Heat-Induced Depyrimidination of Deoxyribonucleic Acid in Neutral Solution†

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ABSTRACT: The rate of hydrolytic release of pyrimidine residues from DNA in neutral aqueous buffers of physiological ionic strength has been measured at 80 and 95°. DNA selectively labeled with ^{14}C in cytosine or thymine residues was prepared from a mutant of *Escherichia coli* B requiring uracil and thymine. During incubation, both pyrimidines were liberated in free form at similar rates: at 95° and pH 7.4, the rate constant for release of thymine from DNA was 2.3×10^{-8} sec^{-1}, and for cytosine 1.8×10^{-8} sec^{-1}. Thus, depyrimidination of DNA is considerably slower than depurination at neutral pH. It is still estimated that DNA depyrimidination may occur at a physiologically significant rate in living cells.

Studies of the hydrolysis of free deoxyribonucleosides have shown that purine and pyrimidine derivatives are degraded in the same fashion, by a mechanism involving direct cleavage of the glycosidic bond without opening of the sugar ring (Shapiro and Kang, 1969; Zoltewicz *et al.*, 1970; Shapiro and Danzig, 1972; Panzicka *et al.*, 1972; Garrett and Mehta, 1972a). While this reaction proceeds more rapidly in acid than in neutral solution for all deoxyribonucleosides, the rates of hydrolysis of pyrimidine derivatives are much less dependent on pH than those of purine derivatives. Thus, deoxycytidine is hydrolyzed at essentially the same rate within the pH range 1–4 (Venner, 1964; Shapiro and Danzig, 1972) while deoxyguanosine and deoxyadenosine are almost 1000 times more stable at pH 4 than at pH 1 (Venner, 1964; Zoltewicz *et al.*, 1970; Hevesi *et al.*, 1972; Garrett and Mehta, 1972a). Similarly, the glycosidic bond of thymidine is cleaved at the same rate at pH 7 as at pH 3 (Shapiro and Kang, 1969), showing that the unprotonated form of this nucleoside is hydrolyzed by a pH-independent reaction with water rather than by acid-catalyzed hydrolysis in neutral and weakly acidic solution. It is clear from these results that the great difference in stability between purine and pyrimidine deoxynucleosides that exists in acid is much less marked at higher pH values. At the macromolecular level, however, Greer and Zamenhof (1962) did not observe heat-induced depyrimidination of DNA at neutral pH, while they could clearly demonstrate depurination under the same conditions. Here it is shown that depyrimidination of DNA does occur at a measurable rate in neutral aqueous buffers, but that it is a considerably slower reaction than depurination.

Materials and Methods

Bacterial Strains. The pyrimidine-requiring, cytidine deaminase-deficient *Escherichia coli* B strain OK305 has been described (Karlström, 1968). A thymine-dependent mutant was obtained from this strain by treatment with aminopterin, and a derivative strain with the ability to grow on low concentrations of thymine was subsequently isolated by direct selection on a medium containing 2 μg/ml of thymine (Karlström, 1968). This strain is referred to as OK308 (*pyr,cdd,tlr*). It was kept at −70° in broth supplemented with 10% glycerol, as it showed poor survival on storage at 4° as a slant culture.

DNA Preparations. Strain OK308 was grown at 37° in a medium containing uracil and thymine as specified below, 1.5 g of NH_4Cl, 6 g of KH_2PO_4, 15 g of K_2HPO_4, 1 g of $Na_2SO_4 \cdot 10H_2O$, 0.2 g of Casamino acids (pretreated with charcoal), 5 g of glucose, and 0.2 g of $MgCl_2 \cdot 6H_2O$ per l. For the preparation of cytosine-labeled DNA, the medium contained 20 μg/ml of thymine and 3 μg/ml of [2-^{14}C]uracil (500 μCi/mg). In late logarithmic phase, nonradioactive uracil (10 μg/ml) was added and growth was continued for 20 min. For thymine-labeled DNA, the medium contained 10 μg/ml of uracil and 3 μg/ml of [2-^{14}C]thymine (400 μCi/mg). In late logarithmic phase, nonradioactive thymine was added to a final concentration of 20 μg/ml, and growth was continued for 20 min. DNA containing 5-bromouracil residues was obtained in the same fashion as thymine-labeled DNA, but the medium contained 10 μg/ml of uracil, 10 μg/ml of [2-^{14}C]5-bromouracil (50 μCi/mg), and 2 μg/ml of thymine. Thymine was included to retain good viability during growth (Hackett and Hanawalt, 1966). In all cases, the bacteria were harvested by centrifugation in the cold, and washed once with a buffer containing 0.15 M NaCl, 0.1 M EDTA (pH 7.0), 20 μg/ml of thymine, and 10 μg/ml of uracil. DNA was then prepared as described (Lindahl and Nyberg, 1972).

