Kinetic Analysis of the Deamination Reactions of Cyclobutane Dimers of Thymidylyl-3′,5′-2′-deoxycytidine and 2′-Deoxycytidylyl-3′,5′-thymidine†

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ABSTRACT: The cyclobutane dimer photoproducts of dTpdC and dCpdT have been produced by acetophenone photosensitization and separated by reverse-phase HPLC. Each dinucleoside monophosphate was shown to produce one cis,syn isomer and two trans,syn isomers. Three of these photoproducts, namely, the cis,syn isomers of dTpdC and dCpdT and one trans,syn isomer (the syn-anti glycosidic isomer) of dTpdC were selected to study the deamination kinetics. Analysis of the pH dependence indicates that the deamination proceeds by the hydrolysis of the imido amide group of the 5,6-saturated cytosine base with the formation of a carbinolamine intermediate. Determination of the kinetic parameters showed that, for these three cyclobutane dimers, the rate-determining step at physiological pH is a nucleophilic attack of hydroxide ion on the protonated 5,6-saturated cytosine base. The kinetic analysis showed that the cis,syn isomers deaminate ~3 times faster than the trans,syn isomer, which is due to a large difference in pK_a of the 5,6-saturated cytosine moiety. An electrostatic interaction between the iminium group of cytosine and the carbonyl group of thymine is proposed to account for the increase in pK_a for the cis,syn isomers relative to the trans,syn isomer. A similar interaction is proposed to explain the relative difference in reactivity between the cis,syn isomers and the trans,syn isomer with regard to the breakdown of the carbinolamine intermediate.

The strong correlation between sunlight exposure and human skin cancer points to the importance of UV-induced mutagenesis. Since DNA modification is considered to be the fundamental event in UV mutagenesis, considerable research effort has gone into determining the structural details, frequency, and site specificity of DNA photoproducts, the mechanisms of DNA repair, and the perturbations that these DNA photoproducts have on transcription and replication mechanisms [for reviews see Cadet and Vigny (1990), Ananthaswamy and Pierceall (1990), Friedberg (1990), and Echols and Goodman (1990)].

A brief survey indicates the disturbing complexity of DNA photochemistry. The type and distribution of photoproducts is dependent upon the spectrum of UV irradiation. UVC (200–290 nm, filtered out at the earth's surface by ozone) and UVB (290–320 nm) produce DNA photoproducts predominantly by direct photolysis, whereas UVA (320–380 nm) causes DNA damage largely through indirect effects. Photoproducts from direct photolysis include (1) pyrimidine cyclobutane dimers formed at dipyrimidine sequences (Cadet & Vigny, 1990), (2) (6–4) photoadducts also formed at dipyrimidine sequences which are largely converted to their Dewar valence isomers by direct secondary photolysis with UVB and UVA radiation (Mitchell & Nairn, 1989; Taylor et al., 1990b), (3) pyrimidine photohydrates predominantly formed at cytosine bases (Boorstein et al., 1989; Weiss et al., 1989), and (4) dimers between adjacent adenines and between thymine and adenine which arise via addition to form azetidine and cyclobutane dimers, respectively (Kumar et al., 1991; Koning et al., 1990). In addition UVB irradiation produces strand breakage which may be caused by direct and/or indirect effects (Francis et al., 1988; Miguel & Tyrrell, 1983).

DNA lesions from UVA irradiation arise from indirect effects wherein endogenous chromophores induce photooxidations through Type I and II photosensitization processes. The lesions formed are strand breaks, DNA–protein cross-linking, and oxidative base damage (Peak & Peak, 1991; Kochevar & Dunn, 1990).

Mutations due to direct absorption of UV radiation by DNA are characteristic, resulting in predominantly C → T transitions at dipyrimidine sites and, to a lesser extent, T → C transitions at dipyrimidine sites, CC → TT double-base mutations, and frameshift mutations in homopyrimidine sequences (Armstrong & Kunz, 1990; Hsia et al., 1989; Keyse et al., 1988; Brash, 1988; Hutchinson, 1987). Since the majority of these mutations occur at dipyrimidine sequences, these mutations are usually explained as being due to errors in translesion synthesis due to DNA photoproducts. Again, due to the involvement of dipyrimidine sequences, pyrimidine cyclobutane dimers and (6–4) photoadducts (as well as Dewar isomers) are believed to be the principal photoproducts responsible for mutations. Evaluations of the mutagenicity of these two types of photoproducts have been undertaken (Zdzienicka et al., 1992; Brash, 1988; Hutchinson, 1987; Armstrong & Kunz, 1990). However, it must be recognized that such generalizations may not be possible. First of all, it was proposed that DNA structural features are a major determinant in the transition mutation since mutation frequency did not correlate with photoproduct frequency (Brash et al., 1987). Another major factor is that the mutagenic potential of both cyclobutane dimers and (6–4) photoadducts may be highly dependent on the dipyrimidine sequence. It has been shown that, with SOS induction, *cis,syn*-TT cyclobutane dimers are replicated in vivo with remarkable fidelity. In contrast, (6–4) TT photoadducts are highly mutagenic, causing specific T → C transitions, with Dewar (6–4) TT photoadducts showing less mutation specificity (LeClerc et al., 1991; Lawrence et al., 1990).

It is of importance to determine how DNA replication enzymes process these photoproducts; i.e., whether the lesions

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are read as noninstructive or misinstructive (Lawrence et al., 1990; Echols & Goodman, 1990). If the photolesions are processed as nonpairing, then the A rule, stating that pdA is preferentially inserted opposite nonpairing lesions by DNA polymerases, accounts very well for the predominant C → T transitions observed. If the lesions are instead mispairing, then the exact structure of the photoproduct is of critical importance. Since the majority of mutations are at cytosine bases and since many photoproducts contain cytosine bases saturated at the 5,6 bond, deamination of the cytosine base to the corresponding uracil base could be the crucial event in UV mutagenesis. The idea that UV-induced C → T mutations could be explained by the correct replicative bypass of deaminated cis,syn cyclobutane dimers has been described in detail (Taylor & O'Day, 1990). In addition, it has been proposed that, before deamination, the replication enzymes are blocked at cytosine-containing dimers but, after deamination, are able to replicate correctly past uracil-containing dimers (Tessmann et al., 1992). Consideration of deamination introduces several complexities into mutagenesis since the facility of deamination may be critically dependent upon the type and dipyrimidine sequence of the photoproduct and also on the DNA structure.

