Effect of pH and Temperature on the Stability of UV-Induced Repairable Pyrimidine Hydrates in DNA†

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ABSTRACT: UV irradiation of cytosine yields 6-hydroxy-5,6-dihydrocytosine (cytosine hydrate) whether the cytosine is in solution as base, nucleoside, or nucleotide or on the DNA backbone. Cytosine hydrate decomposes by elimination of water, yielding cytosine, or by irreversible deamination, yielding uracil hydrate, which, in turn, decomposes by dehydration yielding uracil. To determine how pH and temperature affect these decomposition reactions, alternating poly(dG-[^{3}H]dC) copolymer was irradiated at 254 nm and incubated under different conditions of pH and temperature. The cytosine hydrate and uracil hydrate content of the DNA was determined by the use of *Escherichia coli* endonuclease III, which releases pyrimidine hydrates from DNA by virtue of its DNA glycosylase activity. Uracil content was determined by using uracil-DNA glycosylase. The rate of decomposition of cytosine hydrate to cytosine was determined at 4 temperatures at pH 3.1, 5.4, and 7.4. The E_a was determined from the rates by using the Arrhenius equation and proved to be the same at pH 5.4 and 7.4, although the decomposition rate at pH 5.4 was faster at all temperatures. At pH 3.1, the E_a was reduced. These results suggest that the dehydration reaction is affected by two discrete protonations, most probably of the N-3 and the OH group of C-6 of cytosine hydrate. The deamination of cytosine hydrate to uracil hydrate was maximal at pH 3.1 at all temperatures. The doubly protonated cytosine hydrate probably is the common intermediate for both competing decomposition reactions, explaining why cytosine hydrate is prone to deamination at acid pH. Incubation of irradiated poly(dG-dC) with uracil-DNA glycosylase did not yield significant amounts of uracil unless the DNA was first heated to 70 °C for 16 h. The apparent stability of uracil hydrate in DNA underscores the importance of pyrimidine hydrate DNA glycosylase activities in the repair of UV-induced DNA damage.

Cytosine hydrate (6-hydroxy-5,6-dihydrocytosine) is one of several photoproducts formed in DNA as a consequence of ultraviolet (UV) irradiation (reaction A, Figure 1) (Johns et al., 1965; Grossman & Rodgers, 1968; Kochetkov & Budovskii, 1971). Cytosine hydrates are removed from irradiated DNA via the DNA glycosylase activity of *Escherichia coli* endonuclease III and enzyme activities analogous to endonuclease III which are present in eukaryotic species (Breimer & Lindahl, 1985; Boorstein et al., 1989).

Although the quantum yield of cytosine hydrate is lower than that of dimeric pyrimidine photoproducts (Mitchell et al., 1991), cytosine hydrate is a potentially mutagenic lesion when formed in DNA. This mutagenicity results from two different mechanisms. The first is the ambiguity of cytosine hydrate itself within UV-irradiated template DNA where it pairs with all bases other than guanine during replication of such DNA *in vitro* (Ono et al., 1965; Lecomte et al., 1981). The second mechanism results from the intrinsic instability of cytosine hydrate, which decomposes via two reactions (Johns et al., 1965; Kochetkov & Budovskii, 1971). The first reaction is the elimination of water, yielding the original cytosine (reaction A′, Figure 1). This is a nonmutagenic outcome for the organism. Alternatively, a fraction of cytosine hydrate residues deaminate irreversibly to uracil hydrate (reaction B, Figure 1). If not removed, uracil hydrate leads to mutagenesis because uracil hydrate pairs with adenine during replication (Lecomte et al., 1981). Uracil hydrate, like cytosine hydrate, is removed from DNA via the DNA glycosylase activity of endonuclease III (Boorstein et al., 1989). Also like cytosine hydrate, uracil hydrate eliminates water, yielding uracil (reaction C, Figure 1) (Fisher & Johns, 1976; Boorstein et al., 1989). The persistence of uracil in DNA in place of

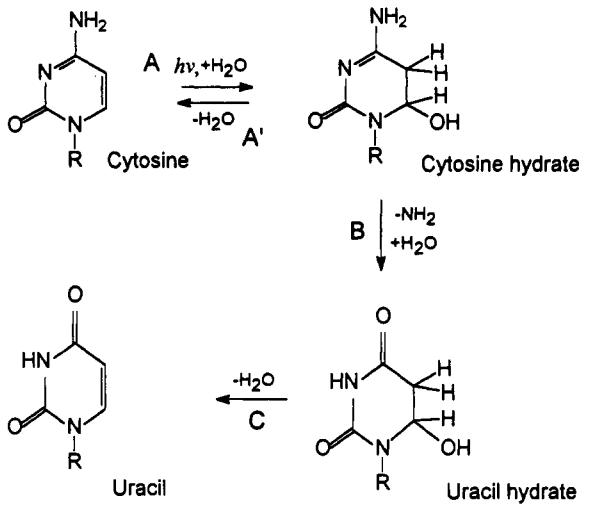

FIGURE 1: Reaction scheme for the formation and decomposition of cytosine hydrate. Reaction A represents the formation of cytosine hydrate by UV irradiation. Reaction A′ represents dehydration of the cytosine hydrate. Reaction B represents deamination of cytosine hydrate to uracil hydrate. Reaction C represents dehydration of uracil hydrate to uracil.

