Paoletti, J., and Le Pecq, J. B. (1971), *Eur. Biophys. Congr. Proc., Protein, Nucleotides, 1*, 391.
Paoletti, C., Le Pecq, J. B., and Lehman, I. R. (1971), *J. Mol. Biol. 55*, 75.
Philipson, L., Albertson, P. A., and Frick, G. (1960), *Virology 11*, 553.
Revet, B. M. J., Schmir, M., and Vinograd, J. (1971), *Nature (London), New Biol. 229*, 10.
Richardson, C. C. (1965), *Proc. Nat. Acad. Sci. U. S. 54*, 158.
Richardson, C. C. (1966), *J. Mol. Biol. 15*, 49.
Richardson, C. C., Inman, R. B., and Kornberg, A. (1964), *J. Mol. Biol. 9*, 46.
Schumaker, V. N., Richards, E. G., and Schachman, H. K. (1956), *J. Amer. Chem. Soc. 78*, 4230.
Signer, R., and Schwander, H. (1949), *Helv. Chim. Acta 32*, 853.
Studier, F. W. (1965), *J. Mol. Biol. 11*, 373.
Thomas, C. A., Jr. (1956), *J. Amer. Chem. Soc. 78*, 1861.
Tomlinson, R. V., and Tener, G. M. (1963), *Biochemistry 2*, 697.
Williams, E. J., Sung, S. C., and Laskowski, M. (1961), *J. Biol. Chem. 236*, 1130.

Rate of Depurination of Native Deoxyribonucleic Acid†

Tomas Lindahl* and Barbro Nyberg

ABSTRACT: The rate of depurination of double-stranded *Bacillus subtilis* DNA, radioactively labeled in the purine residues, has been followed as a function of temperature, pH, and ionic strength. In a Mg^{2+}-containing buffer of physiological ionic strength, the rate constant for depurination of DNA is 4×10^{-9} sec^{-1} at 70° and pH 7.4. The activation energy of the reaction is 31 ± 2 kcal/mole. These data strongly indicate that depurination of DNA occurs at a physiologically significant rate under *in vivo* conditions and consequently that the lesions introduced in this fashion must be repaired.

When neutral DNA solutions are exposed to high temperatures, two kinds of structural changes occur. Within a narrow temperature interval around the helix–coil transition temperature, T_m, the macromolecular structure of the DNA is denatured, and the two strands separate when all hydrogen bonds have been broken (Doty *et al.*, 1960). A different, slower inactivation process also takes place, which is due to heat-induced degradation of the primary structure and may be detected as an irreversible loss of biological activity of transforming DNA (Lerman and Tolmach, 1959; Ginoza and Zimm, 1961; Roger and Hotchkiss, 1961; van Sluis and Stuy, 1962; Strack *et al.*, 1964; Alberts and Doty, 1968). The latter reaction occurs both above and below the T_m, and is faster at low pH (Roger and Hotchkiss, 1961). This irreversible inactivation is probably to a large extent due to depurination of the DNA (Tamm *et al.*, 1952; Greer and Zamenhof, 1962), and/or to the formation of chain breaks (Tamm *et al.*, 1953) and cross-links (Freese and Cashel, 1964) occurring as a consequence of depurination. Other types of heat-induced DNA degradation could also be relevant, *e.g.*, deamination of cytosine residues (Shapiro and Klein, 1966), destruction of deoxyribose residues, and hydrolytic cleavage of pyrimidine–glycosyl bonds (Shapiro and Danzig, 1972).

As these various heat-induced processes are associated with finite activation energies, they should also proceed at physiological temperatures, though at greatly reduced rates. Local denaturation of the DNA double helix, followed by rapid renaturation, has been observed at temperatures well below the T_m, and this structural motility may be of physiological relevance in the interaction of various proteins with DNA (Printz and von Hippel, 1965). Degradation of the covalent DNA structure may also occur at a physiologically significant rate at moderate temperatures.

Greer and Zamenhof (1962) demonstrated that purine bases were released in detectable quantities from DNA at neutral pH and temperatures near the T_m, and that the activation energy for depurination of denatured DNA was approximately 28 kcal/mole in 0.005 M phosphate buffer (pH 6.8). These results indicate that depurination of DNA could take place at a relevant rate also under physiological conditions. However, the liberation of purines in a DNA solution stored for 2 months at 37° was below the limit of chemical detectability. The possibility therefore remained that the rate of depurination of double-stranded DNA would be extremely slow, or show a strong temperature dependence, so that the reaction would be of negligible importance in living cells. For this reason, we have reinvestigated the rate of depurination of double-stranded DNA as a function of temperature and pH, employing bacterial DNA isotopically labeled in the purine residues. The results show that depurination of DNA in solution takes place at a significant rate even under conditions that presumably resemble those *in vivo*.

Materials and Methods

DNA Preparations. A purine-requiring mutant of *Bacillus subtilis* (strain 168 *purB* 6) was obtained from Dr. A. Adams. The bacteria were grown in the competence medium C^+ of O'Sullivan and Sueoka (1967), supplemented with 5×10^{-5} M [8-^{14}C]adenine (20 mCi/mmole). The bacteria were harvested in the late logarithmic growth phase, washed once with 0.15 M NaCl–0.1 M EDTA (pH 7.0) containing 10^{-4} M adenine, suspended in the same solvent, and treated with 1 mg/ml of

† From the Department of Chemistry II, Karolinska Institutet, Stockholm, Sweden. *Received April 25, 1972.* This work was supported by grants from the Swedish Natural Science Research Council, the Swedish Cancer Society (Project No. 362), and the Karolinska Institutet.

lysozyme for 20 min at 37°. Sodium dodecyl sulfate was subsequently added, and DNA was prepared according to the method of Marmur (1961). Two additional purification steps were included in the procedure: after RNase treatment, the DNA was incubated with 100 μg/ml of preincubated Pronase for 1 hr at 37°. It was then chilled to room temperature, and extracted with an equal volume of redistilled, water-saturated phenol. After centrifugation for 10 min at 8000*g* at 4°, the aqueous phase was recovered. The final isopropyl alcohol precipitation step was then repeated twice. The DNA was finally dissolved at a concentration of 0.5 mg/ml in 2 M NaCl–0.01 M Tris-HCl–0.001 M EDTA (pH 8.0), dialyzed against the same solvent for 2 days at 2°, then against solvent without NaCl for an additional day, and stored at 2°.

Nonradioactive *B. subtilis* DNA was prepared from wild-type *B. subtilis* 168, grown in Pen-Assay broth (Difco antibiotic medium no. 3), by the same procedure. DNA was denatured by heating at 100° for 8 min in 0.1 M NaCl–0.01 M sodium phosphate–0.01 M sodium citrate (pH 8.5), followed by quick cooling. Degradation of [^{14}C]purine-labeled *B. subtilis* DNA (0.2 mg/ml) to oligonucleotides with micrococcal nuclease (20 μg/ml) was performed in 0.04 M Tris-HCl (pH 8.0)–0.004 M $CaCl_2$, for 2 hr at 37°. The reaction was stopped by the addition of 0.02 M sodium citrate.