The deamination of cytosine-containing cyclobutane dimers has been reported several times previously. Liu and Yang (1978) reported that the average half-life of their mixtures of *c,s*- and *t,s*-dT[p]dC[1] isomers was 1 h at 50 °C. The deamination of a cyclobutane dimer between cytosine and 5-methoxyuracil (presumably the cis,syn isomer) was measured to have a rate constant of 9×10^{-5} s^{-1} at room temperature (Skalski et al., 1988). Recently it has been reported that the photoproducts of dCpdT deaminate at different rates at room temperature (Douki & Cadet, 1992). The deamination of cytosine dimers in polynucleotides was first reported by Setlow et al. (1965). The in vivo deamination of cytosine-containing dimers has been investigated and different rate constants have been obtained (Fix & Bockrath, 1981; Fix, 1986). However there has been no mechanistic analysis of the deamination reaction, which may explain the rate differences of different photoproducts. With this objective in mind, we set out to examine in detail the deamination mechanism of cytosine-containing photoproducts and herein report the kinetic parameters for the deamination of cyclobutane dimers of dTpdC and dCpdT.

MATERIALS AND METHODS

Chemicals and Equipment. dTpdC and dCpdT were obtained from Sigma Chemical Co. (St. Louis, MO) and were purified by reverse-phase HPLC as described below. Acetonitrile and methanol, HPLC-grade, and acetophenone were used as received (Anachemia, Montréal, Canada). Ammonium acetate, boric acid, hydrochloric acid, monochloroacetic acid, sodium hydroxide, sodium phosphate dibasic, sodium phosphate monobasic (Fisher Scientific), sodium chloride (BDH), and sodium acetate anhydrous (J. T. Baker) were used as received. Water was purified by filtration through ion-exchange filters (Barnstead D0803, D0809) followed by double distillation in a quartz still.

Gradient reverse-phase HPLC was performed on a system consisting of a Model 7125 injector (Rheodyne Inc., Cotati, CA) and two Series 501 pumps and a Model 441 UV detector (Waters Chromatography Division, Millipore Corp., Milford, MA) controlled by an Apple IIE computer via on Adalab data acquisition card, a Chromadapt interface module, and a Chromatochart software (Interactive Microwave Inc., State College, PA). Reverse-phase HPLC was performed on Nucleosil 100A/ODS, 5 μm, 1- × 25-cm columns (CSC Inc., Montréal, Canada).

UV–Vis absorption spectra were obtained on a Cary 2200 spectrophotometer (Varian Associates Inc.) interfaced to a DTK 1630 computer. Mass spectra were acquired using the technique of HPLC/thermospray mass spectrometry as described before (Bérubé et al., 1992). The photolysis system consisted of a 1000-W Xe–Hg lamp (Hanovia) whose output was passed through a 10-cm water filter and dispersed with an 0.25-m UV-blazed grating monochromator (dispersion 1.6 nm/mm, Spectral Energy Co., Hillsdale, NJ). The monochromator output was collimated onto a water-cooled cuvette holder and the fluence rate was determined with a 88XLC radiometer equipped with a Model 200 sensor head (Photodyne Inc., Newbury Park, CA).

Separation of Dinucleoside Monophosphate Photoproducts. The products from acetophenone photosensitization of dinucleoside monophosphates were obtained by 313 ± 4 nm photolysis of aqueous solutions containing 1 mM dinucleoside monophosphate and 15 mM acetophenone with N_2 degassing. The photolysis time was typically 60 min with an average fluence of 5 kJ/m^2. The photoproduct mixture, after lyophilization, was resuspended in 0.1 M ammonium acetate, pH 6.5, and separated by ion-suppression reverse-phase HPLC using a 3 mL/min, 50-min exponential ($y = x^{1.8}$) gradient of 0–20% acetonitrile in 0.1 M ammonium acetate, pH 6.5. Photoproduct fractions, detected at 229 nm, were lyophilized and purified by reverse-phase HPLC using a 2 mL/min, 20-min linear gradient of 0–20% methanol in water.

Preparation of dTpdU and dUpdT. The products from acetophenone photosensitization of dTpdC and dCpdT were deaminated during kinetic measurements. The deamination products, cyclobutane dimers of dTpdU and dUpdT, were photolyzed at 240 nm until the absorbance change at 265 nm was small. The mixture was then lyophilized and subjected to reverse-phase HPLC as described above to obtain purified dTpdU and dUpdT.

Kinetic Measurements. The deamination reactions were carried out in a cylindrical water-jacketed, 5-cm path length quartz cuvette in buffered aqueous solution with 0.4 M NaCl to maintain ionic strength. Buffers used were monochloroacetate, acetate, phosphate, and borate with concentrations varying from 0.01 to 0.08 M. A Corning pH meter 3D equipped with a calomel combination electrode (cat. no. 476183) were used to determine the pH values of the buffer and also of the reaction solutions after kinetic measurements. For the pH dependence, the temperature was maintained at 50 ± 1 °C, and for the determination of Arrhenius parameters, the temperature was varied from 30 to 65 °C at a constant pH 6.0. The thermally equilibrated buffer solution was used to dissolve the lyophilized photoproduct, which was filtered and replaced in the cuvette. After thermal reequilibration for 3 min, the deamination reaction was typically monitored by following the absorbance changes at 240 nm, although occasionally spectra from 210 to 340 nm were recorded. Least-

[1] Abbreviations: Dinucleotides are abbreviated according to Saenger (1984). Therefore dCpdT refers to 2′-deoxycytidylyl-3′,5′-thymidine. Cyclobutane dimer photoproducts of dinucleoside monophosphates are designated X[p]Y according to Cohn et al. (1974), where the prefix indicates the stereochemistry of the cyclobutane ring and the suffix indicates the orientation of the glycosidic bond angles. Therefore *c,s*-dT[p]dC (anti-anti) refers to the cis,syn cyclobutane dimer of dTpdC with anti (dTp-) and anti (-pdT) glycosidic bond angles. R_t, retention time; PB, phosphate buffer.