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cytosine would also result in a mutation were the uracil not removed via the action of the DNA repair enzyme uracil-DNA glycosylase (UDG) (Lindahl, 1974).

The first measurements of the rates of the decomposition reactions of cytosine hydrate were made using cytosine-containing nucleoside monophosphates exposed to UV radiation in aqueous solution (Johns et al., 1965). Those studies revealed that cytidine hydrate monophosphate quickly reverted to cytidine monophosphate, the maximal rate of dehydration being at pH 5.0. Although the rate and actual extent of deamination of cytidine hydrate monophosphate to uridine hydrate monophosphate at each pH could not be measured with precision in those experiments, it was concluded that, unlike dehydration, deamination was "relatively independent of pH" and occurred at a maximum of only 10% of the original hydrated cytosines (Fisher & Johns, 1976).

It had been thought that the relative instability of cytosine hydrate precluded its contributing significantly to the mutagenic properties of UV radiation (Doetsch et al., 1986). However, we demonstrated that the half-life of cytosine hydrate in alternating poly(dG-dC) was 25 h at 37 °C and neutral pH (Boorstein et al., 1990). We also demonstrated the formation of uracil hydrate from cytosine hydrate at neutral pH, proving that deamination of cytosine hydrate occurred in UV-irradiated DNA. Our model system utilized radioactively labeled alternating dPu-dPy double-stranded copolymers (to preclude formation of cyclobutane dimers or 6-4-photoproducts) which were irradiated with 254-nm radiation and then incubated with genetically engineered, purified *E. coli* endonuclease III (Asahara et al., 1989). Analysis of the enzymatically released products was done quantitatively by HPLC to obtain the aforementioned results.

In the current experiments we used our *in vitro* system to investigate the effect of temperature and pH on the decomposition of cytosine hydrate to cytosine and uracil hydrate. In addition, we used genetically engineered UDG to detect uracil residues formed from the subsequent dehydration of uracil hydrate.

MATERIALS AND METHODS

Enzymes. *E. coli* endonuclease III purified from *E. coli* λ N99 (CI857) containing the *nth* gene (Asahara et al., 1989) was stored at −20 °C at a concentration of 5 mg/mL. The storage solution consisted of 100 mM KCl, 50 mM potassium phosphate (pH 6.6), and 50% glycerol. Before use, it was diluted 5-fold into 50 mM Tris·HCl (pH 7.6), 100 mM KCl, 1 mM EDTA, 0.5 mg/mL BSA (molecular grade), 0.1 mM DTT, and 1% glycerol.

E. coli uracil-DNA glycosylase (UDG), purchased from BRL, was stored in a solution of 30 mM Tris·HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM DTT, 0.05% Tween 20, and 50% glycerol at a concentration of 1 unit/μL.

A DNA Pol I/DNase I mixture prepared specifically for nick translation was purchased from BRL and stored in 50 mM Tris·HCl (pH 7.5), 5 mM magnesium acetate, 0.1 mM PMSF, 0.1 mg/mL BSA, and 50% glycerol.

Additional DNA Pol I (10 units/μL) was also purchased from BRL. The storage buffer for the DNA Pol I consisted of 100 mM potassium phosphate (pH 7.0), 1 mM 2-mercaptoethanol, and 50% glycerol. The dilution buffer consisted of 100 mM potassium phosphate (pH 7.0), 1 mM 2-mercaptoethanol, and 10% glycerol.

Radionucleotides. [5-^{3}H]-2′-Deoxycytidine 5′-triphosphate (15–30 Ci/mmol) and [5-^{3}H]-2′-deoxyuridine 5′-triphosphate (5–15 Ci/mmol) were purchased from Amersham.

Copolymers. The alternating double stranded copolymers poly(dG-dC) and poly(dA-dT) were purchased from Pharmacia and stored in crystalline form at −20 °C. Prior to use, the copolymer was dissolved in water and the concentration was determined by measuring absorbency at 260 nm, assuming 50 μg/mL = 1.0 absorbance unit.