Base analysis of DNA was performed by incubation of the DNA (3–5 μg, 10^5 cpm) in a sealed ampoule with 98% formic acid for 30 min at 175°. The hydrolysate was dried over NaOH, dissolved in 100 μl of 10^{-2} M HCl containing 5 μg each of nonradioactive thymine, cytosine, adenine, and guanine, and analyzed by paper chromatography in system III.

Recovery of Material Released from DNA. DNA solutions (usually containing 20 μg/ml of DNA) were incubated at 95 or 80° in either buffer A (0.1 M NaCl–0.01 M sodium phosphate–0.01 M sodium citrate, pH 7.4) or in buffer B (0.1 M KCl–0.05 M *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (Hepes)–KOH–0.01 M $MgCl_2$–0.001 M EDTA, pH 7.4, at 80°) as described (Lindahl and Nyberg, 1972). For studies on denatured DNA at 80°, DNA solutions were incubated at 100° for 10 min prior to transfer to 80°. At the end of an incubation period the following material was added at 0° to an ampoule containing 0.25 ml of heated DNA

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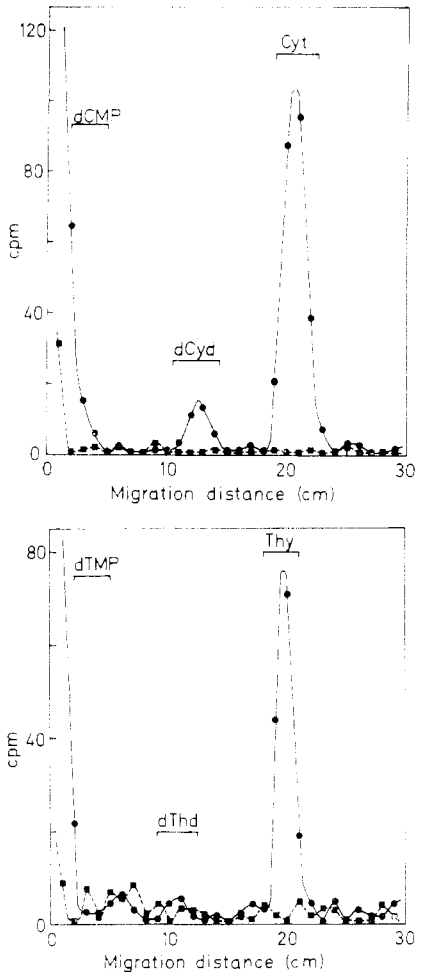

FIGURE 1: Paper chromatography of acid-soluble material released from [^{14}C]pyrimidine-labeled *E. coli* DNA by heating in neutral solution. Single-stranded DNA (5 μg) in 0.25 ml of buffer B was heated at 80° for 14 days prior to analysis: (a, top) cytosine-labeled DNA (165,000 cpm); (b, bottom) thymine-labeled DNA (102,000 cpm); (●) acid-soluble material from heated DNA; (■) acid-soluble material from unheated DNA.

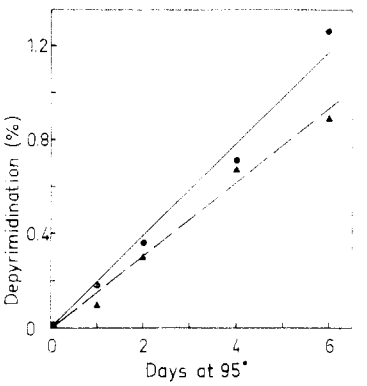

FIGURE 2: Rate of release of free pyrimidines, measured as in Figure 1, from *E. coli* [^{14}C]DNA in buffer B at 95°: (▲) cytosine-labeled DNA; (●) thymine-labeled DNA.

solution: 5 μl of a solution containing 0.5% each of the appropriate base, nucleoside, and nucleotide (*e.g.*, cytosine, deoxycytidine, and dCMP), 60 μl of 0.2% heat-denatured calf thymus DNA, and 80 μl of 2 M $HClO_4$. After 5 min at 0°, the mixture was centrifuged for 10 min at 12,000*g*, and 300 μl of supernatant solution was recovered. This material was neutralized with 2 M KOH, and after 1 hr at 0°, the resulting $KClO_4$ precipitate was removed by centrifugation. The supernatant solution was lyophilized, redissolved in 100 μl of H_2O, and analyzed by paper chromatography in system I.