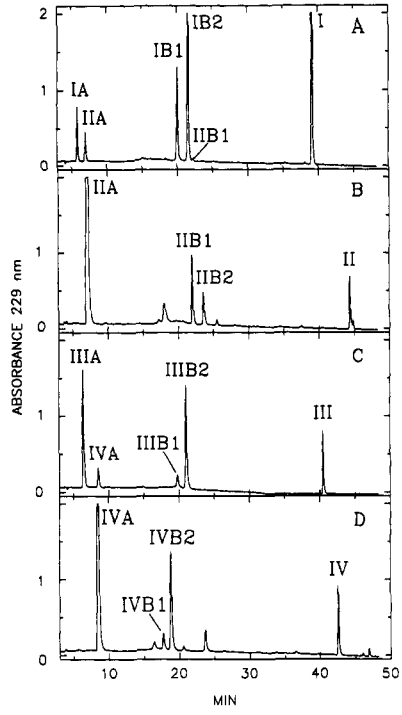


FIGURE 1: Reverse-phase HPLC separation of the products from the acetophenone photosensitization of dinucleoside monophosphates. (A) dTpdC, I; (B) dTpdU, II; (C) dCpdT, III; (D) dUpdT, IV.

squares analysis of the data was performed using the Marquardt algorithm.

RESULTS

Identification and Characterization of Photoproducts. The HPLC chromatograms of the products formed from the acetophenone photosensitization of dTpdC, dTpdU, dCpdT, and dUpdT are shown in Figure 1. Acetophenone photosensitization is known to result exclusively in the formation of cyclobutane dimers by energy transfer to form the excited $^3\pi,\pi^*$ state of the thymine base (Cadet & Vigny, 1990). For dTpdC (I) three major products, IA, IB1, and IB2, are produced (Figure 1A), which display absorbance spectra with maxima at 223 nm in neutral aqueous solution. Separate photolysis of IA, IB1, and IB2 in solution at 240 nm results in photoreversal to I for each photoproduct as determined by HPLC and the appearance of an absorbance band at 268 nm. This photoreversion is a well-characterized reaction of pyrimidine cyclobutane dimers (Patrick & Rahn, 1976; Fisher & Johns, 1976b). The UV absorbance maxima at 223 nm for IA, IB1, and IB2 disappear upon standing in aqueous solution, as shown in Figure 2 for IB2. This spectral change is typical of 5,6-saturated cytosine derivatives which undergo deamination to form the corresponding uracil derivatives (Fisher & Johns, 1976a). This deamination reaction may also be followed by HPLC due to the different retention times of the corresponding uracil derivatives, as shown in Figure 1. These characterizations conclusively identify IA, IB1, and IB2 as the isomeric cyclobutane photodimers of dTpdC, in agreement with previous studies (Liu & Yang, 1978; Koning et al., 1991). Two geometric isomers of the cyclobutane dimer (cis,syn and trans,syn) are possible, and for each geometric isomer there are two possible combinations of N-glycosidic conformations, which result in diastereomers. For the cis,syn dimer the glycosidic bond angles may be anti-anti or syn-syn while for the trans,syn dimer, anti-syn and syn-anti glycosidic conformations are possible (Saenger, 1984).

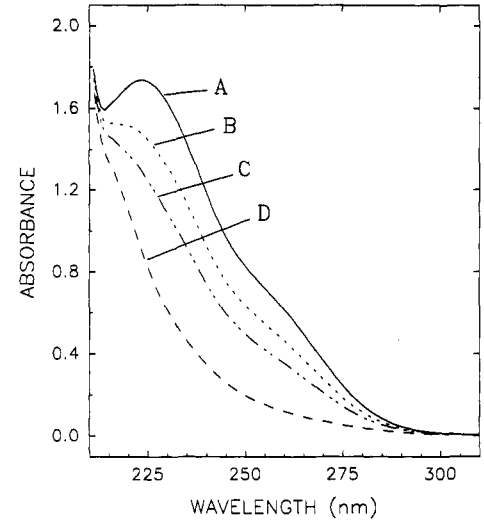
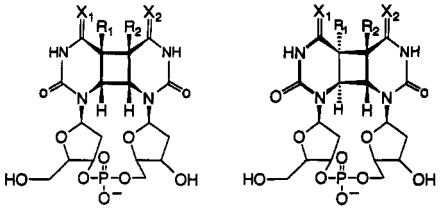


FIGURE 2: Variation of absorbance spectra upon deamination of IB2; conditions, pH = 6.0, 10 mM PB, T = 50 °C. (A) t = 0 min; (B) t = 70 min; (C) t = 140 min; (D) t = 686 min.

Chromatographic studies on dinucleoside monophosphate photoproducts have conclusively shown that ion-suppression reverse-phase HPLC is capable of clearly separating cis,syn from trans,syn dimers, with the more hydrophilic cis,syn dimer eluting before the trans,syn dimer (Demidov & Potaman, 1984; Rycyna & Alderfer, 1985; Cadet et al., 1985; Taylor et al., 1988; Koning et al., 1991). The retention time of fraction IA (5.85 min) agrees very well with that reported for *c,s*-dT[p]dC (R_t = 4.2 min; Koning et al., 1991). All of the above considerations lead us to identify IA as *c,s*-dT[p]dC (anti-anti). NMR studies on the photoproducts of dinucleoside monophosphates have indicated that the cis,syn dimers generally have anti-anti conformations, although there is some flexibility for the 5′ glycosidic bond ranging from high anti to syn depending on the particular cis,syn pyrimidine isomer (Kemmink et al., 1987a; Kan et al., 1988; Koning et al., 1991).