Synthesis of Alternating Poly(dG-[^{3}H]dC) Copolymer. Alternating poly(dG-dC) copolymer was radiolabeled by nick translation. Core reagents from a nick translation kit (NEN) were used in the following protocol. In a microfuge tube, 10 μL of [^{3}H]dCTP at 1 μCi/μL was lyophilized to dryness. From the kit, 5 μL of nick translation buffer and 4 μL of dNTPs (minus the dCTP used for radiolabeling) were added to the tube. Next, H_2O and 0.5 μg of copolymer, in a combined volume of 11 μL, were added. The mixture of DNA Pol I (0.4 unit/μL) and DNase I (40 pg/μL) was used to catalyze the nick translation reaction. The nick translation reaction mixture was incubated at 15 °C for 1 h, at the end of which time an additional 0.5 unit of DNA Pol I in 1 μL was added. After 1 h of additional hour of incubation, the reaction was terminated by addition of 25 μL of 50 mM EDTA. The tube was heated to 72 °C for 10 min to facilitate reaction termination. This step was followed by cooling for 1 h at room temperature to allow DNA reannealing.

Synthesis of an Alternating Poly(dA-dT) Chimeric Copolymer Containing [^{3}H]dU Residues at Intervals of 1 dU/5000 dT Sites. The commercial nick translation kit protocol was followed using alternating poly(dA-dT) copolymer as template DNA with the exception that 4.5 μL of 0.6 uM [^{3}H]dUTP was added to a microfuge tube and lyophilized. After completion of drying, 5 μL of 200 μM dTTP was added to the reaction tube. Nick translation buffer and dNTPs (–dTTP) were then included, followed by 0.5 ug of poly(dA-dT) copolymer. The level of substitution was determined from the specific radioactivity of the [^{3}H]dUTP pool and the amount of radioactivity incorporated per 260-nm absorbance unit.

Purification of Radiolabeled Copolymers. The radioactive copolymers in solution were separated from unincorporated dNTPs by passage through Sephadex G-50 minispin columns (Worthington). The solutions were precipitated in 0.1 vol of 3 M sodium acetate (pH 5.2) and 2.5 vol of ethanol and refrigerated overnight at −20 °C. Following centrifugation (Sorvall RC-5B centrifuge, SS 34 rotor) at 15 000 rpm for 15 min, the supernatants were removed and the precipitates were washed in 500 μL of 70% ethanol. The copolymers were air dried at room temperature for 20 min and then resuspended in TE buffer (10 mM Tris·HCl (pH 8.0) and 1 mM EDTA) to a concentration of 0.02 μg/μL. However, for the experiments in which solution pH was to be adjusted, the poly(dG-[^{3}H]dC) copolymer was resuspended in dilute TE buffer (2 mM Tris·HCl and 0.2 mM EDTA, pH 8.0). The specific radioactivity of copolymers was between 10^7 and 2.0×10^7 dpm/ug. For a chimeric poly(dA-dT) copolymer containing [^{3}H]dU residues (*vide infra*), the specific activity was 10^4 dpm/μg.

Ultraviolet Irradiation of Radiolabeled Substrate DNA. All of the ^{3}H-containing copolymers in TE were irradiated using two unfiltered GE 15-W germicidal bulbs (GISTA) with a primary output at 254 nm (Carrier & Setlow, 1971; Duker & Teebor, 1975). Radiation flux was measured via a UVX radiometer and a UVX25 sensor (UVP, Inc., San Gabriel, CA). All samples were irradiated in weighing boats on ice.

Adjustment of Substrate Solution pH. The pH of the poly(dG-[^{3}H]dC) copolymer containing solutions was adjusted

by addition of 2.5 μL of citrate–sodium phosphate buffer (McIlvaine, 1921) per 5-μL sample. To lower the pH of the solution from 8.0 to 3.1, a buffer of 4 mM citrate and 2 mM sodium phosphate (pH 3.0) was added to the substrate solution. For pH adjustment to 5.4, a buffer concentration of 2.4 mM citrate and 5.1 mM sodium phosphate (pH 5.0) was used. For pH adjustment to 7.4, the buffer concentration was 0.7 mM citrate and 8.7 mM sodium phosphate (pH 7.0). The pH-adjusted substrate solutions were incubated at temperatures ranging from 4 to 55 °C. Samples of each pH-adjusted solution were monitored at all temperatures used to determine whether there were significant changes in pH during incubation. The pH never varied by more than 0.1 unit under any condition used in these experiments. Samples were removed at defined intervals and placed on ice. Tris base solutions were added to the samples to return the pH to 8.0 prior to endonuclease III assay. For the pH 3.1, 5.4, and 7.4 incubations, Tris base was added at concentrations of 95.2, 53.5, and 12 mM, respectively. The pyrimidine hydrate content of the copolymer was then enzymatically determined using endonuclease III.

Assay of Enzymatically Released ^{3}H-Containing Material from Poly(dG-[^{3}H]dC) Copolymer and Poly(dA-dT([^{3}H]-dU)) Copolymer. To assay for endonuclease III repairable pyrimidine hydrates in substrate copolymer, 0.1 μg of irradiated poly(dG-[^{3}H]dC) copolymer in 10 μL was incubated with 1 μg of endonuclease III (1 μg/μL) and 1.22 μL of reaction buffer (500 mM Tris·HCl (pH 7.6), 1 M KCl, 10 mM EDTA, 1 mg/mL BSA, and 1 mM DTT) at 37 °C for 30 min. To assay for uracil in the copolymer, 0.2 μg of copolymer in 20 or 5 μL of reaction buffer (500 mM KCl, 50 mM $MgCl_2$, 20 mM Tris·HCl (pH 8.0), and 20 μL of water) was mixed with 5 μL of UDG (1 unit/μL) and incubated for 30 min at 37 °C.

