]$ peak had a mass of 520.2, whereas there was a $[M + Na^+]$ peak at 542.1. These are the same masses as were measured for the mixture of C7 and C8 in the Tpm^5dC system. These results strongly suggest that acrylamidine products, analogous to **VIII**, are produced because of photolysis of the m^5CpdT system. It is likely that the reason these compounds were not detected by Douki and Cadet is that they adhere strongly to the column and are not eluted until the solvent contains an appreciable concentration of MeOH. The HPLC profile for the irradiated m^5dCpT system, shown in fig. 5 in Ref. 29, was run in an isocratic mode using 25 m*M* ammonium formate as eluent.

In agreement with the results of Douki and Cadet (29), we found that compounds were present in the reaction mixture with the expected HPLC and UV spectral properties of the *c,s* and *t,s* CBD, the (6-4) product and Dewar adduct of m^5dCpT. Using the Capcell column with Gradient A, we isolated the (6-4) adduct; this compound eluted between 1.2 and 1.7 min. We then irradiated the (6-4) product at 312 nm with the BLE-1T158 lamps for 12 min. The HPLC, run on Column A using 25 m*M* ammonium formate at 3 mL/min, displayed a dominant peak eluting at 0.75 min corresponding to the Dewar adduct; a relatively small amount of (6-4) remained after 12 min irradiation. We did not isolate these two compounds from this reaction mixture; however, using the spectral capture capability of the Hewlett-Packard DAD, we obtained the UV spectrum of the (6-4) product and of the Dewar adduct. These spectra are displayed in Panels b and c in Fig. 2, along with the corresponding spectra of the Tpm^5dC products. Both the (6-4) product and the Dewar adduct of m^5dCpT show absorption spectra that are shifted significantly to the red in the region below 260 nm, as compared with the corresponding spectra of the Tpm^5dC products. This would be expected because the m^5dCpT products contain a saturated m^5C ring, whereas the Tpm^5dC products contain a saturated Thy ring.

Comparison of the results of the present study with those of previous work

There are substantial differences in the results of our study of the photochemistry of Tpm^5dC and those obtained by Douki and Cadet (29). (In both studies, experiments for preparative purposes were

done using light from germicidal lamps emitting predominately at 254 nm.) In particular, we have isolated and characterized six photoproducts containing m^5C, as well as one product in which the m^5C has been lost from the dinucleoside phosphate. On the other hand, Douki and Cadet found that the *c,s* CBD and (6-4) adduct of TpT were the major products formed, although two other compounds were present in their reaction mixtures in amounts too small to characterize. In our studies, the TpT *c,s* CBD appears as a secondary product resulting from thermal deamination of the corresponding *c,s* CBD of Tpm^5dC; at most, a trace of the (6-4) adduct of TpT was observed in our photoreacted solutions of Tpm^5dC and then only after high doses had been delivered to the solutions being irradiated.

In the paragraphs below, we will discuss possible answers to the following question: ''Why is there such a disparity between the results of the two sets of experiments?.'' This discussion makes use of some of the results outlined in preceding sections.

One obvious possibility is that the Tpm^5dC used in our experiments differs in some important manner from that of Douki and Cadet. This particular factor is impossible to assess experimentally because samples of the material used by Douki and Cadet in their experiments no longer exist (T. Douki, personal communication). However, control experiments run by Douki and Cadet, using NMR and mass spectrometry, indicated that the Tpm^5dC used for their experiments did not contain significant amounts of TpT, excluding this factor as an explanation for the formation of *c,s* CBD and (6-4) adducts of TpT as the predominant products in their studies of this system (J. Cadet and T. Douki, personal communication). It should be noted that the photochemistry of m^5dCpT reported by Douki and Cadet and that observed in the limited set of experiments we carried out on this compound is quite similar. The main differences in the results of these two studies are that the amount of deaminated CBD product present immediately after irradiation was significantly smaller in our studies than was found in the studies of Douki and Cadet and that we did not observe formation of the deaminated (6-4) adduct of m^5dCpT in our experiments. (Control experiments by Douki and Cadet also showed that their m^5dCpT was did not contain detectable TpT [J. Cadet, personal communication].)