To obtain DNA specifically labeled in 6-methyladenine residues, nonradioactive *B. subtilis* DNA was incubated with phage T4 induced DNA methylase and *S*-adenosyl-[*methyl*-^{14}C]methionine in a total volume of 1 ml as follows: 0.1 M Tris-HCl (pH 8.0), 0.002 M EDTA, 0.001 M dithiothreitol, 0.5 mg/ml of bovine serum albumin, 2×10^{-5} M *S*-adenosyl[^{14}C]methionine (10 mCi/mole), 0.5 mg of DNA, and 0.4 unit of T4 DNA methylase. After 2 hr at 30°, the reaction was stopped by the addition of NaCl to 0.5 M and 2 volumes of ethanol at 0°. The DNA was subsequently repurified by phenol extraction and isopropyl alcohol fractionation. It had a specific activity of 130 cpm/μg of DNA. This DNA (20 μg) was hydrolyzed in 0.1 M HCl at 95° for 1 hr. As determined by paper chromatography, >95% of the radioactivity was present in the form of 6-methyladenine. PM2-[^{32}P]DNA (initially 40,000 cpm/μg of DNA) was prepared according to Masamune *et al.* (1971).

T_m Curves. Absorbance–temperature profiles of DNA solutions were determined in sealed cuvets in a thermostated spectrophotometer. Temperature readings (±0.1°) were obtained with a precalibrated thermistor immersed in one of the blank cuvets. All absorbance values were corrected for the thermal expansion of the solvent.

Heat Treatment. DNA solutions were heated at a concentration of 5 μg/ml in sealed 1-ml glass ampoules, containing 0.3 ml of solution, in the following buffers: (A) 0.1 M NaCl–0.01 M sodium phosphate–0.01 M sodium citrate (pH 4.5–7.4), (B) 0.1 M KCl–0.05 M Hepes[1]-KOH–0.01 M $MgCl_2$–0.001 M EDTA (pH 7.80) (at 25°), (C) 0.1 M KCl–0.05 M Hepes-KOH–0.001 M EDTA (pH 7.80) (at 25°), (D) 0.005 M sodium citrate (pH 5.0) (at 45°), and (E) 0.1 M Tris-HCl–0.01 M sodium phosphate–0.01 M sodium citrate (pH 5.0) (at 25°).

Ampoules containing DNA solutions were immersed in closed, thermostated water baths (Haake). All temperatures were controlled within 0.2°. At the end of a period of heat treatment, the ampoule was removed from the water bath, and opened at room temperature (21°).

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

Analysis of Depurination. To an ampoule containing 0.3 ml of heated DNA, 20 μl of 0.1% adenine in 0.01 M NaOH, 20 μl of 0.1% guanine in 0.1 M NaOH, 10 μl of 0.2% dAMP in 0.02 M Tris-HCl (pH 8.0), and 75 μl of 0.4% heat-denatured calf thymus DNA (Sigma) in buffer A (pH 7.0) were added. The ampoule was transferred to an ice bath, and the DNA was precipitated by one of two different methods. (a) NaCl (60 μl of 2 M) and cold ethanol (0.6 ml) were added, and the sample was left a 0° for 1 hr with intermittent agitation. The solution was transferred to a 3-ml centrifuge tube, and after centrifugation at 10,000*g* for 10 min, 600 μl of the supernatant was recovered. (b) Ice-cold 0.8 M $HClO_4$ (0.3 ml) was added. After exactly 5 min at 0°, the solution was transferred to a chilled centrifuge tube, and centrifuged at 10,000*g* for 10 min at 2°. The supernatant (300 μl) was immediately recovered. With simulated mixtures of DNA and [^{14}C]guanine or [^{14}C]adenine, >95% of the radioactive material was recovered in soluble form by either method after volume corrections. With nonheated [^{14}C]DNA, method a yielded <0.02% soluble radioactive material, and method b 0.28 ± 0.06%. The higher background value in the latter case was due to a small amount of depurination occurring during the acid precipitation step at 0°. The radioactivity of the supernatant solutions were determined by counting in 20 volumes of Aquasol (NEN Chemicals) in a Packard liquid scintillation spectrometer.

The composition of the supernatant solutions was further analyzed by column chromatography on Sephadex G-10 (Pharmacia). Void volumes were determined with Blue Dextran 2000 (Pharmacia). Supernatants obtained by procedure a were evaporated to dryness. The residue was dissolved in 0.5 ml of 0.5 M NaOH at 21°, adjusted to pH 10.5 by addition of 0.12 ml of 2 M glycine, and applied to a column (0.8 × 20 cm) equilibrated with 0.05 M NaCl–0.05 M glycine-NaOH (pH 10.5) at 21°. Supernatants obtained by procedure b were directly applied to columns equilibrated with 0.1 M HCl at 2°. Fractions (0.3 ml) were collected, and the chromatography experiment was completed within 3 hr. In either method, the A_{260} of each fraction was measured, and absorption peaks were identified as dAMP, guanine, or adenine by their spectral properties. The radioactivity of the fractions was determined by counting in Aquasol. When small amounts of radioactive material were analyzed, adjacent fractions were pooled before counting. Recoveries after chromatography in either solvent was >90%.

Paper Chromatography. Adenine and guanine were separated by chromatography in isopropyl alcohol–12 M HCl–water (68:17.6:14.3). 6-Methyladenine was analyzed by chromatography in isopropyl alcohol–water–28% aqueous ammonia (85:15:1.3).

pH Measurements. A Radiometer pH meter 26, equipped with a combined electrode, was used for all measurements. This instrument could be used up to 60°, and was calibrated with a set of commerical standard buffers with known pH at 25, 50, and 60°. The pH of the buffers used in the present work were then determined at all three temperatures. The pH of buffer A showed a small positive temperature coefficient (0.04–0.10 pH unit higher at 60° than at 25° in the pH 5–8 range). Buffers B and C had a negative temperature coefficient (0.28 pH unit lower at 60° than at 25°). Buffers B and C were therefore adjusted to pH 7.80 at 25°, in order to obtain pH 7.45 ± 0.1 at 70°. Buffer E had pH 5.00 at 25° and pH 5.05 at 60°.

Gradient Centrifugation. Centrifugations in linear 5–20% sucrose gradients were for 4 hr at 35,000 rpm and 20° in a

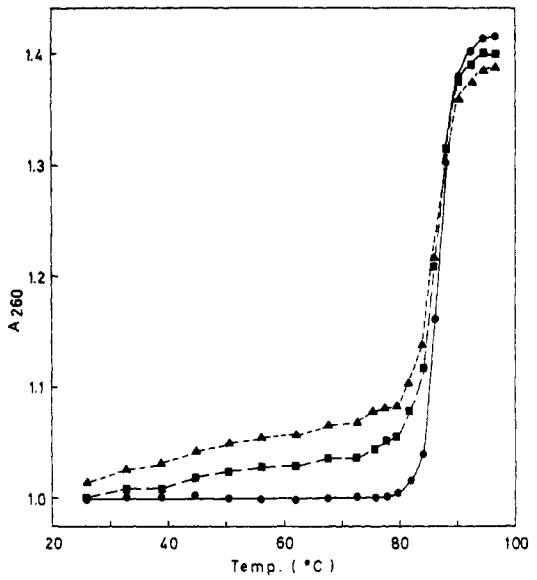

FIGURE 1: Absorbance-temperature profiles of [^{14}C]purine-labeled *B. subtilis* DNA in 0.1 M NaCl–0.01 M sodium phosphate–0.01 M sodium citrate (pH 7.4). (●) Previously unheated DNA; (■) DNA preheated at 80° for 48 hr; (▲) DNA preheated at 80° for 72 hr.