Paper Chromatography. Whatman 3MM paper was used. System I (Reeves *et al.*, 1969) contained: isobutyric acid–water–0.1 M EDTA–concentrated ammonia–toluene (160:22:3:2:20, v/v). Chromatograms were run for 18–24 hr on notched paper (Reeves *et al.*, 1969). System II contained: isobutyric acid–water–0.1 M EDTA–concentrated ammonia (66:33:1:1, v/v). System III (Wyatt, 1951) contained: isopropyl alcohol–concentrated HCl–water (170:41:39, v/v). After drying of the papers, ultraviolet-absorbing material was localized and identified. Strips containing chromatographed samples were then cut transversely in 1-cm pieces. Each piece was 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 by collecting at least 500 cpm in a Packard liquid scintillation spectrometer.

Material. Radioactive pyrimidines were obtained from NEN Chemicals. Hepes was purchased from Sigma.

Results

Properties of DNA Containing [^{14}C]Cytosine or [^{14}C]Thymine. DNA from *E. coli* B has 50% G·C base pairs and does not contain 5-methylcytosine residues (Fujimoto *et al.*, 1965). A uracil- and thymine-requiring mutant, OK308, was isolated from *E. coli* B, and grown in the presence of one radioactive and one nonradioactive pyrimidine. In this way, one or the other of the pyrimidine bases in DNA was selectively labeled. From 150-ml cultures, 200–600 μg of DNA labeled in either cytosine or thymine and containing 20,000–35,000 cpm/μg was obtained. Base analysis of the DNA preparations showed that <0.2% of the label in [^{14}C]cytosine–DNA was present in the form of thymine or purines, and similarly that <0.2% of the label in [^{14}C]thymine–DNA occurred as cytosine or purines. The same method to obtain DNA labeled in pyrimidine residues has recently been used by Ullman and McCarthy (1973) for studies on the degradation of DNA in alkaline solution.

Release of Pyrimidines from DNA. On heating DNA at neutral pH, acid-soluble material is slowly released into solution. This material mainly consists of purine bases and of oligonucleotides. The latter are probably liberated when depurination, followed by chain scission, occurs at two closely located sites (Lindahl and Nyberg, 1972). Here, the acid-soluble substances released from DNA labeled in pyrimidine residues was analyzed by paper chromatography in system I, which provides excellent separation of pyrimidine bases, nucleosides, and mononucleotides (Reeves *et al.*, 1969). Most of the ^{14}C in the low molecular weight fraction (90–95%) remained at the origin, and the amount of such material was that expected due to release of oligonucleotides (Lindahl and Nyberg, 1972). In addition, a distinct peak of radioactivity with the chromatographic properties of a free pyrimidine base appeared both when cytosine- and thymine-labeled DNA were heated (Figure 1). The identity of this material with cytosine or thymine, respectively, was confirmed by paper chromatography in two other solvent systems. The amount of pyrimidines in free form was directly proportional to the time of heating (Figure 2). Both pyrimidine bases were

released at very similar rates, with the liberation of thymine being slightly faster than that of cytosine (Figure 2). The rate constants for release of free pyrimidines from DNA in buffer B at 95° were $k = 2.3 \times 10^{-8}$ sec^{-1} for thymine and $k = 1.8 \times 10^{-8}$ sec^{-1} for cytosine (Figure 2). In buffer A, the rate of depyrimidination at 95° was the same as in buffer B within the experimental error, and it was also found to be independent of the DNA concentration within a range of 5–50 μg/ml of DNA. Depurination of DNA (Lindahl and Nyberg, 1972) and hydrolysis of pyrimidine deoxynucleosides (Shapiro and Kang, 1969) are also independent of buffer composition. These data are consistent with the notion that depurination and depyrimidination occur by the same reaction mechanism (Shapiro and Danzig, 1972). With heated thymine-labeled DNA, no radioactive material other than oligonucleotides and free thymine was detected, but with extensively heated cytosine-labeled DNA, a small amount of deoxycytidine was consistently liberated in addition to free cytosine (Figure 1). The mechanism of release of this deoxynucleoside is unknown, but this pathway of degradation is clearly a minor one in comparison with depyrimidination.

