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Heat-Induced Deamination of Cytosine Residues in Deoxyribonucleic Acid†

Tomas Lindahl and Barbro Nyberg

ABSTRACT: The rate of deamination of cytosine residues in single-stranded and double-stranded *Escherichia coli* DNA, in the polynucleotides poly(dC) and poly(dG)·poly(dC), and in dCMP was investigated as a function of temperature, pH, and buffer composition. For this purpose, nucleic acids and polydeoxynucleotides specifically radioactively labeled in the cytosine residues were prepared. After heat treatment, the polymers were enzymatically degraded to mononucleotides or nucleosides, cytosine and uracil derivatives were separated by paper chromatography, and their radioactivity was determined. Cytosine in single-stranded DNA, poly(dC), or dCMP is similarly susceptible to hydrolytic deamination at pH 7.4, and the reaction proceeds at a rate of $k = 2 \times 10^{-7}$ sec^{-1} at 95°. From measurements at several temperatures it is estimated that the reaction is associated with an activation energy of 29 kcal/mol. These data indicate that a significant amount of conversion of cytosine to uracil occurs during heat denaturation of DNA by standard procedures. The cytosine residues in native DNA are well protected, and are deaminated at <1% of the rate observed with dCMP or poly(dC). In contrast, the cytosine residues in poly(dG)·poly(dC) were deaminated at 75% of the rate of those in poly(dC). The *in vivo* rate of deamination of cytosine residues in DNA is discussed.

If neutral aqueous solutions of DNA are incubated at high temperatures, changes in the covalent structure slowly accumulate. This heat-induced degradation is not indiscriminate. Instead, it is primarily due to hydrolytic attack at a small number of sensitive sites, in particular at the *N*-glycosyl bonds. Free purines and pyrimidines are consequently released at a slow rate from both single-stranded and double-stranded DNA at neutral pH (Greer and Zamenhof, 1962; Lindahl and Nyberg, 1972; Lindahl and Karlström, 1973). The deoxyribose residue at an apurinic or apyrimidinic site in DNA is no longer locked in the furanose form, and will be present in an equilibrium between this form and a reactive free aldehyde form. Several different types of secondary lesions therefore appear at such sites in DNA, including chain breaks (Tamm *et al.,* 1953; Lindahl and Andersson, 1972) and cross-links (Freese and Cashel, 1964; Burnotte and Verly, 1972).

Heat treatment of DNA might also lead to structural changes in base residues, in addition to the loss of occasional bases from DNA. Of the four common bases in DNA, cytosine should be most heat labile, as it is the base most sensitive to hydrolysis in either acid, neutral, or alkaline solution. Shapiro and Klein (1966) found that free cytosine and cytidine are relatively rapidly deaminated by heating in weakly acidic buffers, while there is no detectable deamination of adenosine or guanosine under such conditions, and they proposed that heat-induced cytosine deamination in DNA might have mutagenic implications. When the four common ribonucleotides (which have more stable glycosidic bonds than the deoxyribonucleotides) are incubated separately at 90° in neutral buffers of physiological ionic strength, deamination of CMP to UMP is the prevalent degradative reaction (Lindahl, 1967). Cytosine deamination is also the dominant mode of degradation for DNA in alkaline solution, as it takes place at an approximately tenfold faster rate than other alkali-catalyzed changes in the covalent structure of DNA, *e.g.,* alkali-catalyzed depurination and chain breakage, and imidazole ring opening in adenine residues (Hurst and Kuksis, 1958; Ullman and McCarthy, 1973; Garrett and Mehta, 1972). Further, transition mutations that accumulate spontaneously on prolonged storage of phage T4 suspensions, or on incubation of single-stranded T4 DNA in neutral solution, are apparently due to deamination of hydroxymethylcytosine residues (Drake, 1966; Baltz, 1973). In the present investigation, the rate of deamination of cytosine in DNA at pH values close to neutrality has been followed as a function of DNA conformation, temperature, pH, and buffer composition.