Another alternative to be considered is that the doses, received by the sample in the experiment of Douki and Cadet and by the samples used in our experiments, were dramatically different. For example, it might be postulated that m^5C-containing photoproducts were formed and then destroyed by the absorption of much higher doses of radiation in the system studied by Douki and Cadet. However, the following reasoning rules out that possibility. Douki and Cadet indicate that their sample, irradiated for 1 h with a germicidal lamp, received a total UV dose of 1.5 kJ m^{-2} when expressed in terms of surface area. Thus, the lamp in their experimental setup provided an average irradiance of 0.42 J m^{-2} s^{-1} to the surface of their Tpm^5dC solution. The 25 mL sample used (at a concentration of about 3.6 m*M*) was contained in a 15 cm diameter petri dish; thus their irradiated solution had a surface area of about 1.77×10^{-2} m^2 and a depth of about 1.4×10^{-3} m. Therefore, their sample received an amount of energy corresponding to 26.5 J during its irradiation; on a volume basis, this corresponds to a dose of 1.06×10^6 J m^{-3} = 1.06 J cm^{-3}. The irradiance that our solutions (0.5 m*M*) were exposed to was measured as 1.6 mW cm^{-2} = 16 J m^{-2} s^{-1}; our preparative irradiations were done for 5 h and the surface area of the 3.5 mL cuvettes in which samples were irradiated was 3.5×10^{-4} m^2. Therefore, our samples received 100.8 J over the 5 h period used for preparative irradiations; this corresponds to a dose of 28.8×10^6 J m^{-3} = 28.8 J cm^{-3} (or alternatively, 2.88×10^5 J m^{-2}). Thus, our samples, irradiated for preparative purposes, received a dose about 27-fold larger on a volume basis than the solutions of Douki and Cadet (or 192-fold larger on a surface area basis). Therefore, it cannot be maintained that initially formed m^5C-containing products were initially formed and destroyed because these products are the predominant products in our systems after much higher doses of radiation. A second possibility might be that, for some reason, the two TpT containing products are rapidly produced at small doses, but then the rate of production of m^5C-containing products predominates as the dose increases. However, the results described in a previous section, in which we showed that **II**, **V**, **VI** and the two isomers of **VIII** were primary products, suggest that, even at doses comparable with those of Douki and Cadet, the (6-4) adduct and the *c,s* CBD of TpT are not formed as primary photoproducts. In that section, it was shown that after an average dose of 0.77 J cm^{-3} (7.7 kJ m^{-2} based on average surface dose) as compared with the 1.06 J cm^{-3} received by the sample of Douki and Cadet (surface dose, 1.5 kJ m^{-2}), the only detectable HPLC peaks present were those due to **II**, **V**, **VI** and the two isomers of **VIII**. Thus, the doses received by our sample irradiated for 8 min (either based on average dose per unit volume or per unit area) and the sample irradiated for 1 h by Douki and Cadet are within the same order of magnitude. Hence, it is questionable whether such an explanation can account for the disparateness of the result of Douki and Cadet and those reported in this study.