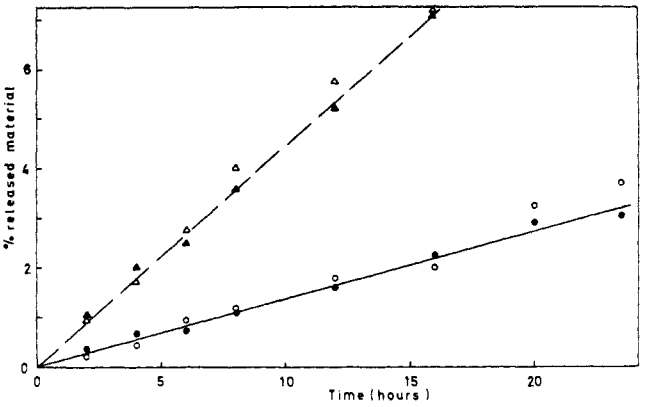

FIGURE 2: Rate of release of material of low molecular weight from *B. subtilis* [^{14}C]DNA in buffer A (pH 5.0) at 70°. Empty symbols: ethanol-soluble material. Filled symbols: acid-soluble material. Circles: native DNA. Triangles: denatured DNA.

SW41 rotor in the Spinco Model L3-50 ultracentrifuge. After completion of a run, fractions were collected from the bottom of the tube, and their radioactivity was determined by counting in Aquasol. PM2 [^{32}P]DNA was added as a marker in all experiments. Neutral sucrose gradients contained 2 M NaCl–0.01 M Tris-HCl–0.01 M EDTA (pH 9.0). Alkaline sucrose gradients contained 0.5 M NaCl–0.3 M NaOH–0.01 M EDTA.

Transformation. B. subtilis transformation tests were performed according to Anagnostopoulos and Spizizen (1961). The recipient stain 168-2 (*ind*$^-$, *leu*$^-$) was transformed to Ind$^+$ at a nonsaturating DNA concentration (0.05 μg/ml).

Material. [8-^{14}C]Adenine (52 mCi/mole), [8-^{14}C]guanine (52 mCi/mmole), and *S*-adenosyl[^{14}C]methionine (40 mCi/mmole) were obtained from NEN Chemicals. [^{32}P]H_3PO_4 (carrier free) was obtained from AB Atomenergi, Studsvik, Sweden. Hepes was purchased from Sigma.

T4 DNA methylase was purified from phage T4 infected *E. coli* B according to Falaschi and Kornberg (1965). Micrococcal nuclease was obtained from Worthington, lysozyme from Sigma, and Pronase from Calbiochem. Pronase was incubated as a 0.5% solution in 0.05 M Tris-HCl (pH 7.5) for 2 hr at 37° before use.

Results

Properties of [^{14}C]*DNA.* DNA was isolated from a *B. subtilis purB*$^-$ mutant grown in a medium containing [^{14}C]-adenine. From 200 ml of bacterial culture, 280 μg of DNA was obtained, with a specific activity of 45,000 cpm/μg. [^{14}C]DNA (3 μg) was incubated in 0.1 M HCl for 1 hr at 95° to release the purine residues. It was then found by paper chromatography that >98% of the radioactive material was in the form of free purines, with 54 ± 2% recovered as adenine and 46 ± 2% as guanine.

When the A_{260} of the *B. subtilis* [^{14}C]DNA was followed as a function of temperature, the total hyperchromicity was 41% (Figure 1). This value is typical of native DNA of the base composition of *B. subtilis* (43 ± 1% G-C base pairs) at moderate ionic strength (Mahler *et al.*, 1964). In the 25–80° temperature range, no hyperchromicity (<0.5%) was observed (Figure 1). As denatured DNA shows an increase in absorption on heating over this temperature interval, this result demonstrates that the [^{14}C]DNA did not contain a significant amount (<1%) of denatured material. The sedimentation coefficient of the [^{14}C]DNA was 34 S in neutral solution, and 38 S in alkaline solution. These data correspond to a double-strand molecular weight of 28 × 10^6, and a single-strand molecular weight of 14 × 10^6 (Freifelder, 1970; Studier, 1965). The [^{14}C]DNA showed transforming activity; 0.15% transformation was obtained at a DNA concentration of 0.05 μg/ml, and in this respect the preparation was indistinguishable from several preparations of nonradioactive *B. subtilis* DNA.

In order to directly measure the release of purines from double-stranded DNA in solution with reasonable accuracy, it is necessary to heat the DNA solutions for relatively long periods of time, particularly at pH values near neutrality. The effect of prolonged incubation below the T_m on the secondary structure of the *B. subtilis* [^{14}C]DNA was therefore investigated. After incubating the DNA for 48 *vs.* 72 hr in buffer A (pH 7.4) at 80° in sealed glass ampoules, followed by storage at 2° for 16 hr, and transfer of the solutions to sealed quartz cuvets, very little hyperchromicity could be detected, <0.5% *vs.* 1%. While this result indicated that the DNA retained its native structure under these conditions, the A_{260} heat denaturation profiles of the preheated DNA solutions were somewhat different from that of unheated DNA (Figure 1). Similar results were obtained with DNA heated for 5 days at 70° in buffer B. Several features of these T_m curves are noteworthy: (a) at 97°, where the secondary structure is fully denatured, the absorption of preheated DNA solutions was 1–2% lower than that of previously unheated DNA. This decrease in absorption at least partly depended on the loss of released guanine residues. Guanine, which is virtually insoluble in water at neutral pH and low temperature, deposited on the walls of the vial when the DNA solutions were chilled after heat treatment, and could be recovered by brief extraction with 0.2 M HCl. (b) The T_m of the DNA solutions showed little change after heat treatment. The previously unheated DNA had a T_m of 86.8° in buffer A, pH 7.4, while the T_m of DNA preheated for 2 days was 86.0°, and for 3 days 85.6°. These results demonstrate that most of the DNA retained a

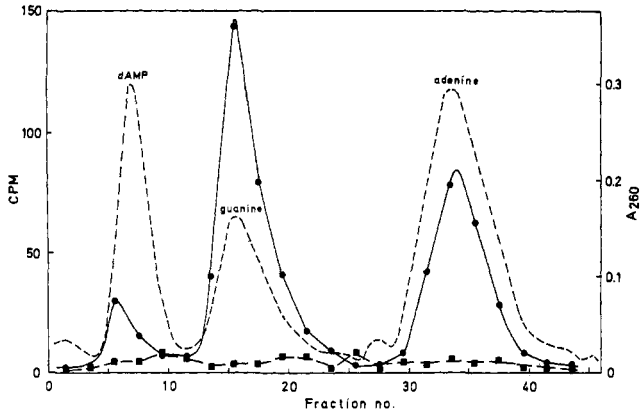

FIGURE 3: Chromatography on Sephadex G-10 at pH 10.5 of material released from *B. subtilis*[^{14}C] DNA by heating for 8 hr at 70° and pH 5.0 in buffer A. The A_{260} of each fraction was determined; the fractions were then pooled two and two, and their radioactivity determined. Two separate chromatography experiments were performed, and the radioactivity profile of the control experiment was superimposed on the other curve. (----) A_{260}; (■) unheated control DNA; (●) heated DNA.