Experimental Section

Materials. Uniformly labeled [^{14}C]dCTP (432 Ci/mol) was

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obtained from the Radiochemical Centre, Amersham. [2-^{14}C]dCMP (20 Ci/mol) was prepared by enzymatic digestion of [2-^{14}C]cytosine-labeled DNA to monucleotides, followed by purification of the dCMP by paper chromatography in an isobutyric acid–NH_4OH based system (see below). Nonradioactive mononucleotides and nucleosides were purchased from Calbiochem. *Escherichia coli* DNA polymerase (5000 units/mg) was purchased from General Biochemicals. Bovine pancreatic DNase I (one crystallization) was obtained from Worthington. It was stored in several small aliquots at −20° as a 0.1% solution in 0.05 M Tris-HCl (pH 7.2) and was not frozen more than once. Snake venom phosphodiesterase was purchased from Sigma. It was further purified according to Sulkowski and Laskowski (1971), and stored as a 0.1% solution at −20°. *E. coli* alkaline phosphatase (chromatographically purified) was obtained from Worthington, and was further purified by heating for 5 min at 95° in 0.15 M KCl–0.01 M $MgCl_2$–0.01 M Tris-HCl (pH 8.2), followed by incubation at 25° for 3 hr (Heppel *et al.*, 1960), and then by chromatography on DEAE-cellulose at 4° (Weiss *et al.*, 1968). *N*-2-Hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (Hepes)[1] was from Sigma.

DNA Preparations. E. coli B DNA, ^{14}C-labeled in the cytosine residues, was obtained from the uracil- and thymine-dependent mutant OK308 (*pyr, cdd, thy, tlr*), grown in the presence of [2-^{14}C]uracil and nonradioactive thymine (Lindahl and Karlström, 1973). Native DNA was isolated according to Marmur (1961), with the addition of treatments with Pronase and phenol, followed by extensive dialysis as previously described (Lindahl and Nyberg, 1972). In buffer A (see below) of pH 7.4, such DNA had a T_m of 89.4°, a total hyperchromicity of 39% at 260 nm on heating from 20 to 98°, and a sedimentation coefficient of 32 S on sucrose gradient centrifugation. The DNA preparations contained 20,000–35,000 cpm/μg of DNA, and had >99% of the total radioactivity present in the form of cytosine residues. *E. coli* B DNA does not contain 5-methylcytosine (Fujimoto *et al.*, 1965). Poly(dG)·poly([^{14}C]dC) preparations, 10,000–20,000 cpm/μg, were made with *E. coli* DNA polymerase according to Radding *et al.* (1962). A typical preparation had a sedimentation coefficient of 10 S, as determined by neutral sucrose gradient centrifugation. Poly([^{14}C]dC) was obtained by equilibrium gradient centrifugation of poly(dG)·poly(dC) in alkaline Cs_2SO_4 (Inman and Baldwin, 1964). Two well-separated peaks with the densities and spectral properties of poly(dG) and poly(dC) were obtained in approximately equimolar proportions after centrifugation. All radioactivity (>99.9%) was associated with the poly(dC) strands, which formed a somewhat broader band than the poly(dG). The isolated poly([^{14}C]dC) had a sedimentation coefficient of 6 S, as determined by alkaline sucrose gradient centrifugation. Polynucleotide concentrations were determined spectrophotometrically (Bollum, 1966).

Solvents. Buffer A contained 0.1 M NaCl–0.01 M sodium phosphate–0.01 M sodium citrate. Buffer B contained 0.1 M KCl–0.05 M Hepes-KOH–0.01 M $MgCl_2$–0.001 M EDTA. All buffers were adjusted to pH 7.40 at the temperature of the actual experiment by employing a Radiometer pH meter 26, equipped with a high-temperature glass electrode and an external temperature compensator. Thus, as the pH of buffers containing Hepes-KOH decreases with increasing temperature, different solutions of buffer B for use at 65–100° had pH 7.8–8.2 at 20°.

Analysis of Deamination. Nucleic acids and mononucleotides were heated at concentrations of 5–50 μg/ml in acid-washed, sealed 1-ml glass ampoules containing 0.1–0.4 ml of solution. In some experiments most of the dissolved O_2 was replaced by N_2, but this procedure was not employed consistently, as it did not lead to detectable changes in the rate of deamination. For heat treatment up to 80°, the ampoules were totally immersed in closed, thermostated water baths (Haake), controlled within 0.2°. Incubations at 95–110° were done in a Gallenkamp OV-330 oven, controlled within 0.5°. When *E. coli* DNA was to be studied in its denatured form at temperatures below 95°, the DNA solutions were first heated for 10 min at 100°, followed by rapid cooling to 0°.