The two other major photoproducts, IB1 and IB2, have been previously identified as trans,syn cyclobutane dimers (Liu & Yang, 1978; Koning et al., 1991). In those studies it was found that the *t,s*-dT[p]dC (syn-anti) isomer is formed in a 3:1 ratio over *t,s*-dT[p]dC (anti-syn). The results shown in Figure 1A indicate that IB2 is formed 2–3 times more efficiently than IB1. However, IB2 elutes later than IB1 under our HPLC conditions, in contrast to the results of Koning et al. (1991) wherein the major *t,s*-dT[p]dC (syn-anti) isomer eluted before the minor *t,s*-dT[p]dC (anti-syn) isomer. Fractions IB1 and IB2 were conclusively identified by examining their deamination products, which coelute with fractions IIB1 and IIB2, respectively, obtained from acetophenone photosensitization of dTpdU (II) (Figure 1B). Fractions IIA, IIB1, and IIB2 were identified as cyclobutane dimers of II using HPLC/thermospray mass spectrometry (Bérubé et al., 1992). NMR analysis of the deamination products of IB1 and IB2 resulted in the assignment of IB1 as *t,s*-dT[p]dC (anti-syn) and IB2 as *t,s*-dT[p]dC (syn-anti) (Tabaczynski et al., 1993).

From the acetophenone photosensitization of dCpdT (III), two major products (IIIA and IIIB2) and two minor products (IVA and IIIB1) are separated by our HPLC conditions (Figure 1C). Fraction IIIA is photoreversed back to III by 240-nm photolysis and undergoes decomposition in aqueous solution with similar spectral changes as shown in Figure 2, resulting in product IVA. This product is also the main product



	X_1	R_1	X_2	R_2
dTpdC	O	CH_3	NH	H
dCpdT	NH	H	O	CH_3

FIGURE 3: Structures of the cyclobutane dimers of dTpdC and dCpdT. The cytosine bases are shown in their imino tautomeric form, and for the trans,syn isomer the syn-anti glycosidic diastereomer is shown.

from acetophenone photosensitization of dUpdT (IV) (Figure 1D), and products IVA, IVB1, and IVB2 were identified as cyclobutane dimers of IV by HPLC/thermospray mass spectrometry. These characteristics identify IIIA as a cyclobutane dimer photoproduct of III and its short HPLC R_t is in agreement with that reported for *c,s*-dC[p]dT (anti-anti) (Koning et al., 1991).

Products IIIB1 and IIIB2 display the same characteristics as described above for photoproducts IB1 and IB2, and their late HPLC R_t's and greater relative stability to deamination strongly indicate that they are the trans,syn isomers. The deamination products of IIIB1 and IIIB2 are IVB1 and IVB2, respectively. Previous studies have reported that only one trans,syn isomer is produced from acetophenone photosensitization of dCpdT, which was identified after deamination as *t,s*-dU[p]dT (syn-anti) (Koning et al., 1991). Our results indicate that fraction IIIB2 is formed with a ratio of 10:1 in comparison with fraction IIIB1. It is presumed that the major fraction IIIB2 is *t,s*-dC[p]dT (syn-anti) and therefore fraction IIIB1 would be the previous unidentified other trans,syn diastereomer, *t,s*-dC[p]dT (anti-syn).

The structures of these cyclobutane dimers of dTpdC and dCpdT are shown in Figure 3.

Deamination of Cytosine-Containing Cyclobutane Dimers. The cyclobutane dimers of dTpdC and dCpdT are characterized by an absorbance maximum at 223 nm which disappears upon deamination, a reaction which is faster for the cis,syn isomers than for the trans,syn isomers. This spectral change can be easily monitored to determine the kinetics of the deamination reaction. Three cyclobutane dimer products were selected to investigate the reaction in detail: *c,s*-dT[p]-dC (IA), *c,s*-dC[p]dT (IIIA), and *t,s*-dT[p]dC (syn-anti) (IB2). The first two are chosen since they correspond to the main photoproducts at TC and CT sequences in DNA, and the last product was chosen to compare cis,syn and trans,syn isomers.

The spectral absorbance changes were found to always strictly follow first-order kinetics independent of the observation wavelength or solution composition. The absorbance changes for all kinetic runs were followed for a minimum of 4 half-lives and the pseudo-first-order rate constants were determined by a nonlinear least-squares analysis of the data. The effect of buffer at all pH values and for all three compounds were determined and an example is shown in Figure 4 for phosphate catalysis of *c,s*-dT[p]dC deamination. Linear regression analysis of the data, as shown in Figure 4, was used to determine the rate constants corrected for buffer catalysis, and these extrapolated rate constants with their associated standard errors are plotted as a function of pH for all three

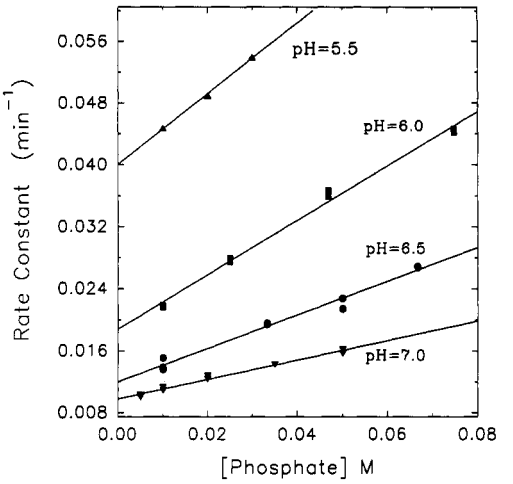


FIGURE 4: Change in the measured deamination rate constant for IA as a function of pH and buffer concentration.

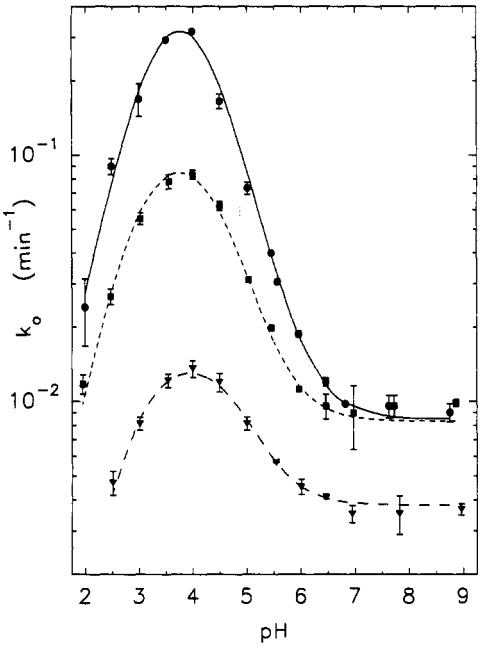


FIGURE 5: pH–rate profile for the hydrolysis of IA (●), IIIA (■), and IB2 (▼), corrected for buffer catalysis. The curves are obtained from nonlinear regression analysis using eq 1.

compounds in Figure 5. The bell-shaped curves thus obtained are characteristic for reactions involving hydrolysis of imines (Kayser & Pollack, 1977; Cordes & Jencks, 1963). The deamination of saturated cytosine bases can be described as a hydrolysis of the imido amide functional group, for which the mechanism is depicted in Figure 6.