[^{3}H]dU-containing poly(dA-dT) chimeric copolymer and irradiated poly(dG-[^{3}H]dC) copolymer were assayed for uracil content with UDG. The chimeric copolymer was constructed as a substrate to determine the sensitivity of UDG as a reagent enzyme when very small amounts of uracil were present in the irradiated copolymer. Copolymer (0.2 μg) in 20 or 5 μL of reaction buffer (500 mM KCl, 50 mM MgCl, and 20 mM Tris·HCl (pH 8.0)) was mixed with 20 μL of water and 5 μL of UDG (1 unit/μL), and the mixture was incubated for 30 min at 37 °C.

All reactions were terminated by addition of 1 vol of BSA (10 mg/mL) and 5.5 vol of acetone, and reaction mixtures were allowed to stand overnight at −20 °C. The mixtures were then centrifuged at 8000 rpm for 15 min at 4 °C. The supernatant fluids were transferred to fresh tubes, and the acetone was evaporated under nitrogen. The dried material was then resuspended in 200 μL of water for analysis by reverse phase HPLC.

Heating of Samples To Convert Uracil Hydrate to Uracil To Facilitate Chromatographic Resolution during HPLC Analysis. Uracil hydrate elutes before cytosine, and the fractions in which it was detected often contained several hundred counts of background radioactivity. Therefore, to improve the quantitation of uracil hydrate released by endonuclease III, uracil hydrate was converted to uracil (which elutes later than cytosine) prior to HPLC analysis of the material released by endonuclease III. Following resuspension in 200 μL of water, as described in the preceding paragraph, the samples were incubated for 1 h at 90 °C in sealed tubes (Sinsheimer & Hastings, 1949). The cytosine hydrate and uracil hydrate content of the copolymer was estimated to be twice the amount of radioactive material recovered as cytosine and uracil, respectively. This is based on the assumption that the enzymatically released cytosine hydrate and uracil hydrate, labeled with tritium in the C-5 position, randomly lose tritium or hydrogen from that position during the E_1 type dehydration–elimination reaction. We have previously used this estimate in measuring the cytosine hydrate and uracil hydrate content of irradiated copolymers (Boorstein et al., 1990).

High-Pressure Liquid Chromatography Analysis of Enzymatically Released ^{3}H-Containing Material. Unlabeled cytosine and uracil, 1.0 μg of each, were added as UV markers prior to injection of the sample into the HPLC column to assist in identification of ^{3}H-containing material released by either endonuclease III or UDG. Samples were filtered through 0.2-μm centrifuge filters (Centrex) and analyzed using a 5-μm 0.46 × 25 cm Ultrasphere ODS column (Beckman). The eluent consisted of 35 mM ammonium formate (pH 3.5) and 0.5% methanol at 1 mL/min for 10 min. The column was washed with 100% methanol for 30 min after every other sample run. The elution of the radiolabeled cytosine and uracil was quantified using a Radiomatic Flo-One in-line detector.

RESULTS

Cytosine Hydrate Stability as a Function of pH and Temperature. To determine the effect of pH on cytosine hydrate decomposition, irradiated poly(dG-[^{3}H]dC) copolymer was incubated at either pH 3.1, 5.4, or 7.4. At each pH, irradiated copolymer was incubated at a constant temperature (4, 15, 25, 37, 45, or 55 °C) for a specified interval. Following the incubation, the copolymer solution was readjusted to pH 8.0 and the hydrate content of the copolymer was determined by an endonuclease III assay.

Plots of the log percent cytosine hydrate remaining in the copolymer versus time are illustrated in Figure 2. Rate constants for the stability of cytosine hydrate were calculated using the first-order equation $\ln(N_t/N_0) = -kt$, where N_t is the cytosine hydrate concentration after a specific time interval, N_0 is the cytosine hydrate concentration at time zero, k is the rate constant, and t is the time (s). The rate constants in the irradiated copolymer at pH 3.1 for 4, 15, 25, and 37 °C are 0.026, 0.067, 0.11, and 0.3 h^{-1}, respectively. The rate constants at pH 5.4 for 4, 15, 25, and 37 °C are 0.012, 0.051, 0.19, and 0.72 h^{-1}, respectively. The rate constants at pH 7.4 for 25, 37, 45, and 55 °C are 0.019, 0.04, 0.12, and 0.36 h^{-1}, respectively. Thus, at the lower incubation temperatures of 4 and 15 °C, the rate constants for cytosine hydrate decomposition are greater at pH 3.1 than at pH 5.4. In contrast, above 15 °C, rate constants for pH 5.4 are greater than at pH 3.1. Rate constants were obtained for all three pH values at temperatures of 25 and 37 °C. At these temperatures, the cytosine hydrate was maximally stable at pH 7.4.