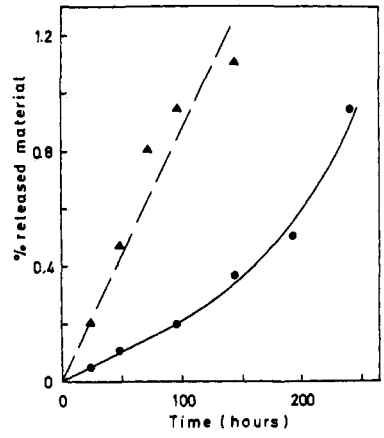

FIGURE 4: Rate of release of ethanol-soluble material from *B. subtilis* [^{14}C]DNA in 0.1 M KCl–0.05 M Hepes-KOH–0.01 M $MgCl_2$–0.001 M EDTA (pH 7.4) (buffer B), at 70°. (●) Native DNA; (▲) denatured DNA.

double-stranded structure after prolonged heat treatment at 80°. (c) The preheated DNA solutions showed an increasing absorption over the 25–80° interval, indicative of the presence of a small amount of single-stranded DNA in the DNA solutions. This is probably the consequence of chain breaks occurring at apurinic sites, followed by the release of the oligonucleotide sequences between closely located strand interruptions. From Figure 1, it may be estimated that by heating DNA for 2 days at 80° in buffer A (pH 7.4) the DNA is gradually converted to a form that can be represented as a mixture of 91% double-stranded and 9% single-stranded DNA at that temperature. To ensure that all subsequent experiments were performed at submelting temperatures, the T_m of *B. subtilis* DNA was also determined in the following buffers: in buffer A (pH 5.0), the T_m was 81.9°; in buffer A (pH 4.5), T_m = 77.0°; and in buffer D, T_m = 58.5°.

Release of Purines from DNA. The buffer employed for most experiments was buffer A (0.1 M NaCl–0.01 M sodium phosphate–0.01 M sodium citrate). This solvent has good buffering capacity over a wide pH range, its pH changes very little as a function of temperature, and it retains a similar ionic strength at different pH values (μ = 0.133 at pH 5.0; μ = 0.156 at pH 7.4).

In a typical experiment, [^{14}C]purine-labeled *B. subtilis* DNA was heated in buffer A at pH 5.0 and 70°. Under these conditions, radioactive material was found to be released from the DNA in an ethanol-soluble or acid-soluble form (Figure 2). The initial rate of release of such material was identical when ethanol or dilute cold perchloric acid was used to precipitate the DNA, and was independent of the DNA concentration (between 0.3 and 30 μg per ml). If the DNA was heat denatured prior to the incubation at pH 5.0, the rate of release of radioactive material was 3.3 times higher. The rate constant for the initial rate of relase of ethanol-soluble material from double-stranded DNA under these solvent conditions was $k = 3.8 \times 10^{-7}$ sec^{-1}.

The nature of the released material was investigated by separating oligonucleotides, mononucleotides, nucleosides, and free purines from each other by column chromatography on Sephadex G-10 (Sweetman and Nyhan, 1971). Purine bases are adsorbed to the dextran gel matrix, and are therefore eluted much later than nucleotides. Under the conditions used here, little separation of oligonucleotides, mononucleotides, free pyrimidines, and pyrimidine deoxynucleosides was obtained, while purine deoxynucleosides were eluted between the nucleotides and the free purines. Because of the poor solubility of guanine at neutral pH, the chromatography experiments were done either at pH 10.5 (at 21°) or at pH 1 (at 2°). At the former pH, adenine is eluted well separated from guanine. In contrast, at low pH the free purines are eluted together, but well separated from purine deoxyribonucleosides.

When [^{14}C]DNA was heated at pH 5.0 and 70° as described above, followed by precipitation of the DNA with ethanol (or acid) and chromatography of the released material at pH 10.5 (or pH 1), >90% of the radioactive material was found to consist of guanine and adenine (Figure 3). No detectable amount of purines was observed when unheated [^{14}C]DNA was processed in the same fashion. Guanine was released approximately 1.5 times faster than adenine from the DNA, as previously noted by others (Tamm *et al.*, 1952; Greer and Zamenhof, 1962). In contrast to these earlier observations, however, we have been unable to detect any selective release of either guanine or adenine during early stages of hydrolysis; the proportion of guanine to adenine remained constant when released material was analyzed after 2–20 hr of heat treatment.

Similar results on the release of purines from DNA were obtained over the entire pH range 4.5–7.4 in buffer A (see below). At pH 7.4, the rate of depurination was much slower than at pH 5.0, but most of the low molecular weight material released from DNA by heat was again accounted for as free purines.

In an attempt to mimic *in vivo* conditions, a Mg^{2+}-containing buffer was also employed. This solvent, buffer B (0.1 M KCl–0.05 M Hepes-KOH–0.01 M $MgCl_2$–0.001 M EDTA), showed a larger variation in pH as a function of temperature than buffer A, and was therefore only used at 70 and 80°, at pH 7.40 ± 0.15. The initial rate of release of material of low molecular weight from DNA in this Mg^{2+}-containing buffer was only 70% of that observed in buffer A (pH 7.4) or in buffer B without $MgCl_2$ (buffer C). This effect was probably due to the stabilizing effect of Mg^{2+} ions on the secondary

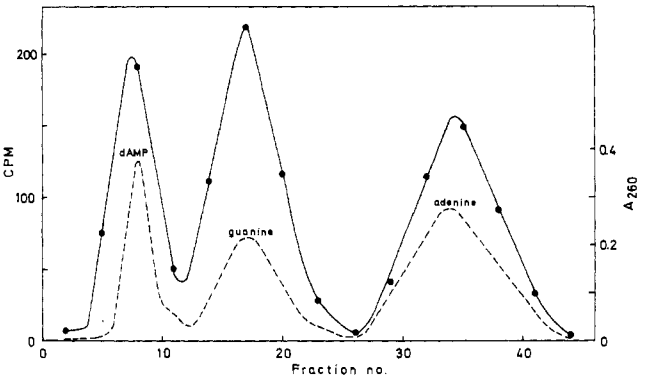

FIGURE 5: Chromatography on Sephadex G-10 at pH 10.5 of ethanol-soluble material released from *B. subtilis* [^{14}C]DNA by heating for 8 days in buffer B at 70°. (----) A_{260}; (●) radioactivity.

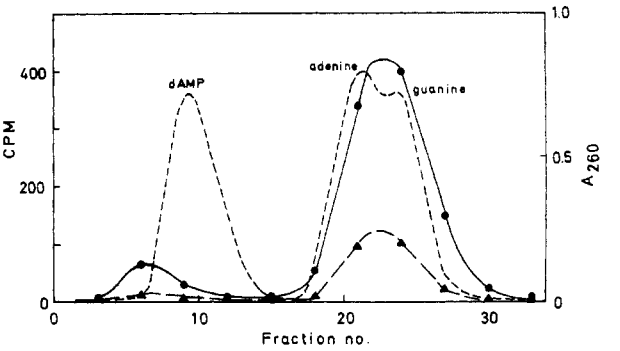

FIGURE 6: Chromatography on Sephadex G-10 at pH 1 of acid-soluble material released from *B. subtilis* [^{14}C]DNA by heating for 8 days in buffer B at 70°. (----) A_{260}; (●) radioactivity, heated DNA; (▲) radioactivity, unheated control DNA.

structure of the DNA, as it is known that Mg^{2+} ions interact with the phosphate residues in DNA, but not directly with the purine bases (Lyons and Kotin, 1965). The results obtained in buffer B at 70° are presented in Figures 4–6. During the first 4 days of heat treatment, radioactive material was released at a constant rate from double-stranded DNA (Figure 4), but at prolonged heating the rate increased, presumably due to partial loss of the secondary structure of the DNA (see Figure 1). The rate of release of ethanol-soluble material from denatured DNA was 4 times faster than from native DNA (Figure 4). At both 4- and 8-days incubation at 70°, ~70% of the released radioactive material was in the form of free purines, and ~30% in the form of oligonucleotides, as determined by chromatography experiments. The data in Figure 5 show a chromatography experiment on ethanol-soluble material released from DNA. Both guanine and adenine had obviously been released, in approximately the same proportions as in buffer A at pH 5.0. No detectable radioactivity was found in a soluble form when unheated [^{14}C]DNA was precipitated with ethanol under the same conditions. In Figure 6, acid-solubilized material is analyzed in a similar fashion, by chromatography at pH 1. The acid was more effective than ethanol for precipitating the oligonucleotides released during the heat treatment, so a larger proportion of the analyzed material consisted of free purines in this case. A small amount of depurination of the control DNA was also seen in this experiment, occurring as a consequence of the acid solvent conditions during the precipitation and chromatography of the material. However, much more radioactivity was found in the purine peak when heated DNA was chromatographed, and the difference between the two radioactivity curves in Figure 6 agrees well with the amount of purines detected at pH 10.5 (Figure 5). Further, the data in Figure 6 demonstrate that no deoxyribonucleosides were released, as they would have eluted between the dAMP peak and the purine peak (data not shown), *i.e.*, in a region where no radioactive material was found. From these results, it is estimated that the observed rate constant for depurination of double-stranded DNA in buffer B at 70° is 4.5×10^{-9} sec^{-1}.