The observed rate constant, which is derived from the depicted mechanism using a steady-state approximation, is a function of the acid dissociation constant (K_a) and the individual rate constants k_1, k_{-1}, k_2, and k_3:

$$k_0 = \frac{k_1[H^+][H_2O] + K_w k_2}{([H^+] + K_a)([H^+](k_{-1}/k_3) + 1)} \quad (1)$$

where k_0 is the deamination rate constant corrected for buffer catalysis and K_w is the ion product of water at 50 °C. The four parameters in eq 1 (K_a, k_1, k_2, and k_{-1}/k_3) were determined by a weighted (reciprocal square of standard errors), unconstrained, least-squares fitting of the data. Consistent values were obtained using a wide variety of starting

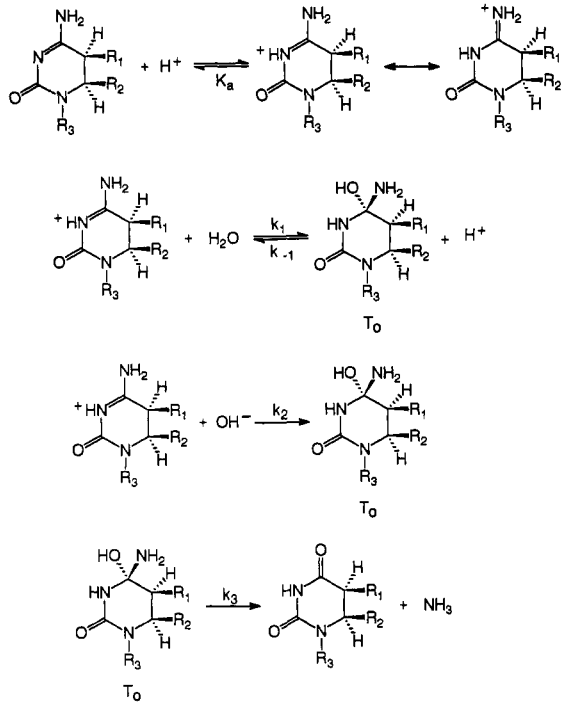


FIGURE 6: Mechanism of hydrolytic deamination for 5,6-saturated cytosine bases.

Table I: Kinetic Parameters from Nonlinear Regression Analysis of Observed Deamination Rate Constants[a]

parameters	*c,s*-dC[p]dT IA	*c,s*-dT[p]dC IIIA	*t,s*-dT[p]dC IB2
K_a (M)	$(3.45 \pm 1.70) \times 10^{-5}$	$(6.12 \pm 1.89) \times 10^{-5}$	$(1.18 \pm 0.41) \times 10^{-3}$
pK_a	4.46 ± 0.23	4.21 ± 0.14	2.93 ± 0.16
k_1 (M^{-1} s^{-1})	$(0.36 \pm 0.12) \times 10^{-4}$	$(1.74 \pm 0.47) \times 10^{-4}$	$(3.66 \pm 1.57) \times 10^{-4}$
k_2 (M^{-1} s^{-1})	$(0.87 \pm 0.49) \times 10^{5}$	$(1.58 \pm 0.54) \times 10^{5}$	$(1.37 \pm 0.47) \times 10^{6}$
k_{-1}/k_3 (M^{-1})	960 ± 650	1940 ± 1150	$65\,000 \pm 15\,000$

[a] Values given with 95% confidence intervals.

parameters and these values are listed in Table I with the calculated curve fits shown in Figure 5.

Our limited investigation of buffer catalysis shows four distinct regions for all three dimers: (1) above pH 7, there is no buffer catalysis; (2) between pH 5 and 7, there is general acid catalysis as depicted in Figure 4; (3) between pH 3.5 and 4.5, there is a small amount of catalysis with no definite trend; and (4) below pH 3.5, there is no buffer catalysis. Below pH 2.0, absorbance measurements become very difficult due to the loss of the absorbance maximum upon protonation of the cytosine.

In addition, the temperature dependence of the deamination rate constant was determined at pH 6.0 and 5 mM acetate for IA and IB2. The calculated Arrhenius parameters were $\ln k = 17.25$ and $E_a = 57.2 \pm 1.2$ kJ mol^{-1} for IA and $\ln k = 19.55$ and $E_a = 67.5 \pm 3.0$ kJ mol^{-1} for IB2.

DISCUSSION

The maximum observed in the pH–rate profiles for all three dimers is characteristic for the hydrolysis of imines and indicates the formation of a carbinolamine intermediate with a change in rate-determining step as a function of pH (Cordes & Jencks, 1963; Jencks, 1969; Kayser & Pollack, 1977; Okuyama et al., 1982). Below the pH maximum of 3.8–4.0, breakdown of carbinolamine intermediate (T_0 in Figure 6) is rate-determining, and above the pH maximum, the formation of T_0 is rate-determining. For amidine compounds this pH dependence of the hydrolysis rate is not observed, although a detailed investigation did indicate a two-step mechanism with formation of a carbinolamine intermediate (Robinson & Jencks, 1967a,b). Various properties of the imine functional group such as pK_a, susceptibility to H_2O and OH^- attack, intramolecular interactions, and basicity of the leaving group will influence the role of each individual reaction in determining the rate-determining step, and an examination of buffer catalysis helps to unravel the importance of each individual reaction in Figure 6 as a function of pH.