At 80°, the rate of depyrimidination of denatured DNA was approximately 20 times slower than at 95°; the rate constants for release at this temperature were $k = (1.2 \pm 0.2) \times 10^{-9}$ sec for thymine and $k = (1.0 \pm 0.2) \times 10^{-9}$ for cytosine. It should be noted that denatured DNA at this temperature contains hydrogen-bonded, renatured regions and therefore is not directly comparable to the fully single-stranded DNA present at 95°. For native DNA at 80°, the depyrimidination reaction was markedly slower than for denatured DNA, and apparent initial rate constants of $k = (2–5) \times 10^{-10}$ sec^{-1} were obtained. As prolonged heating of the DNA at 80° was necessary to liberate detectable amounts of pyrimidines from double-stranded DNA, and such heating does generate some single-stranded sequences (Lindahl and Nyberg, 1972), these data should be regarded as upper estimates rather than representing the true rate of depyrimidination of native DNA. For comparison, it may be noted that at neutral pH and 80°, depurination of native DNA is four times slower than depurination of denatured DNA (Lindahl and Nyberg, 1972).

Bromouracil-Containing DNA. DNA containing [2-^{14}C]5-bromouracil residues was prepared, and the depyrimidination of this material during heating in the dark was followed by the methods described above. Heat-induced release of free 5-bromouracil was observed. The depyrimidination reaction proceeded at a somewhat faster rate ($k \approx 10^{-8}$ sec^{-1} with denatured DNA in buffer B at 80°) than the liberation of cytosine and thymine. This is in agreement with the studies on deoxynucleosides, which have shown that at neutral pH the glycosidic bond of 5-bromodeoxyuridine is more labile than that of thymidine (Shapiro and Kang, 1969). However, degradation of DNA by cleavage of glycosidic bonds at bromouracil residues still appears to be a slow reaction. In addition to free bromouracil, other degradation products were also formed at a relatively rapid rate in this case, indicating that cleavage of the glycosidic bond is not the main pathway of hydrolytic degradation of BrdUMP residues. For this reason, the depyrimidination of bromouracil-containing DNA was not studied in detail. Garrett *et al.* (1968) have shown that 5-halouracils, *e.g.*, 5-bromouracil, are much more sensitive than uracil or 5-methyluracil (thymine) to hydrolysis in neutral solution, and that degradation of the chromophoric ring structure occurs by general base catalysis.

Discussion

Depyrimidination of DNA occurs in neutral water solution at high temperatures. The rate of release of pyrimidines is, however, markedly slower than the previously determined rate of hydrolytic release of purines under the same conditions (Lindahl and Nyberg, 1972). In comparison with our estimates for the rates of cleavage of glycosidic bonds in single-stranded DNA, the rates of hydrolysis of free deoxynucleosides are 10–50 times higher at pH 7.4 (Shapiro and Kang, 1969; Garrett and Mehta, 1972b). Similar observations have recently also been made for DNA *vs.* free deoxynucleosides in 80% H_2SO_4 (Shapiro and Danzig, 1973). Glycosidic bonds in deoxymononucleotides are hydrolyzed more slowly than in the corresponding deoxynucleosides, but considerably more rapidly than in DNA (Shapiro and Chargaff, 1957; Venner, 1966; Shapiro and Danzig, 1973).

In addition to thymine and cytosine, 5-methylcytosine is present as a minor base residue in DNA from many sources. The rate of release of this pyrimidine from DNA was not investigated, but it seems unlikely that it would be faster than that observed for cytosine and thymine. Thus, at neutral pH, the glycosidic bond in 5-methyldeoxycytidylic acid is cleaved at approximately the same rate as that in dCMP (unpublished observations). Further, 5-methyldeoxycytidine is hydrolyzed at a similar but slightly slower rate than deoxycytidine at pH 6 (Shapiro and Kang, 1969).