After incubation of nucleic acids in buffer A, $MgCl_2$ was added to a final concentration of 5×10^{-3} M. To nucleic acids in buffer B, $CaCl_2$ was added to a final concentration of 6×10^{-3} M. DNA solutions of high ionic strength, or of pH values outside the 7–8 range, were dialyzed against buffer B after heat treatment. To all samples, pancreatic DNase I was added to a concentration of 100 μg/ml, followed by incubation at 30° for 4 hr. Venom phosphodiesterase was then added to a concentration of 60 μg/ml, and the incubation was continued for 2 hr. For analysis of mononucleotides, the reaction was stopped by addition of EDTA to 0.04 M, followed by heating at 100° for 2 min to inactivate the enzymes. The resulting small precipitate was removed by centrifugation. For analysis of nucleosides, hydrolysates in buffer B were further degraded by incubating the samples with alkaline phosphatase for 30 min at 37° with 10 μg/ml of enzyme, followed by renewed addition of the same amount of phosphatase and incubation for 30 min at 65°.

Enzymatic digests were mixed with nonradioactive carrier mononucleotides or nucleosides, applied to Whatman 3 MM paper, and analyzed by descending paper chromatography. Chromatography of mononucleotides was performed in isobutyric acid–water–concentrated NH_4OH–0.2 M EDTA, 132:66:2:1, or in 2-propanol–concentrated HCl–H_2O, 170:41:39 (Wyatt, 1951). The following R_F values were observed in the latter system: dCMP, 0.64; dUMP, 0.78; dTMP, 0.83; deoxycytidine, 0.50; cytosine, 0.50. For analysis of nucleosides, a solvent system containing the upper phase from a mixture of ethyl acetate–1-propanol–H_2O, 4:1:2 (Hall, 1971), was used. The R_F values in this system were: deoxycytidine, 0.06; deoxyuridine, 0.23; thymidine, 0.32; uridine, 0.13; cytidine, 0.04; cytosine, 0.06; dCMP, 0.00. In the experiments described below, the paper was notched before use to reduce the flow rate of the solvent (Reeves *et al.*, 1969), and the solvent front was allowed to run off the paper in order to obtain better resolution of slowly migrating substances. After drying of chromatograms, ultraviolet absorbing material was localized and identified. Strips containing individual samples were then cut transversely in 1-cm pieces. Each such piece was subsequently cut into small fragments, which were transferred to a scintillation counting vial containing 2 ml of H_2O. After elution at room temperature overnight, 15 ml of Aquasol (NEN chemicals) was added, and the radioactivity of the fraction was determined in a Packard liquid scintillation counter.

For spectrophotometric determinations of the deamination of mononucleotides, spectra of untreated and heated nucleotide solutions were obtained in the 230–300-nm range with a Cary 14 spectrophotometer at 20°.

Results

Deamination of Cytosine Residues in Single-Stranded DNA. E. coli DNA, ^{14}C-labeled in the cytosine residues, was enzymatically degraded to mononucleotides and analyzed by paper chromatography. Approximately 99.5% of the recovered

[1] Abbreviation used is: Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid.

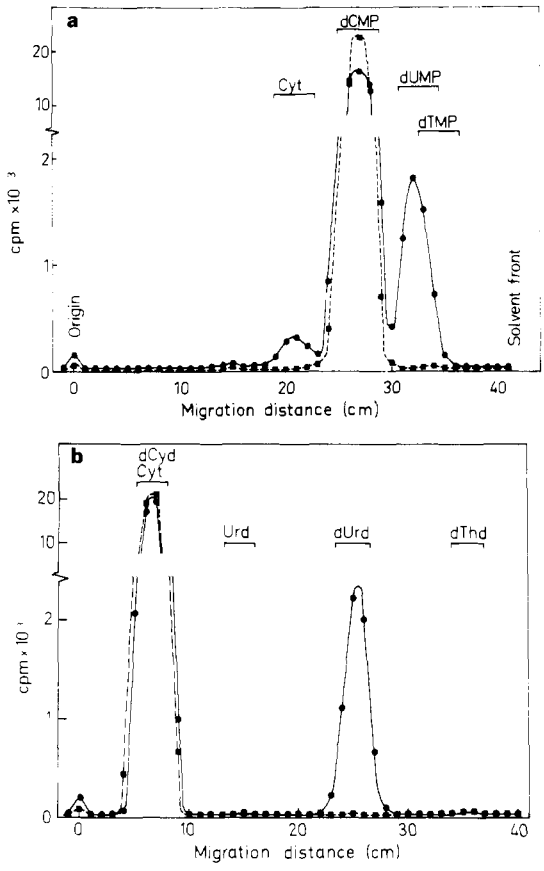

FIGURE 1: Paper chromatographic analysis of deamination of heat-treated [^{14}C]cytosine-labeled *E. coli* DNA. The DNA was enzymatically degraded to mononucleotides or nucleosides before chromatography: (■ - - - - ■) control DNA; (● —— ●) DNA heated for 6 days at 95° and pH 7.4 (buffer B) prior to analysis; (a) mononucleotides chromatographed in 2-propanol–concentrated HCl–H_2O, 170:41:39; (b) nucleosides chromatographed in ethyl acetate–1-propanol–H_2O, 4:1:2.