The Arrhenius plots of Figure 3 were prepared with data from the three pH values. Energies of activation and their standard errors were calculated by linear regression according to the relationship $E_a = -mR$, where m is the slope and R is the gas constant. The E_a for pH 3.1 was 12.3 kcal/mol (n = 4; 2SE = 0.86, where SE is the standard error). For pH 5.4 and 7.4, the E_a was 21.2 kcal/mol (n = 4; 2SE = 0.15) and 19.3 kcal/mol (n = 4; 2SE = 2.12), respectively. Thus, the E_a at pH 3.1 is significantly lower than the E_a at pH 5.4 and 7.4.

Formation and Stability of Uracil Hydrate as the Deamination Product of Cytosine Hydrate. Following irradiation, the poly(dG-dC) was incubated at pH 3.1, 5.4, or 7.6 at 4 or 37 °C, and the amount of uracil hydrate present in the polymer

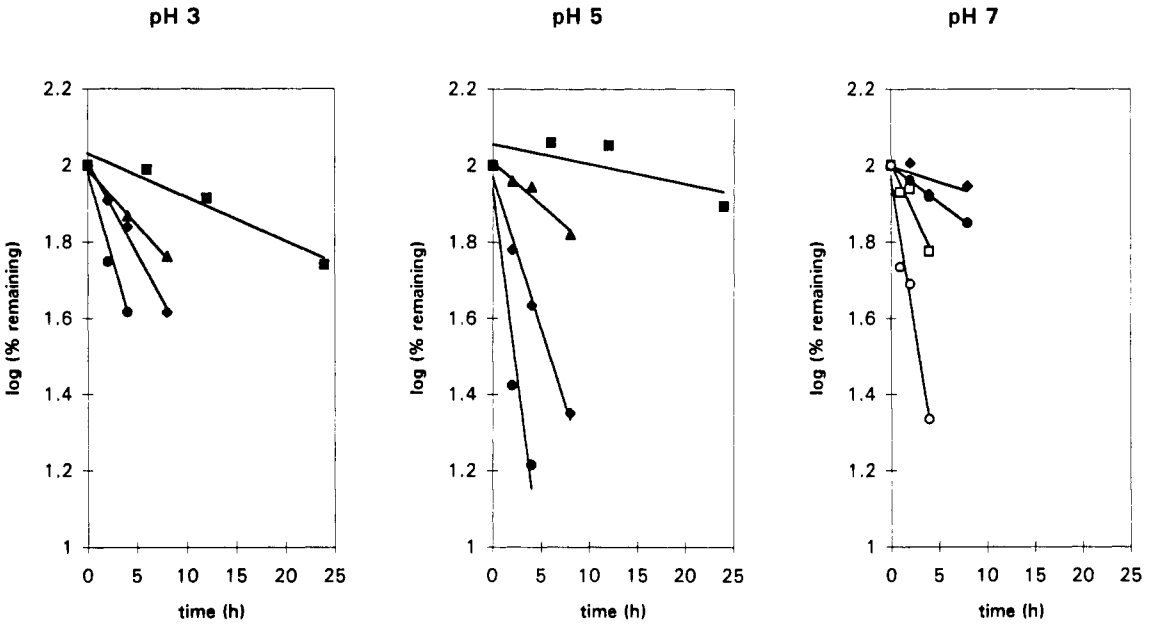

FIGURE 2: Rate of decomposition of cytosine hydrate in poly(dG-[^{3}H]dC) as a function of pH and temperature. The copolymer was UV irradiated, and the pH was adjusted to either 3.1, 5.4, or 7.4. The copolymer was then incubated at temperatures of 4 (■), 15 (▲), 25 (♦), 37 (●), 45 (□), or 55 °C (○). After indicated intervals of 0–24 h, the solution pH was readjusted to 8.0 and the copolymer was incubated with endonuclease III. Cytosine release was quantified using reverse-phase HPLC. Each point represents the average of duplicate determinations.