A potential source of error in the data obtained at pH 7.4 would be a selective, relatively rapid depurination of the simultaneously released oligonucleotide material. To investigate this point, [^{14}C]DNA was degraded with micrococcal nuclease to 59% acid solubility. This enzymatically degraded DNA was subsequently heated at 80° for 48 hr in buffer A (pH 7.4). After precipitation with 0.4 M $HClO_4$, the acid-soluble material was chromatographed on Sephadex G-10 (Figure 7). The amount of purines released from this oligonucleotide material by heat treatment was obviously small (<2%), and apparently similar to that obtained with denatured high molecular weight DNA (0.9%) under the same conditions. This observation is in agreement with the finding of Greer and Zamenhof (1962) that depurination of nucleotide residues at DNA chain ends does not occur at a measurably higher rate than depurination of internal residues. As denatured DNA is depurinated at approximately four times the rate of native DNA at pH 7.4 (Figure 4), and the DNA contained at most 10% denatured material after 2 days at 80° or 5 days at 70° (Figure 1), the observed rates of depurination of native DNA at pH 7.4 could only be slightly too high (<20% overestimation). In conclusion, from the results above, the correct rate constant for the initial rate of depurination of native DNA in buffer B (pH 7.4) at 70°, is estimated to be $k = 4 \times 10^{-9}$ sec^{-1}.

Release of 6-Methyladenine. In several gram-negative bacteria, both 6-methyladenine and 5-methylcytosine are present to a minor extent in the DNA, while only the latter base has been found in the DNA of higher organisms (Dunn and Smith, 1958). Like other pyrimidines, 5-methylcytosine is not released from DNA by gentle acidic hydrolysis (Tamm *et al.*, 1952). To study the rate of release of 6-methyladenine from DNA at high temperatures, nonradioactive *B. subtilis* DNA was incubated with *S*-adenosyl[^{14}C]methionine and phage T4 induced DNA methylase. This enzyme exclusively methylates adenine residues in DNA (Haussmann and Gold, 1966). The radioactive DNA, freed from the enzyme by phenol extraction, contained 0.02% 6-methyladenine residues. It was incubated in buffer A (pH 5.0) at 70°, and the rate of release of radioactive material in an ethanol-soluble and acid-soluble form was measured. The observed rate constant was $k = 1.1 \times 10^{-6}$ sec^{-1}, and for denatured DNA under the same conditions $k = 2.6 \times 10^{-6}$ sec^{-1}. It thus appears that 6-methyladenine residues are lost from DNA two to three times more rapidly than the common purine bases. As ~3% of the adenine residues in many gram-negative bacteria, *e.g.*, *E. coli*, are methylated, the rate of depurination of such DNA would be slightly faster than that of DNA devoid of 6-methyladenine residues.

Temperature Dependence of Depurination. [^{14}C]Purine-labeled *B. subtilis* DNA was heated for various times in buffer A (pH 5.0) at 5° intervals between 45 and 80° to produce 0.3–3% depurination, and data similar to those in Figure 2

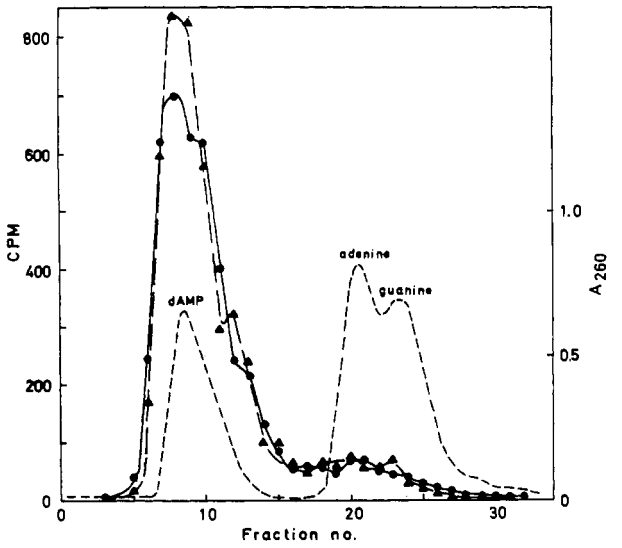

FIGURE 7: Chromatography on Sephadex G-10 at pH 1 of a micrococcal nuclease digest of *B. subtilis* [^{14}C]DNA before and after heating for 48 hr at 80° in buffer A (pH 7.4). (----) A_{260}; (▲) unheated DNA; (●) heated DNA.

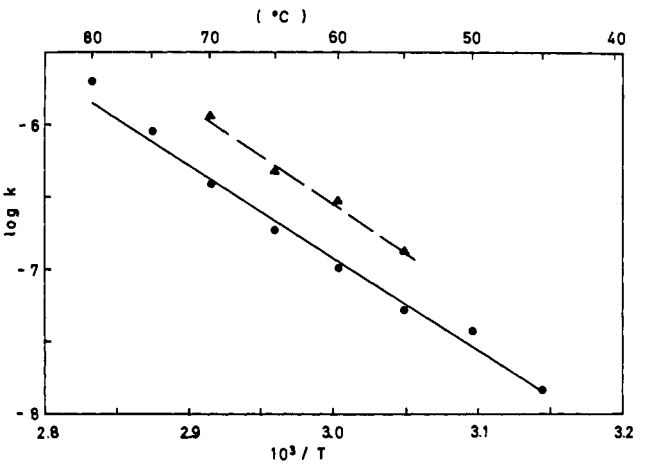

FIGURE 8: Arrhenius plot. Rate (sec^{-1}) of depurination of *B. subtilis* [^{14}C]DNA in buffer A (pH 5.0) as a function of temperature. (●) Native DNA; (▲) denatured DNA.

were obtained. The rate constant of depurination was determined at each temperature. The results are summarized in the form of an Arrhenius plot (Figure 8). From the slope of the line, the activation energy of depurination was directly obtained as 29 ± 2 kcal/mole. A more limited set of data for denatured DNA under the same solvent conditions also gave an activation energy of 29 ± 3 kcal/mole (Figure 8). Moreover, the activation energy for depurination of double-stranded DNA at pH 6.0 was found to be the same as at pH 5.0, as determined from kinetic experiments in buffer A (pH 6.0) at 60, 65, 70, and 75°.

Buffer A shows a very slight increase in pH with increasing temperature. Because of this effect, the activation energies observed here should be 5–10% too low, as the rate of depurination of DNA decreases with increasing pH. It is therefore estimated that the correct activation energy of depurination of double-stranded DNA in solution is 31 ± 2 kcal/mole.