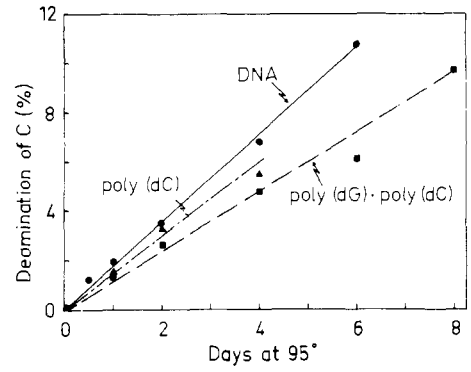

FIGURE 2: Rate of deamination of cytosine residues in *E. coli* DNA and in polydeoxyribonucleotides at 95° and pH 7.4 (buffer B): (● — ●) denatured *E. coli* DNA; (■ - - ■) poly(dG)·poly(dC); (▲ — · — · ▲) poly(dC).

radioactivity (80–100% yield in different experiments) was in the form of dCMP (Figure 1a, dashed line). In addition, 0.1–0.2% of the material remained at the origin, and 0.1–0.3% was found as dTMP. DNA preparations from bacteria harvested in the logarithmic growth phase contained less [^{14}C]dTMP than DNA from cells grown to stationary phase. Similar results were obtained when the [^{14}C]DNA was degraded to nucleosides before the chromatographic analysis. More than 99% of the material was then present in the form of deoxycytidine (Figure 1b, dashed line); 0.1–0.5% of the radioactivity remained at the origin in different experiments, and 0.1–0.3% was present as thymidine. Of other nucleosides, ~0.01% uridine was detected, showing that the DNA preparations were essentially free from RNA. Small quantities of deoxyuridine (0.02–0.03%) were also consistently observed. The origin of the deoxyuridine derived from unheated DNA is unknown; possibly it was due to traces of deaminase activity in the partly purified venom phosphodiesterase preparations employed. This background of minute amounts of deoxyuridine was the limiting factor in attempts to detect very small amounts of heat-induced cytosine deamination. In general, analysis of deoxynucleosides was more sensitive than that of deoxynucleotides because of the very different R_F values of deoxycytidine, deoxyuridine, and thymidine in the solvent system employed.

When [^{14}C]DNA preparations were heated at pH 7.4 for 6 days at 95° in buffers of physiological ionic strength before analysis, 10–11% of the radioactive mononucleotides migrated as dUMP instead of dCMP in two different chromatography systems. Analogous results were obtained when the heated DNA was analyzed as deoxynucleosides. Thus, when two equal aliquots of a heated DNA sample were chromatographed as nucleotides *vs.* nucleosides, 10.3% of the radioactive mononucleotides was accounted for as dUMP, and 10.6% of the nucleosides as deoxyuridine (Figure 1a, and b, solid lines). In the analysis of the mononucleotides, 1.4% of the radioactive material was also observed to migrate as cytosine or deoxycytidine (Figure 1a). The identity of this material with free cytosine, released by heat-induced cleavage of the glycosidic bond of dCMP residues in DNA, was confirmed by further chromatographic analysis (Lindahl and Karlström, 1973). No degradation products other than those due to deamination and depyrimidination were detected in these experiments. This demonstrates the specific and limited attack of water on dCMP residues in DNA at neutral pH. Thus, no significant quantities of pyrimidine dimers or similar products were formed, as they would have been released as oligonucleotides during the enzymatic digestion and subsequently detected as radioactive material remaining at the origin in the paper chromatographic analysis (Dellweg and Wacker, 1966). Further, no indications of a significant amount of heat-induced cleavage of the chromophoric pyrimidine ring structure were observed.