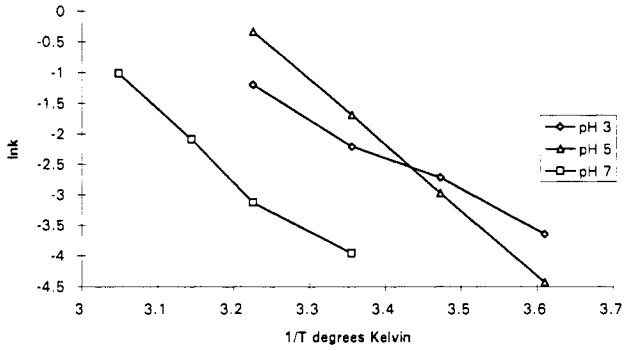

FIGURE 3: Arrhenius plots of rate constants of cytosine hydrate decomposition. For each pH, the natural log of each rate constant was plotted vs the reciprocal of the temperature. Energies of activation were determined from the slopes of each pH plot.

was determined. The net formation of uracil hydrate as a function of time is shown in Figure 4. The net formation of uracil hydrate is the sum of three reactions. First, uracil hydrate is formed during UV irradiation of poly(dG-dC) by a mechanism which is not yet fully understood (Boorstein et al., 1989). That portion of uracil hydrate is the "0" time value shown in Figure 4. Second, uracil hydrate is formed from the deamination of cytosine hydrate, a reaction which, as has been shown here, is favored at acid pH and is in competition with the dehydration of cytosine hydrate back to cytosine. Finally, the net amount of uracil hydrate decreases as a consequence of its decomposition to uracil via the dehydration reaction. Thus, the data of Figure 4 represents the sum of these three reactions at each condition described in the figure caption. At temperatures of 4 and 37 °C, the greatest net formation of uracil hydrate was seen at the lowest pH of 3.1. After 24 h at pH 3.1 the deamination fraction was 30% and 20% at 4 and 37 °C, respectively, in contrast to the maximum of 10% previously reported for the deamination of cytidine hydrate monophosphate (Johns et al., 1965; Fisher & Johns, 1976).

Stability of Uracil Hydrate in Alternating Poly(dG-[^{3}H]-dC) following UV Irradiation. We previously reported finding a small amount of uracil released by UDG from UV-irradiated alternating poly(dG-dC) which had been incubated at 37 °C and pH 7 for 24 h. Those measurements were using made the UDG activity of a partially purified calf thymus preparation and showed a 10-fold variability (Boorstein et al., 1990). In the current experiments, we utilized genetically engineered *E. coli* UDG in an attempt to determine the effect of pH and temperature on the dehydration of uracil hydrate to uracil in UV-irradiated alternating poly(dG-[^{3}H]dC) copolymer. An aliquot of sample from every condition point of Figure 1 was treated with UDG at the same time a portion was being analyzed with endonuclease III. After the UDG reaction was stopped with cold acetone, HPLC analysis of the acetone-soluble material failed to reveal uracil in any consistent fashion. The lower limit of detection of this assay was 100 dpm, which would represent about 3% of the maximal uracil hydrate content of the copolymer as determined by analysis with endonuclease III.

To prove that UDG-mediated release of uracil from a copolymer could be detected were uracil present in extremely small quantities, chimeric alternating poly(dA-dT) copolymer was prepared with [^{3}H]dU substitutions at a ratio of 1 dU for every 5×10^3 dT residues. From a potential 2000 dpm of [^{3}H]uracil present in 0.2 μg of chimeric copolymer, 1000 dpm (50%) was released in 1 h at 37 °C by 5 units of UDG. This demonstrated that UDG could release half the uracil from an alternating dPu-dPy copolymer if the uracil were present at a level as low as 0.02%. Thus, if the lower limit of detection of any peak using an on-line scintillation counter, as was done in all of these experiments, is 100 dpm, the efficiency of UDG in releasing rare uracil residues is about 50% and the specific activity of the uracil residue is half that of uracil hydrate as a result of tritium exchange; the minimum detectable 100 dpm represents the deamination product of 400 dpm of uracil hydrate within the same polymer.

We could reproducibly prove that uracil hydrate opposite guanine dehydrated to uracil only under extreme conditions. After UV irradiation of alternating poly(dG-[^{3}H]dC), the pH was lowered to 3.1 and the copolymer was incubated for 24 h at 4 °C, conditions which maximize formation of uracil hydrate (see Figure 4). After 24 h, the pH was readjusted