Temperature Dependence. Each reaction step depicted in Figure 6 will have a temperature dependence which may be determined by measuring the entire pH–rate profile at several temperatures. Measurement of the temperature dependence at one pH value and buffer concentration gives only the Arrhenius parameters for the overall rate constant. Approximate values for the observed deamination rate constant at 25 °C and pH 7 for IA and IB2 were determined by using the Arrhenius parameters and correcting for the pH. These calculated values of $k(c,s\text{-dT[p]dC}) = 1.5 \times 10^{-3}$ min^{-1} and $k(t,s\text{-dT[p]dC}) = 3.9 \times 10^{-4}$ min^{-1} agree very well with the deamination rate constants recently reported for the cis,syn and trans,syn cyclobutane dimers of dCpdT under similar conditions (Douki & Cadet, 1992).

More interestingly, analysis of the thermal resistance of UV-induced glutamine t-RNA mutations in *Escherichia coli* strains gave estimates of the activation energies and rate constants for the mutation reactions (Fix, 1986). The mutations were proposed to be due to the deamination of cytosine in cytosine-containing cyclobutane dimers due to the suppression caused by photoreactivation, and values of 17 ± 3 kcal mol^{-1} for the activation energies of two mutation reactions with different rates were measured. This value is in better agreement with the value for the trans,syn isomer, IB2 ($E_a = 16.1 \pm 0.7$ kcal mol^{-1}), compared to the value ($E_a = 13.7 \pm 0.3$ kcal mol^{-1}) measured for the cis,syn isomer, IA, although the cis,syn dimer has been shown to be the predominant cyclobutane dimer formed in DNA (Patrick & Rahn, 1976). In comparison, an activation enthalpy of 15.2 kcal mol^{-1} was determined for Schiff base hydrolysis (Ehrhardt et al., 1983). These activation energies for deamination of cytosine-containing cyclobutane dimers are much lower than the value of 29 kcal mol^{-1} determined for the deamination of cytosine in DNA (Lindahl & Nyberg, 1974; Frederico et al., 1990).

Dissociation Constants. The dissociation constants for the conjugate acids of the cytosine moiety of the photoproducts (K_a) are given in Table I. The pK_a's are not significantly different for *c,s*-dC[p]dT and *c,s*-dT[p]dC, but there is a significant difference between the cis,syn and trans,syn isomers. The values for the cis,syn isomers are very similar to the pK_a's determined for cytosine derivatives: cytidine, 4.29; 3′-CMP, 4.32; and 5′-CMP, 4.43. These pK_a values increase due to the electrostatic attraction of the negatively charged phosphate group at different distances from the base (Clauwaert & Stockx, 1968). For photoproducts IA, IB2, and IIIA, it is assumed here that the stabilizing effect of the phosphate group is the same for all three isomers. The similar pK_a values for the cis,syn isomers and 3′- and 5′-CMP was unexpected since saturation of the 5,6 bond of cytosine should cause the loss of resonance stabilization of the positive charge of the conjugate acid and therefore a lower pK_a. A similar effect is seen in the increase of pK_a between uracil and 5,6-dihydrouracil due to a loss of resonance stabilization of the negative charge of the conjugate base (Saenger, 1984). Therefore, the similar pK_a

values indicate that there must be a compensation for the loss of resonance and an examination of the structure of the cis,-syn cyclobutane dimer shows the proximity of the N4 amino group on the cytosine moiety to the C4-O4 carbonyl group of the thymine moiety [see Koning et al. (1991)]. Therefore, the possibility of stabilization of the positively charged conjugate acids by hydrogen bonding immediately comes to mind as an explanation for the higher than expected pK_a's. However, consideration of the geometry of the cytosine amino group in relation to the thymine carbonyl in the cis,syn cyclobutane isomer [including deformation by cyclobutane ring puckering (Kim & Alderfer, 1992; Kim et al., 1993)] indicates that the angle between the N–H bond and the oxygen lone pair electrons would not fall in the range where hydrogen bonding is possible (Luck, 1976). Another possibility is a π hydrogen bond between N–H and the electrons in the carbonyl double bond (Kollman & Allen, 1972). Again, however, this does not seem likely since it would require rotation of the C4–N4 bond of cytosine, which is hindered by its partial double bond character. It seems then best to explain the stabilization by an electrostatic dipole interaction between the negative end of a thymine carbonyl group and a positive iminium group (R=N^+H–) of cytosine. Since the trans,syn isomer has these groups on opposite faces of the cyclobutane ring, this interaction is not possible, and therefore its lower pK_a value should more accurately reflect the unperturbed acidity of the imido conjugate acid in 5,6-saturated cytosine derivatives.

pH Dependence and Buffer Catalysis. Above pH 7, the hydrolysis rate constant is pH-independent and no buffer catalysis is observed. This behavior can be explained by referring to eq 2, which is derived from eq 1 by assuming $[H^+]k_{-1}/k_3 \ll 1$ at pH > 5:

$$k_{obs} = (k_1[H_2O] + k_2[OH^-])\left(\frac{[H^+]}{[H^+] + K_a}\right) \quad (2)$$

Analysis of eq 2 shows clearly that the rate-determining step for deamination is nucleophilic attack of H_2O and OH^- on the protonated imine group. The kinetic parameters from Table I show that, above pH 7, the $k_2[OH^-]$ term predominates over the $k_1[H_2O]$ term. The pH independence is explained by an increase in OH^- concentration being compensated for by a decrease in concentration of the protonated cytosine and a lack of buffer catalysis due to the high nucleophilic reactivity of OH^- (Jencks, 1969).

Between pH 5 and 7, the hydrolysis rate constant increases with decreasing pH and general acid catalysis is observed (Figure 4). This behavior is explained by the increasing importance of H_2O attack on the protonated 5,6-saturated cytosine, and the observed catalysis is actually specific acid plus general base catalysis. The observed rate constant at pH 5–7 can be described by a modification of eq 2 to account for buffer catalysis:

$$k_{obs} = (k_1[H_2O] + k_2[OH^-] + k_b[B][H_2O])\left(\frac{[H^+]}{[H^+] + K_a}\right) \quad (3)$$

By use of eq 3, k_b's for phosphate and acetate catalysis were determined for all three cyclobutane dimers and then combined with k_1 for solvent catalysis (Table I) in order to calculate the Bronsted β coefficient, which was found to be 0.27. This value is close to the β values of 0.30–0.45 found for general base catalyzed H_2O attack on cationic Schiff bases (Kayser & Pollack, 1977; Okuyama et al., 1982).