The heat-induced conversion of dCMP to dUMP residues in denatured DNA was linear with time (Figure 2), and was independent of the DNA concentration within the range studied (5–50 μg/ml). The rate constant for deamination of cytosine residues in single-stranded DNA at 95° and pH 7.4 was $k = 2 \times 10^{-7}$ sec^{-1}. Under the same conditions, the uracil–deoxyribose bonds in the partly deaminated DNA were hydrolytically cleaved at an approximate rate of $k = 6 \times 10^{-8}$ sec^{-1}, so the amount of free uracil released from DNA was insignificant in the present experiments.

Deamination of Mononucleotides. [^{14}C]dCMP was heated at 95° for various times, and then analyzed by paper chromatography. This compound was found to be deaminated at a slightly faster rate (15% rate difference) than the cytosine residues in single-stranded DNA at pH 7.4 in two buffers (buffers A and B). Thus, there was little difference in sensitivity between the monomeric and the polymeric dCMP residues under these conditions. Ullman and McCarthy (1973) have previously observed that in 1 M NaOH, deamination of deoxycytidine is more rapid than deamination of dCMP, which in turn is a slightly faster reaction than the deamination of cytosine residues in DNA. When the spectral properties of dCMP in the near-ultraviolet region were followed as a function of incuba-

TABLE I: Rate Constants of Deamination of Cytosine Residues in Single-Stranded *E. coli* DNA in Different Buffers at 100° and pH 7.4.

Solvent	k (sec^{-1})
0.05 M Hepes–KOH–0.001 M EDTA	3.5×10^{-7}
+ 0.1 M NaCl	3.7×10^{-7}
+ 0.35 M NaCl	4.1×10^{-7}
+ 0.35 M NaOAc	4.3×10^{-7}
+ 1 M NaOAc	4.1×10^{-7}
+ 0.15 M sodium phosphate	3.7×10^{-7}
+ 0.4 M sodium phosphate	4.2×10^{-7}
+ 0.01 M $MgCl_2$	3.7×10^{-7}

tion time at pH 7.4 and 95°, changes in absorption consistent with a conversion of pure dCMP to a mixture of dCMP and dUMP were observed. From the time-dependent change of the A_{280}/A_{260} ratio, the rate constant of deamination of dCMP was estimated to be $k = 2 \times 10^{-7}$ sec^{-1} at 95° (buffer A), in good agreement with the results from the paper chromatographic analysis ($k = 2.3 \times 10^{-7}$ sec^{-1}, buffer A). The chromatographic method was the more accurate one, because the spectra were not corrected for the simultaneously occurring cleavage of dCMP to cytosine and 2′-deoxyribose 5′-phosphate.

The rate of deamination of 5-methyldeoxycytidylic acid at pH 7.4 was also studied by spectrophotometry. On heating at 95°, spectral changes indicative of the formation of dTMP were observed. The rate of deamination was estimated to be $k = 9 \times 10^{-7}$ sec^{-1} in buffer A and $k = 1.1 \times 10^{-6}$ sec^{-1} in buffer B, *i.e.*, the rate of deamination of the 5-methyl derivative is approximately four times faster than that of dCMP itself at neutral pH.

Solvent Effects. The deamination of cytosine residues in single-stranded DNA at pH 7.4 and 95° proceeded at a similar rate in several buffers of approximately the same ionic strength. In buffer A (0.1 M NaCl–0.01 M sodium phosphate–0.01 M sodium citrate) the rate constant of deamination was $k = 2.0 \times 10^{-7}$ sec^{-1}. In the Mg^{2+}-containing buffer B (0.1 M KCl–0.05 M Hepes–KOH–0.01 M $MgCl_2$–0.001 M EDTA) the rate of deamination was $k = 2.2 \times 10^{-7}$ sec^{-1}, and the same rate was observed when $MgCl_2$ was excluded from this buffer.

The effect of buffer solutions of increasing ionic strength on the deamination rate was investigated at 100°. Cytosine residues in *E. coli* DNA were deaminated at a rate close to $k = 4 \times 10^{-7}$ sec^{-1} under all solvent conditions studied (Table I). A small, positive salt effect apparently was present on addition of either NaCl, sodium acetate, or sodium phosphate to the DNA solutions. This small promotion of the rate of deamination was close to the experimental error in these determinations, and there was no clear difference between the effects of the various buffer anions.