PH Dependence of Depurination. Native [^{14}C]DNA was heated at 70° in buffer A at several different pH values, and the rate of depurination was determined at intervals of 0.5 pH unit as described above. The rate constants obtained are plotted as a function of pH in Figure 9. The rate of depurination decreases with increasing pH values in the pH range studied. From kinetic data on the hydrolysis of purine deoxyribonucleosides, it has been concluded that cleavage of the purine–glycosyl bond occurs by specific acid-catalyzed hydrolysis, involving preequilibrium protonation of the purine followed by rate-limiting bond cleavage (Zoltewicz *et al.*, 1970). The corresponding nucleotides are probably hydrolyzed in the same fashion (Venner, 1966). In this type of hydrolysis, the reaction rate should decrease one order of magnitude for each increase of one pH unit, and the present data in the pH 4.5–6.0 range are in good agreement with this prediction. Above pH 6.0, the pH dependence of the reaction decreases, indicating that in addition to acid-catalyzed hydrolysis, depurination by a pH-independent "water" reaction (Bender, 1971) also occurs to a significant extent. Thus, in comparison to the data obtained at pH 5.5, the rate observed at pH 7.4 was fivefold higher than expected for a reaction proceeding only by specific acid-catalyzed hydrolysis (Figure 9).

Salt Effects. When the [^{14}C]DNA was heated at 45° in buffer A (pH 5.0) and in buffer D, the rate of depurination was found to be sevenfold more rapid in the latter solvent. The rate of depurination of native DNA thus proceeds more rapidly in solvents of low ionic strength. Similar results have been obtained earlier by Greer and Zamenhof (1962).

The same rate of depurination (<20% rate difference) was observed for native DNA at 70° in buffer A (pH 5.0) and in buffer E, in which the NaCl of the solvent had been replaced by Tris-HCl. The depurination reaction thus was not detectably influenced by the presence of this amine. Strauss and Hill (1970) found that the rate of release of alkylated purine residues from DNA was the same in buffers with and without primary amino groups, and the present results are in agreement with their data.

Transformation. When native DNA is heated below the T_m in neutral solution, the transforming activity decreases slowly. In Figure 10, the rate of inactivation of nonradioactive *B. subtilis* DNA (mol wt 25 × 10^6) in buffer A (pH 7.4) has been determined at 80°. A pseudo-first-order type of curve is observed for the first order of magnitude of inactivation, but on further incubation the rate decreases progressively. Inactivation curves of similar shapes have been observed for transforming DNA exposed to ultraviolet or ionizing irradiation (Lerman and Tolmach, 1959; Scholes *et al.*, 1967).

The initial rate of inactivation of transforming DNA in buffer A (pH 7.4) was measured as a function of temperature at 5° intervals between 65 and 85°. An activation energy of 28 ± 2 kcal/mole was obtained for the reaction. In buffer A (pH 5.0) the rate of inactivation of transforming activity was found to be 32 times higher than at pH 7.4. However, the temperature dependence of the process is the same at pH 5.0 (Lindahl, 1971) as at pH 7.4. A markedly lower pH dependence, and a slightly lower temperature dependence was thus observed for the irreversible loss of transforming activity in comparison with depurination.

From the data on the rate of depurination and the rate of loss of transforming activity, it is clear that both at pH 5.0 and 7.4, several depurination events are necessary to cause one inactivating hit in a transforming DNA molecule. In buffer A, one inactivating hit (63% inactivation) was obtained in 9.5 hr at pH 7.4 and 80°, while ~20 purine bases had been lost per DNA molecule within the same time interval. At

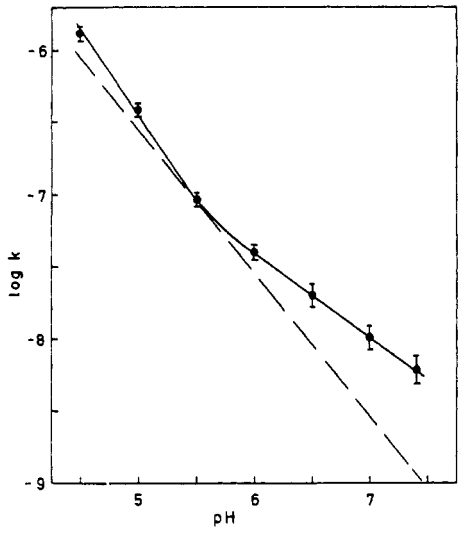

FIGURE 9: pH dependence of the rate of depurination of native *B. subtilis* [^{14}C]DNA in buffer A at 70°. The dashed line is the one expected for specific acid-catalyzed hydrolysis, using the rate constant obtained at pH 5.5 as a reference point.

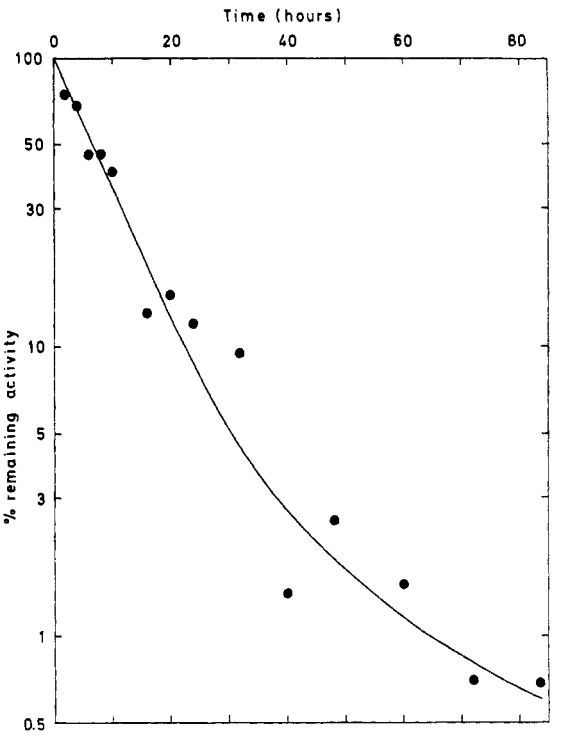

FIGURE 10: Rate of inactivation of transforming *B. subtilis* DNA in buffer A (pH 7.4) at 80°.

pH 5.0 and 70°, 50 ± 5 purine bases had been lost per transforming DNA molecule during the time period required for the introduction of one inactivating hit.

The rate of inactivation of transforming activity was found to change in a similar fashion as the rate of depurination under a variety of solvent conditions, *e.g.*, in buffer D, the rate of inactivation was ninefold faster than in buffer A (pH 5.0). These data show that the main cause of irreversible inactivation of transforming DNA depends on a reaction that proceeds more rapidly at low pH and low ionic strength. While the results are consistent with the hypothesis that this reaction is identical with depurination (Greer and Zamenhof, 1962), it seems likely that secondary events at apurinic sites are also important in this regard, and that alkali-catalyzed chain breakage at apurinic sites contributes significantly to the inactivation of transforming DNA (van Sluis and Stuy, 1962). The transforming activity is known to be very sensitive to the introduction of single-strand interruptions in the DNA (Bodmer, 1966). Moreover, the pH dependence of the inactivation of transforming DNA was found to be smaller than for depurination as such, and this difference may be rationalized by assuming that at least part of the inactivation is due to chain breakage.