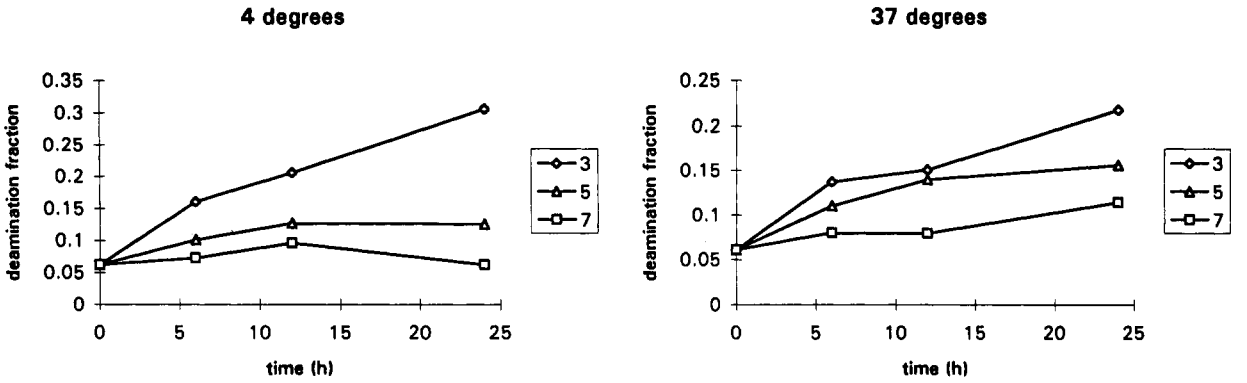

FIGURE 4: Net formation of uracil hydrate from cytosine hydrate following irradiation of poly(dG-[^{3}H]dC). Following UV irradiation, the pH of alternating poly(dG-[^{3}H]dC) copolymer was adjusted to 3.1 (◇), 5.4 (△), or 7.4 (□). The copolymer was then incubated at 4 or 37 °C. After 0, 6, 12, or 24 h of incubation, the copolymer solution was readjusted to pH 8.0 and the substrate was assayed for uracil hydrate with endonuclease III. Samples were prepared by adding 1.0 μg of enzyme per 0.1 μg of DNA. Acetone-soluble counts from the enzymatic release were heated at 90 °C for 1 h to generate uracil by dehydration. Uracil was measured quantitatively using reverse-phase HPLC as described in Materials and Methods. As previously reported and discussed, a small amount of uracil hydrate was detected at time 0 (Boorstein et al., 1989).

to 8.0 and samples of the copolymer were incubated with endonuclease III to determine hydrate content. A second aliquot of identically treated copolymer was heated to 70 °C for 16 h to dehydrate as much uracil hydrate to uracil as possible and then incubated with either endonuclease III or UDG. After 24 h of incubation at pH 3.1, 3500 dpm of uracil hydrate was released by endonuclease III. However, after heating the copolymer at 70 °C prior to endonuclease III assay, only 330 dpm of uracil hydrate were released. This proved that heating of the copolymer caused the decomposition of uracil hydrate to something which no longer was a substrate for endonuclease III.

To determine whether heating at 70 °C converted uracil hydrate to uracil, the copolymer was incubated with UDG. Six hundred disintegrations per minute of uracil, representing the dehydration product of 1200 dpm of uracil hydrate, was released by UDG. Since the efficiency of the UDG in releasing uracil under these conditions was only 50%, an additional 600 dpm of uracil was presumably also present, representing a total of 2400 dpm worth of dehydrated uracil hydrate. This value agrees reasonably well with the 3170 dpm difference between the uracil hydrate content before and after heating. This result showed that it was possible to convert uracil hydrate to uracil within the copolymer by heating and that uracil residues could be released by UDG.

These last experiments demonstrated that, be it under physiologic conditions or extremes of pH with temperatures up to 55 °C, uracil hydrate formed from the deamination of cytosine hydrate in alternating poly(dG-[^{3}H]dC) copolymer did not dehydrate to uracil to an appreciable degree.

DISCUSSION

Cytosine hydrate, the major enzymatically repairable nondimeric derivative of cytosine produced by UV irradiation of DNA, has a half life of 25 h *in vitro* at physiologic temperature and pH in alternating poly(dG-dC) copolymers (Boorstein et al., 1990). Ultimately, cytosine hydrate decomposes by dehydration to cytosine or by deamination to uracil hydrate. Previously, the effect of pH and temperature on the rates of these decomposition reactions could only be studied for cytosine hydrate moieties as cytosine base, nucleoside, or mono- and dinucleotides exposed to UV radiation in solutions (Johns et al., 1965; Hariharan & Johns, 1968; DeBoer et al., 1970). The availability of endonuclease III and UDG as specific reagent enzymes coupled with the resolving power of HPLC linked to on-line liquid scintillation counting permit measurement of the rates of the decomposition reactions of cytosine hydrate residues formed in alternating poly(dG-dC) copolymers.

The rate for the dehydration reaction can be expressed as a function of temperature via the Arrhenius equation (Weston & Schwarz, 1972). In the current experiments, the E_a of 12 kcal/mol for the dehydration of cytosine hydrate to cytosine at pH 3.1 differed significantly from the E_a of 21 and 19 kcal/mol obtained from the plots of the reaction rates at pH 5.4 and pH 7.4. Although our previous estimate of the E_a at pH 7.4 for this reaction in alternating poly(dG-[^{3}H]dC) copolymer was 16 kcal/mol (Boorstein et al., 1990), the difference between that value and the 19 kcal/mol obtained here is a reflection of experimental variation. In the current experiments, all of the measurements made were done simultaneously using the same batch of endonuclease III. Thus, although repeat experiments might yield slightly different measurements of E_a at each pH because of the intrinsic variability of the measurements, the relative difference between pH 3.1 and pH 5.4 and 7.4 measurements would persist. Johns et al. (1965) determined that the E_a for the dehydration of the hydrate of CMP was unchanged in the pH range of 4.5–8.0. This data is consistent with our finding of an unchanged E_a between pH 5.4 and 7.4.