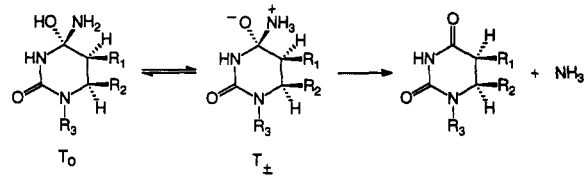


FIGURE 7: Breakdown of the carbinolamine intermediate T_0 via formation of a zwitterion, $T_\pm$.

Logarithmic plots of the rate constants for H_2O and OH^- attack versus the K_a of the cationic imines are generally linear and the slope of this Hammett-like correlation is interpreted as indicating the relative position of the transition state (Cordes & Jencks, 1963; Kayser & Pollack, 1977; Okuyama et al., 1982). It has been generally found that a lower slope is observed for OH^- attack compared to H_2O which is consistent with the concept that the transition state for H_2O attack on the cationic imine is more productlike than the transition state for OH^- attack due to different nucleophilic reactivity. Although only three substrates were examined here, a reasonable linear correlation was obtained for OH^- attack (r = 0.998). The slope of this correlation (–0.76) is appreciably greater than that found for protonated Schiff bases and would indicate a lesser susceptibility to nucleophilic attack for the protonated cytosine. No linear correlation was observed for H_2O attack.

In the pH range 3.5–4.5, a maximum in the hydrolysis rate constant is observed and a small amount of buffer catalysis occurs with no clear trend. These features are explained by a changeover in the rate-determining step which in this pH range is determined by the combination of the formation and breakdown of the carbinolamine intermediate.

For pH values below 3.5, the hydrolysis rate constants decrease with decreasing pH and no buffer catalysis is observed. Breakdown of the carbinolamine intermediate is rate-determining in this pH region, and the rate of hydrolysis is determined by a competition in the breakdown of the carbinolamine between water and ammonia expulsion. No buffer catalysis is observed since, in the case of strongly basic amine leaving groups, such as here, breakdown of the carbinolamine with amine expulsion takes place only through the dipolar form, $T_\pm$ (Figure 7), with no proton transfer (Jencks, 1969; Rosenberg et al., 1974; Sayer et al., 1974). Inhibition occurs in acidic solution because protonation of T_0 or $T_\pm$ removes the dipolar form and inhibits amine expulsion.

Considering this mechanism, the different values of k_{-1}/k_3 obtained for the different cyclobutane dimers may be evaluated. It is obvious that the water expulsion relative to ammonia expulsion is greatly favored for the trans,syn isomer compared to the cis,syn isomers. Steric hindrance may be considered to explain this difference. For the trans,syn isomer, the amine group on the carbinolamine will be interacting with the methyl group on the neighboring thymine and this steric repulsion may destabilize T_0, favoring water expulsion. For the cis,syn isomers the amine group on the carbinolamine will sterically interact with the carbonyl group on the neighbouring thymine and this steric hindrance should be similar to that in trans,syn isomer [see Koning et al. (1991)]. However, since ammonia expulsion proceeds through the dipolar intermediate, $T_\pm$, a π hydrogen-bonding interaction and/or electrostatic stabilization between the amino group and the neighboring carbonyl group may facilitate the proton transfer to form $T_\pm$ and/or stabilize $T_\pm$. Therefore, it is proposed that the higher ratio for ammonia expulsion relative to water expulsion for cis,syn isomers, compared to trans,syn isomers, may be

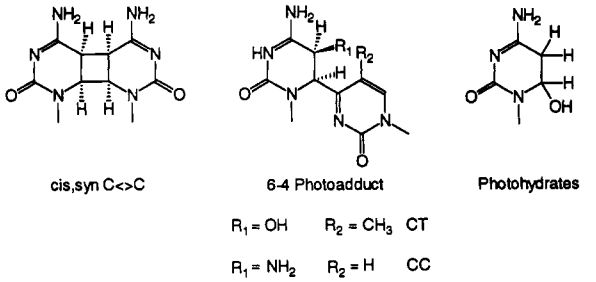


FIGURE 8: Photoproducts containing 5,6-saturated cytosine bases from the direct photolysis of DNA.

explained by this interaction between the amino group in the carbinolamine intermediate and the neighboring carbonyl group on thymine which aids in formation of $T_{\pm}$.

Deamination Rates of Other Cytosine Photoproducts. The detailed analysis of the mechanism of deamination for cytosine-containing cyclobutane dimers indicates the factors important in determining the deamination rate of similar cytosine photoproducts in DNA. These photoproducts include (1) the cis,syn cyclobutane dimer of CC, (2) the (6–4) and Dewar (6–4) photoadducts of CC and CT, and (3) cytosine photohydrates. The structures of these compounds at the base level are shown in Figure 8.

The kinetic parameters determined for the cyclobutane dimers of TC and CT show that, at pH ≥ 7.0, nucleophilic attack on the 5,6-saturated protonated cytosine is rate-determining with only OH^- attack being important. Using the values from Table I in eq 2, it is seen that the deamination rate constant for the trans,syn isomer is 3 times smaller than for cis,syn isomers due to the large difference in K_a values even though the rate constants for nucleophilic attack are higher for the trans,syn isomer. For the cis,syn cyclobutane dimer of dCpdC, the deamination rate constant for one cytosine moiety should be lower than that determined for *c,s*-dT[p]dC and *c,s*-dC[p]dT. This is predicted because the pK_a of one cytosine moiety in *c,s*-dC[p]dC should be lower than the pK_a values of the other cys,syn isomers, because the neighboring amino group should not be able to stabilize the iminium group by dipole interactions as well as a carbonyl group. The deamination rate constant for the second cytosine corresponds to deamination of *c,s*-dC[p]dU or *c,s*-dU[p]dC, and it is expected to be similar to the rate constants observed for *c,s*-dC[p]dT and *c,s*-dT[p]dC since the interaction with the neighboring carbonyl group is possible and the pK_a values should be similar.