Discussion

Small but detectable amounts of purine bases are continuously released at elevated temperatures from double-stranded DNA in buffers of physiological ionic strength at pH 7.4. This observation is in agreement with the previous data of Greer and Zamenhof (1962). At 70°, >15° below the T_m, the rate of release of purines from DNA was found to proceed at an initial rate of $k = 4 \times 10^{-9}$ sec^{-1} in a Mg^{2+}-containing buffer. At lower ionic strength, the rate of depurination is higher, and our rate estimates under such conditions are in general agreement with the data obtained for calf thymus DNA by Greer and Zamenhof (1962). This shows that the depurination studied here does not reflect some peculiar property of *B. subtilis* DNA, but presumably occurs in DNA from all sources. Moreover, the rate of depurination of phage PM 2 DNA at the 0.01% level, as measured by the introduction of alkali-labile sites in the circular DNA molecules, was the same as that found here for *B. subtilis* DNA at the 0.1–5% level (Lindahl and Andersson, 1972). These observations indicate that the depurination rate is not markedly dependent on the DNA sequence.

From the results obtained, it appears that as a consequence of the widely used denaturation procedure for DNA of heating at 100° for 10 min in 0.15 M NaCl–0.015 M sodium citrate (pH 7.0) approximately 1 apurinic site will be introduced per 10^4 base pairs. As previously pointed out by Alberts and Doty (1968), such unwanted degradation may be avoided by performing the heating step at a higher pH.

The temperature dependence of the rate of depurination of native DNA was associated with an activation energy of 31 ± 2 kcal/mole at both pH 6.0 and 5.0, and the same activation energy was also observed for denatured DNA at pH 5.0. The temperature dependence of the rate of irreversible inactivation of native transforming DNA, which probably mainly depends on the introduction of many apurinic sites in the DNA molecule, was found to be 28 ± 2 kcal/mole at both pH 7.4 and 5.0. These data are in fair agreement with similar estimates by others. Greer and Zamenhof (1962) determined the activation energy for the release of adenine from denatured calf thymus DNA in 0.005 M phosphate buffer (pH 6.8) to be 28 kcal/mole. Ginoza and Zimm (1961) found the irreversible loss of the residual transforming activity of *B. subtilis* DNA, heated above the T_m, to be associated with an activation energy of ~34 kcal/mole in 0.1 M phosphate buffer (pH 7.25). As it would be difficult to directly determine the very slow rate of depurination at 37° and pH 7.4 by the techniques employed in the present work, this rate constant has instead been estimated from the experimentally determined data for the rate of depurination at pH 7.4 and 70°, in a Mg^{2+}-containing buffer of physiological ionic strength,

and for the activation energy of the reaction. The value obtained in this fashion for depurination of native DNA at 37° and pH 7.4 is $k = 3 \times 10^{-11}$ sec^{-1}. As straight lines were obtained in the Arrhenius plots of data covering a relatively wide range of submelting temperatures, it appears implausible for thermodynamic reasons that a "threshold" temperature exists below which depurination of DNA would not occur.

The hydrolytic cleavage of the glycosidic bond of purine nucleotides in DNA should proceed at the same rate both *in vitro* and *in vivo*, unless DNA exists inside cells in some previously unrecognized, partly dehydrated state. This appears unlikely for several reasons. Wilkins and his associates have studied the conformation of the DNA in bacteriophage, spermatozoa, and cell nuclei, and found the highly hydrated B form to be present (Fuller *et al.*, 1965). In solutions containing high concentrations of neutral polymers, DNA is driven into a more compact tertiary structure due to excluded volume effects, and this structure may be similar to the *in vivo* conformation of DNA. However, the DNA is still fully hydrated in this compact form (Jordan *et al.*, 1972). While DNA can exist in solution in a partly dehydrated state without losing its double-helical structure under unusual solvent conditions, *e.g.*, in a four-component system comprised of DNA, salts, water, and high concentrations of ethylene glycol (Eliasson *et al.*, 1963), there is thus no evidence that similar solvent conditions would occur intracellularly. Different photoproducts are induced in DNA after irradiation with ultraviolet light in the fully hydrated and partly dehydrated states, and the photoproducts found in DNA after irradiation *in vivo* are those that are characteristic of fully hydrated DNA (Hieda, 1971). Further, the rate of depurination of alkylated DNA, obtained by treatment of either cells or purified DNA with monofunctional alkylating agents, is identical *in vivo* and *in vitro* at pH 7.3 (Lawley *et al.*, 1969).

It is concluded from the arguments above that depurination of DNA probably occurs to a significant extent under *in vivo* conditions. It is therefore of interest to estimate the amount of DNA depurination that would occur during one cell generation in different organisms. Several bacteria exist that grow actively at 70°, *e.g.*, *Bacillus stearothermophilus* and *Bacillus acidocaldarius* (Darland and Brock, 1971). If the size of the replicating chromosome of a thermophilic bacterium is taken as 5×10^6 base pairs, and the generation time at 70° is 40 min (Darland and Brock, 1971), it may be concluded that ~50 purine residues are lost from the bacterial chromosome in each generation. This estimate only involves the assumptions that DNA is fully hydrated *in vivo*, and that the Mg^{2+}-containing buffer employed for the experiments at 70° and pH 7.4 is representative of intracellular solvent conditions. To evaluate the rate of depurination of DNA at 37°, it is necessary to make use of the experimentally determined activation energy of the process. It may then be predicted that an *E. coli* cell, growing with a generation time of 40 min at 37°, should lose 0.5 purine/chromosome in each generation. For a mammalian cell, which contains 800 times more DNA than *E. coli* and grows with a generation time of 20 hr, 12,000 purines should be lost from the DNA in each cell generation due to hydrolysis. However, as at least 50% of the DNA is present as nucleohistone in this case (Clark and Felsenfeld, 1971) and therefore may be protected, it is possible that 2,000–10,000 depurination events per generation would be a better estimate. In the same fashion, a long-lived, nongrowing mammalian cell, *e.g.*, a human nerve cell would lose ~10^8 purines from its DNA during the lifetime of the individual, or ~3% of its total amount of purine residues in DNA. As a depurination event causes a local loss of genetic information, these estimates for the rate of depurination of DNA suggest that it would be of great advantage to cells to be able to repair apurinic sites in their DNA.

The rate of depurination of DNA *in vivo* is strongly increased after treatment of cells with alkylating agents. Such treatment results in the formation of purine derivatives in DNA with very labile glycosidic bonds, such as 3-methyladenine and 7-methylguanine, which are relatively rapidly released by hydrolysis (Brookes and Lawley, 1963). The lesions introduced by treatment with monofunctional alkylating agents can be repaired *in vivo* (Strauss *et al.*, 1968; Lawley *et al.*, 1969). Possibly repair of alkylation damage in DNA is an additional form of expression of cellular DNA repair mechanisms, which primarily have evolved as a safeguard toward spontaneous, heat-induced degradation of the DNA.

References

Alberts, B. M., and Doty, P. (1968), *J. Mol. Biol. 32*, 379.

Anagnostopoulos, C., and Spizizen, J. (1961), *J. Bacteriol. 81*, 741.

Bender, M. L. (1971), Mechanisms of Homogenous Catalysis from Protons to Proteins, New York, N. Y., Wiley-Interscience, p 38.

Bodmer, W. F. (1966), *J. Gen. Physiol. 49*, 233.

Brookes, P., and Lawley, P. D. (1963), *Biochem. J. 89*, 138.

Clark, R. J., and Felsenfeld, G. (1971), *Nature (London), New Biol. 229*, 101.

Darland, G., and Brock, T. D. (1971), *J. Gen. Microbiol. 67*, 17.

Doty, P., Marmur, J., Eigner, J., and Schildkraut, C. (1960), *Proc. Nat. Acad. Sci. U. S. 46*, 453.

Dunn, D. B., and Smith, J. D. (1958), *Biochim. Biophys. Acta 68*, 627.