The results of our experiments reveal that at least two factors contribute to the increased rate of dehydration of cytosine hydrate to cytosine with decreasing pH. One component lowers the E_a between pH 5.4 and 3.1 as reflected by the difference in the slopes of the plot of log k vs $1/T$ at those two pH values. The second factor markedly increases the rate of dehydration when the pH is lowered from 7.4 to 5.4 but is independent of temperature and thus, by definition, does not affect the E_a. This indicates that the temperature-independent "A factor" of the Arrhenius equation is pH dependent for the dehydration of cytosine hydrate within a DNA-like polymer.

The two pH-dependent components affecting the rate of dehydration presumably represent two distinct protonations of the cytosine residue. The initial protonation accelerates the dehydration reaction between pH 7.4 and 5.4 and does not affect E_a. The second protonation, occurring at pH 3.1, might lower the E_a by destabilizing the starting material, perhaps by charge repulsion. Likely protonation sites are the N-3 atom of the pyrimidine ring and the OH group of the ring C-6 atom (DeBoer et al., 1970).

Formation of uracil hydrate was favored at pH 3.1 > 5.4 > 7.4 at both 4 and 37 °C. Unfortunately, the energetics of the deamination reaction cannot be determined reliably in this system. To determine the temperature dependence of the deamination of cytosine hydrate to uracil hydrate, it would be necessary for the concentration of cytosine hydrate to remain fixed, except for that lost through deamination. However, as the temperature increases, cytosine hydrate is siphoned off via the quantitatively more significant dehydration reaction, which yields cytosine. Even if it were argued that initial measurements of uracil hydrate content might be extrapolated to estimates of the rate of deamination, these measurements would not be reliable because the initial amount of uracil hydrate is so small and cannot be determined experimentally with a high degree of reliability. This contrasts with the dehydration of cytosine hydrate to cytosine where the initial concentration of cytosine hydrate is high and decreases with time. For these two reasons, temperature-dependent deamination rates could not be measured accurately and only the net deamination at various temperatures and pH was determined.

Nevertheless, despite ambiguities in the determination of the extent of deamination of cytosine hydrate, the pH dependence of the deamination reaction as measured at two temperatures has implications for understanding why cytosine hydrate is prone to deamination. Like dehydration, the deamination reaction is also favored by protonation at N-3. This protonated form is resonance stabilized by its ability to delocalize the positive charge to C-4. At lower pH, as the OH group on C-6 also becomes protonated, the doubly protonated cytosine hydrate becomes unstable by virtue of charge repulsion. Decomposition occurs either by loss of the 6-OH_2^+, yielding cytosine, or by addition of water to the positively charged C-4 followed by loss of ammonia, yielding uracil hydrate. Although the dehydration of cytosine hydrate is the favored reaction, the extent of deamination is considerable at acidic pH.

In these experiments, the dehydration of uracil hydrate to uracil was a relatively rare event. These results suggest that the biologically significant consequences of the formation of cytosine hydrate within DNA result primarily from its tendency to mispair and from its propensity to deaminate to premutagenic uracil hydrate (Lecomte et al., 1981). Endonuclease III repairs both cytosine hydrate and uracil hydrate and is the only repair enzyme of *E. coli* known to have DNA glycosylase activity directed against hydrated pyrimidines (the recently described endonuclease VIII was only tested against oxidized and reduced thymine moieties in DNA (Melamede et al., 1994)). We also detected pyrimidine hydrate DNA glycosylase activity in HeLa extracts (Boorstein et al., 1989), and it is likely that the many so-called UV endonuclease activities described within the past 20 or more years in different species and cell types are analogous to endonuclease III. Their widespread phylogenetic distribution suggests that the removal of pyrimidine hydrates is of importance in maintaining genomic integrity in all organisms exposed to UV radiation. Since, as we have shown here, relatively little uracil is formed in DNA as a consequence of the dehydration of uracil hydrate, UDG seems to have a more limited back-up responsibility in the repair of this type of monomeric UV damage.

In summary, these experiments measured the progress of the three simultaneous reactions shown in Figure 1. These were reaction A′, the dehydration of cytosine hydrate to cytosine; reaction B, the deamination of cytosine hydrate to uracil hydrate; and reaction C, the dehydration of uracil hydrate to uracil. The first two reactions were affected by changes in temperature and pH. The third reaction did not seem to be of great significance in the context of these experiments. Although cytosine hydrate is formed at about 1–2% the frequency of cyclobutane dimers (Mitchell et al., 1991), its relative intrinsic stability in DNA, its potential for mispairing, and its tendency to deaminate to uracil hydrate all suggest that, in the absence of enzymatically mediated repair, pyrimidine hydrates could exert considerable mutagenicity leading to deleterious effects upon the organism exposed to UV irradiation.

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