For the (6–4) and Dewar (6–4) photoadducts of dCpdC and dCpdT, the saturated cytosine has a pyrimidone ring at the C6 position and either a hydroxy group for dCpdT or an amino group for dCpdC at the C5 position. These groups are not expected to influence the pK_a of the saturated cytosine, which should be similar to that for *t,s*-dT[p]dC. As well, the rate constants for nucleophilic attack are expected to be similar. No intramolecular catalysis by the C5 OH group is expected since it has been shown that intramolecular general base catalysis by an ionized OH group in the ortho position does not occur in the hydrolysis of Schiff bases derived from salicyaldehyde and from 3-hydroxypyridine-4-carboxaldehyde (Reeves, 1965; French et al., 1965). Therefore, the deamination rate constants for the (6–4) and Dewar (6–4) photoadducts of dCpdT and dCpdC are expected to be similar to that of *t,s*-dT[p]dC. This prediction is supported by recent experimental results which indicate that the deamination rate constants for *t,s*-dC[p]dT and (6–4) dCpdT are similar (Douki & Cadet, 1992).

The same considerations apply to the deamination of cytosine photohydrates as described above for (6–4) photoadducts, and therefore, the deamination of the cytosine photohydrates should have a similar rate constant as for *t,s*-dT[p]dC. This deamination reaction is in competition with the dehydration reaction across the C5–C6 bond, which is more rapid (Fisher & Johns, 1976a; Cadet & Vigny, 1990).

Deamination in DNA. Consideration of the deamination mechanism of cytosine photoproducts of dinucleoside monophosphates indicated the importance of the photoproduct structure in determining the hydrolysis rate. However, in DNA other factors may influence the deamination.

One factor to consider is the accessibility of the nucleophilic attack of H_2O and OH^-. Electrostatic repulsion by the phosphodiester backbone of double-stranded DNA should considerably inhibit hydroxide ion attack. In addition, the restricted motion of water in the primary hydration shell around DNA is expected to reduce the ability of H_2O to react. These two factors along with base stacking and hydrogen bonding are expected to considerably reduce the deamination rate in double-stranded DNA. It is rather surprising, then, that the values measured here for cis,syn dimers of TC and CT ($\sim 8.0 \times 10^{-3}$ min^{-1}) are only different by a factor of 2–3 from the values measured in vivo ($\sim 3.0 \times 10^{-3}$ min^{-1}) (Fix, 1986) although the pH and temperature are similar. In comparison, the rate of deamination of a single cytosine residue is 2 orders of magnitude greater for single-stranded DNA than for double-stranded DNA (Frederico et al., 1990). The presence of the cyclobutane dimer will disrupt the DNA double helix although experiments indicate that the DNA remains double-stranded (Kemmink et al., 1987b; Taylor et al., 1990a). It therefore would seem that the rate of deamination of cytosine-containing cyclobutane dimers is higher than expected in DNA. Two factors may explain this.

The first factor is the possible internal catalysis by a phosphate group. The nucleophilic attack of H_2O is subject to general base catalysis and therefore may be catalyzed by a phosphate group in DNA. Intramolecular general base catalysis of Schiff base hydrolysis by internal amino and carboxylate groups has been demonstrated to increase the rate constant for H_2O attack by 1–3 orders of magnitude (Kayser & Pollack, 1977; Okuyama et al., 1982). The possible effect of intramolecular general base catalysis by phosphate may be investigated by measuring the deamination rate constant–pH profile for dinucleotides. Inspection of models indicates that a phosphate group at the 5′ end should be in a favorable position to catalyze addition of water on a saturated cytosine base at the 5′ end, while the position of a phosphate group at the 3′ end does not seem to be as favorable to catalyze water attack on cytosine bases at the 3′ end. Therefore, intramolecular catalysis may cause the deamination of TC and CT dimers, which for dinucleoside monophosphates is the same at physiological pH, to be different in DNA. In addition, the deamination of different photoproducts such as trans,syn dimers of TC and CT and the (6–4) adducts of CT and CC may be affected differently by this intramolecular phosphate catalysis.

Another important factor which may change the deamination rate in DNA is the polarity of the environment. The effect of solvent hydrophobicity on the hydrolysis of Schiff bases has been carefully investigated by Pollack and coworkers, who showed that solvent polarity affects the deamination in three ways: (1) change in the pK_a of the protonated imine, (2) change in the rate constant for H_2O attack, and

(3) change in the rate constant for general base catalysis of H_2O attack (Pollack et al., 1977).

Increase of solvent hydrophobicity was shown to increase the rate constants of nucleophilic attack on protonated Schiff bases by factors of 3–9 for H_2O and 1.5–5 for OH^- (Pollack & Brault, 1976; Pollack et al., 1977; Ehrhardt et al., 1983). It was shown that changes in the Schiff base pK_a can adequately account for the rate constant variations due to Hammett correlations discussed before. It was also shown that the rate constants for intermolecular general base catalysis showed a greater enhancement relative to k_1 due to solvent change and this was attributed to the different nature of the transition state (Pollack & Brault, 1976). The effect of solvent was also to enhance the *intra*molecular catalysis, which was shown to result in a larger increase in k_1 than could be accounted for by a change in pK_a.

Therefore, it is possible that the effect of intramolecular phosphate catalysis on deamination may be relatively greater in DNA compared to a simple 5′-dinucleotide in aqueous solution.

In summary, the pH dependence of the deamination kinetics of cyclobutane dimer photoproducts of dTpdC and dCpdT indicates that the hydrolysis proceeds via the formation of a carbinolamine intermediate. For both the cis,syn and trans,-syn isomers, the rate-determining step for deamination at physiological pH is the nucleophilic attack of OH^- on the protonated 5,6-saturated cytosine moiety to form the carbinolamine intermediate. The difference in deamination reactivity between the cis,syn and trans,syn isomers is caused by a large increase of pK_a for the cis,syn isomers relative to the trans,syn isomer, and this increase is attributed to a stabilization of the protonated 5,6-saturated cytosine base by an electrostatic interaction with the carbonyl group on the neighboring thymine base. This deamination mechanism indicates that, for dinucleoside monophosphates, the cis,syn cyclobutane dimers should deaminate more rapidly than (6–4) and Dewar (6–4) photoadducts at physiological pH. However, since the formation of the carbinolamine intermediate is affected by solvent hydrophobicity and general base catalysis, it is not clear whether the difference in deamination efficiency for the different photoproducts will be increased or reduced in DNA.

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