The detailed mechanism of the hydrolytic deamination of cytosine residues in DNA is unknown. It has been proposed (Shapiro and Klein, 1966, 1967; Notari, 1967) that free cytidine is deaminated in neutral and weakly acidic solutions by a mechanism involving specific hydrogen ion catalysis combined with buffer-catalyzed general acid-base catalysis. This would lead to the transient saturation of the 5,6-double bond by addition of buffer ion, hydrolytic deamination of the dihydrocytosine intermediate, and elimination of the buffer ion from the dihydrouridine intermediate to yield free uridine. Such dihydropyrimidine reaction intermediates would have very short lifetimes at the high temperatures employed in our experiments

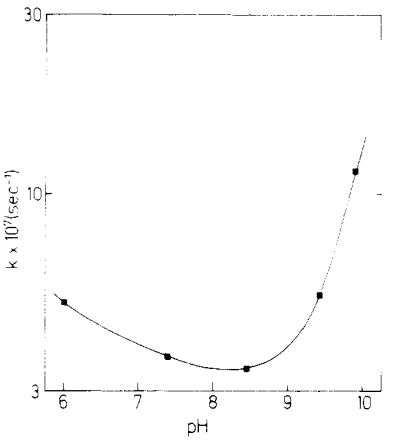

FIGURE 3: pH dependence of the rate of deamination of cytosine residues in *E. coli* DNA at 100°. The buffers contained 0.1 M NaCl, 10^{-3} M EDTA, and either 0.05 M Hepes–KOH (pH 6.0–7.4 at 100°) or 0.05 M sodium borate (pH 8.45–9.95 at 100°).

(Johns *et al.*, 1965; Vanderhoek and Cerutti, 1973) and would therefore not have been detected. However, if this mechanism were the correct one for the hydrolytic deamination of cytosine residues in DNA at neutral pH, a marked promotion of the reaction by high concentrations of phosphate buffer would be expected (Notari, 1967), but such a rate increase was not observed (Table I). Possibly, deamination events still occur by an addition–elimination mechanism, but with water rather than buffer anion as the principal base catalyzing the reaction (Shapiro and Klein, 1967; Hauswirth *et al.*, 1972).

pH Dependence. Aliquots of [^{14}C]cytosine-labeled *E. coli* DNA were incubated at 100° for 12, 24, and 96 hr in a series of buffers of pH 6–10, and the rates of cytosine deamination were determined. The reaction showed a relatively small pH dependence in the pH range 6–9, but proceeded more rapidly above pH 9 (Figure 3). This is consistent with the rapid rate of deamination of DNA cytosine residues in strong alkaline solution (Ullman and McCarthy, 1973). For single-stranded DNA, this rate is approximately 1000 times faster in 1 M NaOH than at pH 7.4. The deamination of dCMP showed a pH dependence similar to that of single-stranded DNA, and proceeded three times faster at pH 5.0 than at pH 7.4. A shallow pH dependence with a maximum at pH 3.5 has previously been observed for the deamination of free cytidine in the pH range 2.5–6.0 (Shapiro and Klein, 1966). As the acid-catalyzed cleavage of the glycosidic bond of dCMP shows a much stronger pH dependence than the deamination reaction in neutral and weakly acidic solutions, it becomes the predominant pathway of degradation of dCMP below pH 6, but in neutral and alkaline solution the deamination reaction is the more important one.

The rate of deamination of cytosine residues in DNA is slowest at pH 8.0–8.5 (Figure 3). It is of interest that the cleavage of purine–deoxyribose bonds also apparently proceeds at a minimal rate in this pH range, as the hydrolysis of deoxyadenosine is mainly acid-catalyzed below pH 8.0 and mainly alkali-catalyzed above 8.5 (Garrett and Mehta, 1972). It seems likely that the covalent structure of DNA has its maximum stability in aqueous solution at pH 8.0–8.5.

Rate of Deamination of Cytosine Residues in Double Helices. The cytosine residues in poly([^{14}C]dC) were deaminated at a similar or slightly lower rate than those in single-stranded DNA at 95° (Figure 2). The cytosine residues in the polynucleotide poly(dG)·poly([^{14}C]dC), ($T_m \approx 110°$) were also read-

ily deaminated at 95° (Figure 2). Mixtures of poly(dG) and poly([^{14}C]dC) in the proportions 50:50, 60:40, and 70:30 were deaminated at similar rates as poly(dG)·poly([^{14}C]dC) preparations which had not undergone strand separation, so this relatively rapid deamination did not seem to depend on the presence of single-stranded sequences of poly(dC) in the preparations used. Further, Radding *et al.* (1962) observed that of 16 preparations of poly(dG)·poly(dC) synthesized in the same way as here with the *E. coli* DNA polymerase I, seven contained the two chains in equimolar proportions and nine contained an excess of poly(dG), but no preparations with an excess of poly(dC) were found.