Still another possibility to be considered is that the distribution of absorption events within the solutions being irradiated might be quite different, in some way affecting the products being formed. For example, absorption of a significantly higher number of photons near the solution surface in the more concentrated solution of Douki and Cadet could lead to a higher percentage of excited states being produced near the surface that could, in some manner, react differently (*e.g.* deaminate more rapidly) than molecules in bulk solution, thus promoting formation of **III** and the (6-4) adduct of TpT. Indeed, the premise for such an argument can be shown to hold. The 254 nm radiation incident on the samples in both our experiments and in those of Douki and Cadet was essentially completely absorbed. The ratio of the ε value at 254 nm for Tpm^5dC to that of ε_{max} (at 271 nm) can be measured to be 1.38. Thus, ε_{254} can be evaluated, using the estimated value of ε_{max} of 18 150 given previously, to be about 13 150. The absorbance of a 0.5 m*M* solution at 254 nm along a 1 cm path length of our irradiation cell can be calculated to be about 6.6, whereas a similar calculation for the 3.6 m*M* solution of Douki and Cadet along a path length of 0.14 cm produces an absorbance of 6.2. However, the fraction of incident radiation that was absorbed along a given distance of passage through the two irradiated solutions was quite different, being about 7.2-fold higher for the solution of Douki and Cadet. This implies that the number of photons absorbed and number of excited states produced in this latter solution, at any given depth traversed by the light beam, should be about 7.2-fold higher, suggesting that the number of excited state molecules produced near the surface in the solution of Douki and Cadet were 7.2-fold higher than the number produced near the surface in our solutions. Although the considerations outlined in this paragraph do establish one significant distinction between conditions holding in our irradiated solutions and those of Douki and Cadet, the outlines of a mechanism describing how this difference could

account for the discrepancy between the two sets of results are murky.

Another possibility that can be considered is that the Tpm^{5}dC starting material of Douki and Cadet (or the aqueous medium in which it was dissolved) contained a small amount of impurity (other than TpT) that, in some manner, promoted direct formation of the *c,s* CBD and (6-4) products of TpT or, alternatively, dramatically increased the rate of deamination of the initially formed Tpm^{5}dC *c,s* CBD and (6-4) adduct. For example, such an agent might promote highly efficient deamination of the m^{5}C residue in the excited state of Tpm^{5}dC, resulting in formation of the *c,s* CBD and (6-4) adducts of TpT as the dominant products. However, this must be considered a highly speculative idea that because of the non-accessibility of the parent starting material(s) cannot be tested.

There are several other potential causes for the differences in our results and those of Douki and Cadet. These workers did not buffer their solution during irradiation (T. Douki, personal communication) and did not indicate the pH of the unbuffered solution in which reaction occurred. Thus, it is possible that pH differences might play a role in explaining the disparity between our results. In our studies, we have examined in detail at the photochemistry of Tpm^{5}dC at pH 4.6 and 7.4 in the absence of added buffer and at pH 7.55 in 5 m*M* phosphate buffer (see above). In each of these cases, we found that the Tpm^{5}dC products described above were formed as primary photoproducts; however, the stability of the *c,s* CBD of Tpm^{5}dC, once formed, is dependent on pH, being considerably more susceptible to deamination at the lower pH. We did not find evidence for formation of either the *c,s* CBD or the (6-4) adduct of TpT as primary photoproducts at pH 7.4 in unbuffered solution or in the phosphate-buffered solution. At pH 4.6, there was always some *c,s* TpT CBD present in freshly irradiated solutions; however, this likely resulted from deamination of parent Tpm^{5}dC *c,s* CBD. The (6-4) adduct of TpT was observed only as a trace photoproduct at pH 4.6 and then only after high doses of 254 nm radiation.

The limited scope of our studies, on the effect of pH on the photochemistry of Tpm^{5}dC, do not allow us to rule out the possibility that this factor plays a role in explaining the disparity between our results and those of Douki and Cadet. A significantly different photochemistry, as compared with that occurring in our studies, might result in solutions irradiated at pH values significantly higher or lower than the ones we used in our studies.

Another possibility to consider is that the temperature of irradiation could affect the photochemistry. Douki and Cadet did not control the temperature during their irradiations (T. Douki, personal communication), whereas our preparative irradiations were done at about 4°C. We made a comparative study of the photochemistry of Tpm^{5}dC at room temperature (about 22°C) with that occurring at 4°C when the two samples are irradiated under otherwise identical conditions. There was not a significant difference in the amount of *c,s* TpT CBD formed in the two experiments. There was no evidence of formation of significant amounts of the TpT (6-4) adduct at either temperature. These results suggest that temperature of irradiation is probably not important in explaining the dissimilarities of our results and those of Douki and Cadet.