The slow, spontaneous depyrimidination of DNA in neutral aqueous solution described here appears to be of no obvious practical use for the removal of pyrimidines from DNA *in vitro*, as much better methods exist for this purpose, *e.g.*, hydrazinolysis (Türler and Chargaff, 1969). However, the biological consequences of the reaction are of interest. The data presented here indicate that at 80 and 95° and at pH 7.4, depyrimidination occurs at approximately 5% of the rate of depurination. The activation energy for hydrolysis of pyrimidine deoxyribonucleosides has been found to be 31–34 kcal/mol for several different nucleosides (Garrett *et al.*, 1966; Shapiro and Danzig, 1972), which is very similar to the value of 31 kcal/mol observed for depurination of DNA (Lindahl and Nyberg, 1972). We have previously estimated that for a growing mammalian cell, several thousand purines should be released from the DNA by spontaneous hydrolysis during one cell generation (Lindahl and Nyberg, 1972), and it now appears that a few hundred pyrimidine residues might also be liberated within the same time period. In both cases, the lesion in DNA is essentially the same one, a sugar–phosphate residue in the DNA "backbone" lacking an attached base residue. Therefore, the lesion could presumably be corrected *in vivo* by the same excision–repair mechanism (Lindahl and Andersson, 1972; Verly *et al.*, 1973).

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Characterization of Two Complementary Polypeptide Chains Obtained by Proteolysis of Rabbit Muscle Phosphorylase†

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ABSTRACT: A limited proteolysis of rabbit muscle phosphorylase by subtilisin results in a nick in the protomer which yields two reasonably homogeneous complementary fragments; one, of mol wt 30,000, containing the phosphoserine residue corresponds to the NH_2-terminal third of phosphorylase; the other, of mol wt 70,000, containing the lysine residue which binds pyridoxal 5'-phosphate, corresponds to the COOH-terminal two-thirds of phosphorylase. Information about the tertiary and quaternary structure of phosphorylase derived from the kinetics of this proteolytic treatment is discussed.

Several structural studies of proteins based on their sensitivity to proteolytic enzymes have been made. Linderström-Lang (1952) has proposed two models to describe the degradation by proteases: (a) the "one by one" model—the first break produces an overall destabilization of the protein structure and is followed by a fast and extensive degradation of the polypeptide chain; (b) the "zipper" model—the first cleavage in the protein is much faster than the other breaks.

Rabbit muscle phosphorylase *a* seems to be degraded by various proteases according to the zipper-type mechanism (Nolan *et al.*, 1964). From their observations, it can be concluded that one, or very few, limited parts of the protein backbone are sensitive to proteolysis and that the fragments thus formed are degraded very slowly. We were interested in further investigating the specific sensitivity of phosphorylase to limited proteolytic degradation to see if such an approach would provide information about the structure of this enzyme. We were mainly interested in the phosphorylase *b* to phosphorylase *a* conversion. The two forms of the enzyme were submitted to a mild proteolytic treatment and the degradation products examined. Subtilisin was chosen as the proteolytic enzyme for two reasons. First, it is a rather nonspecific protease (Harris and Roos, 1959) and the susceptible bonds depend more on their exposure than on the neighboring residues. Second, Graves *et al.* (1968) have extensively studied phosphorylase *b'*, a protein obtained by mild proteolytic treatment of phosphorylase *a* by trypsin, and it seemed interesting to compare the results obtained with trypsin and subtilisin.

Materials and Methods

Activity and Concentration of Phosphorylase. Phosphorylases *a* and *b* were assayed according to Helmreich and Cori (1966). Enzyme concentration was determined spectrophotometrically; the extinction coefficient at 280 nm used was $E_{1\,cm}^{1\%}$ 13.2 (Buc and Buc, 1968).

Phosphorylases. Rabbit muscle phosphorylase *b* was purified according to Fischer *et al.* (1958). Phosphorylase *a* labeled with ^{32}P was obtained by phosphorylation of phosphorylase *b* according to Krebs *et al.* (1964). Reduced phosphorylase *b* was prepared by reduction of the Schiff base between pyridoxal-P and the enzyme according to Strausbauch *et al.* (1967), except for the buffer which was replaced by 0.05 M Tris-acetate (pH 8.5). (All pH measurements were performed at room temperature.)

Carboxymethylation was performed in 0.1 M Tris-acetate (pH 8.1) and 6 M guanidine·HCl at room temperature. Phosphorylase *b* (*ca.* 10 mg/ml) was first denatured by incubation in this buffer for 20 min in the presence of dithiothreitol (2 mol/mol of cysteine residue). [^{14}C]Iodoacetic acid was then

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