Eliasson, R., Hammarsten, E., Lindahl, T., Björk, I., and Laurent, T. C. (1963), *Biochim. Biophys. Acta 68*, 234.

Falaschi, A., and Kornberg, A. (1966), *Proc. Nat. Acad. Sci. U. S. 54*, 1713.

Freese, E., and Cashel, M. (1964), *Biochim. Biophys. Acta 91*, 67.

Freifelder, D. (1970), *J. Mol. Biol. 54*, 567.

Fuller, W., Wilkins, M. H. F., Wilson, H. R., and Hamilton, L. D. (1965), *J. Mol. Biol. 12*, 60.

Ginoza, W., and Zimm, B. (1961), *Proc. Nat. Acad. Sci. U. S. 47*, 639.

Greer, S., and Zamenhof, S. (1962), *J. Mol. Biol. 4*, 123.

Haussmann, R., and Gold, M. (1966), *J. Biol. Chem. 241*, 1985.

Hieda, K. (1971), *Mutation Res. 12*, 365.

Jordan, C. F., Lerman, L. S., and Venable, Jr., J. H. (1972), *Nature (London), New Biol. 236*, 67.

Lawley, P. D., Lethbridge, J. H., Edwards, P. A., and Shooter, K. V. (1969), *J. Mol. Biol. 39*, 181.

Lerman, L., and Tolmach, L. (1959), *Biochim. Biophys. Acta 33*, 371.

Lindahl, T. (1971), *in* Molecular and Cellular Repair Processes, Beers, Jr., R. F., and Herriott, R. M., Ed., Baltimore, Md., Johns Hopkins University Press, p 4.

Lindahl, T., and Andersson, A. (1972), *Biochemistry 11*, 3618.

Lyons, J. W., and Kotin, L. (1965), *J. Amer. Chem. Soc. 87*, 1781.

Mahler, H. R., Kline, B., and Mehrotra, B. D. (1964), *J. Mol. Biol. 9*, 801.

Marmur, J. (1961), *J. Mol. Biol. 3*, 208.

Masamune, Y., Fleischman, R. A., and Richardson, C. C. (1971), *J. Biol. Chem. 246*, 2680.
O'Sullivan, A., and Sueoka, N. (1967), *J. Mol. Biol. 27*, 349.
Printz, M., and von Hippel, P. (1965), *Proc. Nat. Acad. Sci. U. S. 53*, 362.
Roger, M., and Hotchkiss, R. (1961), *Proc. Nat. Acad. Sci. U. S. 47*, 653.
Scholes, C. P., Hutchinson, F., and Hales, H. B. (1967), *J. Mol. Biol. 24*, 471.
Shapiro, R., and Danzig, M. (1972), *Biochemistry 11*, 23.
Shapiro, R., and Klein, R. S. (1966), *Biochemistry 5*, 2358.
Strack, H. B., Freese, E. B., and Freese, E. (1964), *Mutation Res 1*, 10.
Strauss, B. S., Coyle, M., and Robbins, M. (1968), *Cold Spring Harbor Symp. Quant. Biol. 33*, 277.
Strauss, B. S., and Hill, T. (1970), *Biochim. Biophys. Acta 213*, 14.
Studier, F. W. (1965), *J. Mol. Biol. 11*, 373.
Sweetman, L., and Nyhan, W. L. (1971), *J. Chromatogr. 59*, 349.
Tamm, C., Hodes, E., and Chargaff, E. (1952), *J. Biol. Chem. 195*, 49.
Tamm, C., Shapiro, H., Lipshitz, R., and Chargaff, E. (1953), *J. Biol. Chem. 203*, 673.
van Sluis, C. A., and Stuy, J. H. (1962), *Biochem. Biophys. Res. Commun. 7*, 213.
Venner, H. (1966), *Hoppe-Seyler's Z. Physiol. Chem. 344*, 189.
Zoltewicz, J. A., Clark, D. F., Sharpless, T. W., and Grahe, G. (1970), *J. Amer. Chem. Soc. 92*, 1741.

Rate of Chain Breakage at Apurinic Sites in Double-Stranded Deoxyribonucleic Acid†

Tomas Lindahl* and Annika Andersson

ABSTRACT: Several apurinic sites were introduced in the covalently circular, double-stranded DNA from phage PM2 by heating the DNA below the T_m at pH 5. The rate of chain cleavage at apurinic sites in such circular molecules, at pH 7.4 and physiological ionic strength, was subsequently measured by sedimentation velocity experiments. The presence of Mg^{2+} ions and/or amines increases the rate of chain breakage. Of several small molecules tested, putrescine (1,4-diaminobutane) was the most effective in this regard. In a Mg^{2+}-containing buffer, the average lifetime of the DNA chain at an apurinic site is 190 hr at 37°. The chain breaks are predominantly introduced at the 3′ side of the apurinic sugar moiety between this sugar and the phosphate residue. The DNA chain was more alkali resistant than expected at the apurinic sites; quantitative chain cleavage at such sites was only obtained after incubation for several hours at pH 12.8 and 25°.

Purine bases are released from DNA in acidic solutions (Feulgen, 1918; Tamm *et al.*, 1952a). Depurination of DNA also occurs at a very slow but physiologically significant rate at neutral pH (Greer and Zamenhof, 1962; Lindahl and Nyberg, 1972). Exposure of DNA *in vivo* or *in vitro* to alkylating agents or to nitrous acid leads to the formation of purine nucleotide derivatives with a labile purine–sugar glycosidic linkage, such as the nucleotide derivatives of 3-methyladenine, 7-methylguanine, and xanthine, and thus to great increases in the rate of DNA depurination (Lawley and Brookes, 1963; Burnotte and Verly, 1971).

The DNA chain is susceptible to alkaline hydrolysis at apurinic sites, and chain breakage at neutral pH occurs preferentially at such sites (Tamm *et al.*, 1953; Adamiec and Shugar, 1959). While such chain cleavage is still a slow reaction in neutral phosphate buffer at 37° (Laurence, 1963; Lawley *et al.*, 1969), the reaction is more rapid in the presence of Mg^{2+} ions (Tamm *et al.*, 1952b), primary amines (Tamm *et al.*, 1953; Strauss and Hill, 1970), high concentrations of basic proteins (McDonald and Kaufmann, 1954), or several aldehyde reagents (Livingston, 1964).

In the present work, small numbers of apurinic sites were introduced without accompanying chain breakage in circular DNA from phage PM2. Because of the unusual hydrodynamic properties of double-stranded, covalently closed circular DNA molecules (Vinograd *et al.*, 1965), it was possible to measure by sedimentation analysis the rate of introduction of a single-strand interruption in such a molecule under different solvent conditions. The results indicate that spontaneous chain cleavage at an apurinic site in double-stranded DNA would not occur in growing cells for several cell generations.

Materials and Methods

DNA. Phage PM2 and its *Pseudomonas* host were kindly provided by Dr. R. Espejo. PM2 [^{32}P]DNA preparations were made according to Masamune *et al.* (1971). The specific activity of this DNA was initially 40,000 cpm/μg. *Escherichia coli* DNA (mol wt 25 × 10^6) was prepared according to Marmur (1961).

Enzymes. Polynucleotide kinase was prepared from phage T4 infected *E. coli* according to Richardson (1965). A gel

† From the Department of Chemistry II, Karolinska Institutet, Stockholm, Sweden. *Received April 25, 1972.* This work was supported by grants from the Swedish Natural Science Research Council, the Swedish Cancer Society (Project No. 362), and the Karolinska Institutet.