In summary, although a number of factors can be suggested (and a number discounted) as potential reasons for the disparity in the photochemistry of Tpm^{5}dC described by the experiments reported in this study and by those of Douki and Cadet, it is not possible to make any definitive statements concerning the reason(s) for the deviations in the two sets of results. The possibility remains that the reaction conditions used by Douki and Cadet differ in some unidentified manner from those we used and that this divergence is responsible for the differing results.

One general conclusion that can be drawn, on the basis of the studies described in this article, is that the type of photoinduced deamination process suggested by Douki and Cadet (*i.e.* deamination of Tpm^{5}dC in its excited state, leading to formation of the *c,s* CBD and (6-4) adduct of TpT as predominant primary photoproducts, rather than the corresponding Tpm^{5}dC compounds) does not act as an important mechanism under the reaction conditions we used. However, the operation of such a mechanism cannot be ruled out under conditions that were not encompassed by our studies.

SUMMARY AND CONCLUSIONS

We have shown that UV irradiation of the biologically relevant dinucleoside monophosphate Tpm^{5}dC leads to formation of eight products. Two of these are CBD of Tpm^{5}dC, namely the *c,s* and *t,s* isomers (**II** and **V**). The (6-4) product (**VI**) and Dewar adduct (**VII**) of Tpm^{5}dC have also been isolated and characterized. Two acrylamidine products (**VIII**) are formed, presumably via reaction of the m^{5}C moiety with water. We have also isolated a product (**IX**) in which the dinucleotide has lost its m^{5}C moiety via a depyrimidination reaction. The *c,s* CBD of TpT (**III**) is formed as a secondary thermal reaction product, resulting from deamination of **II**. Finally, small amounts of TpT (**IV**) were isolated.

The distribution of photoproducts is dependent upon the wavelength of light used for promoting photoreaction. Irradiation with either unfiltered or Vycor filtered lamps emitting predominately at 254 nm leads to preferential formation of the (6-4) product, the Dewar adduct, the acrylamidine products and the depyrimidinated dinucleotide. Irradiation with lamps that emit primarily at 312 nm yields the two CBD of Tpm^{5}dC as predominant products, although the Dewar adduct and the two acrylamidine products are also present in significant amounts in the resultant photoreaction mixture. The acrylamidine products, analogous to **VIII**, and the depyrimidinated photoproduct **IX** have not been previously observed in the context of dinucleotide photochemistry.

The *c,s* CBD of Tpm^{5}dC undergoes thermal deamination to form the corresponding dimer of TpT. Rate constants for this process have been measured at four different temperatures and the enthalpy and entropy of activation for the deamination reaction have been evaluated at pH 7.4. These results are likely to be relevant to understanding the mutational hot spots that are observed at Tpm^{5}dC sites in UV-irradiated eukaryotic DNA (Ref. 13 and references therein). The rate of the deamination reaction of **II** appears to be considerably enhanced at the acidic pH of 4.6, as compared with pH 7.4, which has implications for the design of studies to detect **II** itself in UV-irradiated DNA and chromatin.

As mentioned above, the *c,s* Tpm^{5}dC dimer is thought to play a role in UV-induced mutagenesis. However, the other products characterized here may also have significance for understanding the deleterious effects of UV radiation on eukaryotic cellular systems, particularly that in the UV-B region. If such products are indeed produced photochemically at key Tpm^{5}dCpdG sites (*e.g.* in regions of chromosomal DNA associated with transcriptional regulation), then incomplete repair (*e.g.* replacement of the damaged m^{5}C with Cyt without subsequent methylation to restore the original base sequence) could induce alterations in gene expression with attendant biological consequences.

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