Cytosine residues in poly(dC) and poly(dG)·poly(dC) were deaminated 15 times more slowly at 70 than at 95° (Figures 2 and 4). Similar results were also obtained with dCMP (Table II). A much larger difference was observed with denatured *E. coli* DNA, which was deaminated 180 times more slowly at 70 than at 95° (Figures 2 and 4). Denatured DNA is single-stranded at 95° but partially renatures to a double-stranded form at 70° (Marmur *et al.*, 1963). These results therefore indicate that the secondary structure of DNA, in contrast to that of poly(dG)·poly(dC), offers good protection against the deamination of cytosine residues. This notion was directly confirmed by measurements of the rate of cytosine deamination in native *E. coli* DNA at 70°. The reaction clearly was very slow, and initially proceeded at <1% of the rate observed for poly(dC) or dCMP at the same temperature (Figure 4). The apparent increase in reaction rate after 30 days of incubation at 70° (Figure 4) was probably due to generation of single-stranded sequences by heat-induced depurination and subsequent chain breakage (Lindahl and Nyberg, 1972).

Temperature Dependence of the Reaction. The deamination of cytosine in different forms was followed at several temperatures. The results are summarized in Table II. The data obtained with poly(dG)·poly(dC), poly(dC), and dCMP show that the deamination process in these cases is associated with an activation energy of approximately 29 kcal/mol. A temperature dependence of this magnitude is typical for the hydrolysis of small molecules or residues in polymers not undergoing conformational changes. For comparison it is noted that depurination of both native DNA and denatured DNA in neutral solution is associated with an activation energy of 31 ± 2 kcal/mol (Lindahl and Nyberg, 1972).

Cytosine deamination in denatured DNA showed a large temperature dependence within the range 70–95°, but outside this range the rate of deamination was less strongly temperature-dependent (Table II), and thus more similar to the results obtained with polynucleotides and dCMP. This is consistent with the notion that the changes in secondary structure that occur in denatured DNA when the temperature of the solution is decreased from 95 to 70° lead to protection of the cytosine residues.

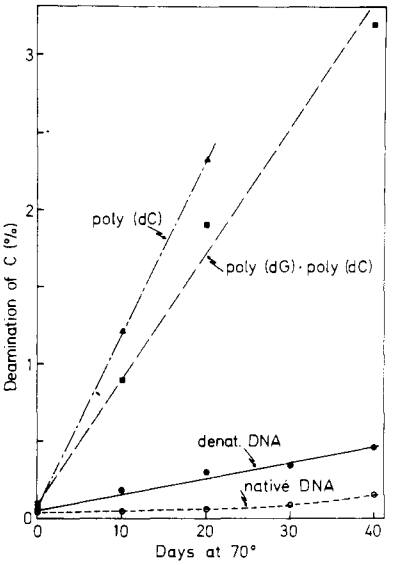

FIGURE 4: Rate of deamination of cytosine residues in different polymers at 70° and pH 7.4 (buffer B): (○ - - - - ○) native *E. coli* DNA; (● —— ●) denatured *E. coli* DNA; (■ - - ■) poly(dG)·poly(dC); (▲ — · — · ▲) poly(dC).

Discussion

Heat-induced deamination of cytosine residues in single-stranded DNA takes place at an easily detectable rate in aqueous buffers at pH 7.4. It seems likely that this reaction and the cleavage of glycosidic bonds are the two most common forms of hydrolytic degradation of the primary structure of DNA under such solvent conditions. Denaturation of the secondary structure of DNA by heat, or by alkali treatment, will consequently lead to the conversion of a minority of the cytosine residues to uracil, and after subsequent renaturation double-helical heterozygous molecules containing rare guanine·uracil base pairs will be obtained. From the data in Table II, we estimate that in DNA heated at 100° for 10 min at neutral pH, 1 uracil residue will be present per 5000 cytosine residues.

For deamination of cytosine residues in double-helical structures, we unexpectedly found that there was little inhibition of hydrolytic attack at such residues in poly(dG)·poly(dC), while they were well protected in *E. coli* DNA. Possibly, steric factors account for the difference in reactivity of cytosine residues in these two helical forms. Double-stranded polydeoxynucleotides containing all the purines in one of the two strands and the pyrimidines in the other strand have a helical structure with slightly tilted base pairs that differs from the classical B

TABLE II: Rate Constants (sec^{-1}) of Deamination of Cytosine Residues in Different Forms at Various Temperatures.[a]

Cytosine in	Temp (°C)				
	65	70	80	95	110
(a) Denatured *E. coli* DNA	4.9×10^{-10}	1.2×10^{-9}	1.3×10^{-8}	2.2×10^{-7}	1.5×10^{-6}
(b) Native *E. coli* DNA (initial rate)		$<1 \times 10^{-10}$	$<4 \times 10^{-10}$		
(c) Poly(dC)		1.3×10^{-8}		1.9×10^{-7}	
(d) Poly(dG)·poly(dC)		9.5×10^{-9}	3.3×10^{-8}	1.5×10^{-7}	
(e) dCMP		1.6×10^{-8}		2.6×10^{-7}	
(f) 5-Methyl-dCMP				1.1×10^{-6}	

[a] Solvent: buffer B (0.1 M KCl–0.05 M Hepes–KOH–0.01 M $MgCl_2$–0.001 M EDTA (pH 7.4)).

structure of DNA (Langridge, 1969). This unusual secondary structure is also less resistant to heat denaturation than would be predicted from the base composition (Wells *et al.*, 1970). Another possibility is that the greatly different rates of cytosine deamination depend on differences in conformational motility between DNA and poly(dG)·poly(dC). Double-helical nucleic acids undergo transient local structural distortions in solution, "breathing," that lead to hydrogen exchange of interstrand hydrogen-bonded hydrogens with the surrounding water. Such opening events occur also at low temperatures, and represent a reaction associated with a low activation energy that is different from the heat-induced denaturation of the double helix (McConnell and von Hippel, 1970). Thus, there is no correlation between the T_m and the rate of hydrogen exchange at temperatures well below the T_m for different double-stranded helices, and poly(dG)·poly(dC) has been found to exhibit a several times higher rate of hydrogen exchange than native DNA from natural sources (Englander *et al.*, 1972). A third possibility is that poly(dG)·poly(dC) rearranges into a triple helical structure at elevated temperatures in the buffers used here, with an accompanying decrease in the protection of the cytosine residues.

The different rates of hydrolytic deamination of cytosine in single-stranded *vs.* double-stranded DNA and in polydeoxynucleotides may be compared with the different reaction rates observed in studies of the accessibility of cytosine residues in double-helical nucleic acids to group-specific reagents. The reaction rates with different reagents vary considerably, primarily depending on which reagent is used. Cytosine residues in poly(I)·poly(C) are readily deaminated by treatment with nitrous acid, the reaction proceeding only 2–3 times more slowly than the analogous reacion with single-stranded poly(C) (Shapiro and Yamaguchi, 1972). However, cytosine residues in poly(I)·poly(C), or in native DNA, are efficiently protected against deamination by treatment with bisulfite, possibly because of steric hindrance in the double helix to the approach of this bulky reagent (Shapiro *et al.*, 1973). Methoxyamine has been reported to react 400 times more slowly with cytosine residues in native DNA than with free cytidine (Cashmore *et al.*, 1971). Formaldehyde reacts extremely slowly with cytosine residues in native DNA, compared with those in denatured DNA, and the reaction apparently only takes place at transiently open segments in native DNA molecules (von Hippel and Wong, 1971). In the heat-induced hydrolytic degradation of cytosine residues in DNA, water may be regarded as a reagent of small size that is already present in the grooves of the DNA double helix (Tunis and Hearst, 1968). It is therefore not obvious why the cytosine residues in native DNA are so well protected against hydrolytic attack. Heat-induced hydrolytic cleavage of glycosidic bonds occurs at only a fourfold slower rate in native DNA than in denatured DNA (Lindahl and Nyberg, 1972).

In living cells, a small but significant fraction of the bases in the DNA may be directly accessible to the surrounding water as a consequence of local structural fluctuations in the DNA helix (von Hippel and Wong, 1971) and transient generation of single-stranded sequences during DNA replication and transcription (Bick *et al.*, 1972). Such regions would be potential targets for hydrolytic deamination of cytosine residues, especially in thermophiles. Consequently, cellular repair mechanisms may well exist that specifically convert guanine–uracil base pairs in DNA back to guanine–cytosine base pairs.

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