# Effect of $Mn^{2+}$ on the in Vitro Activity of Human Deoxyribonucleic Acid Polymerase $\beta$†

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ABSTRACT: Optimum reactivity of human DNA polymerase $\beta$ with DNA typically requires the presence of $Mg^{2+}$ and high concentrations of activated DNA primer-template (>300 $\mu$M in nucleotide) and deoxyribonucleoside triphosphates (100 $\mu$M in each). In studies with defined linear and circular duplex DNA primer-template molecules, with which it is impractical to reach the concentration of 3′-hydroxyl primer termini that is achieved in the routine incubation, the enzyme is essentially inactive under standard assay conditions. In an effort to circumvent this difficulty we have observed that substitution of the divalent cation $Mn^{2+}$ for $Mg^{2+}$ has a profound effect on the kinetic parameters of the polymerase reaction. At activated DNA concentrations >100 $\mu$M in nucleotide, $Mg^{2+}$ is the preferred cation, but at DNA concentrations <100 $\mu$M, $Mn^{2+}$ is preferred. With defined primer-template molecules at low concentrations DNA synthesis can be detected only in the presence of $Mn^{2+}$. The apparent $K_m$ for activated DNA with $Mn^{2+}$ is <10 $\mu$M, while in reactions with $Mg^{2+}$, the apparent $K_m$ is ~300 $\mu$M. The effects of these divalent cations on the $K_m$ values for deoxynucleoside triphosphate substrates are qualitatively similar. Thus, under appropriate reaction conditions with DNA, $Mn^{2+}$ may be as effective as $Mg^{2+}$, strongly preferred or absolutely required for the demonstration of polymerase $\beta$ activity. During the course of these studies we have developed a new method for the purification of DNA polymerase $\beta$ from normal adult human liver. Detailed examination of the physical and enzymatic properties of the human hepatic enzyme revealed no significant differences from those we have previously described for the near homogeneous polymerase isolated from transformed human KB cells.

The standard in vitro assay of DNA polymerase activity requires the presence of a divalent cation and an appropriate primer-template. The former requirement can generally be satisfied only by $Mg^{2+}$ or $Mn^{2+}$, while the latter is most often accommodated by a suitably treated DNA preparation or a variety of synthetic oligomer-initiated homopolymer systems (Bollum, 1974; Loeb, 1974; Kornberg and Kornberg, 1974; Weissbach, 1975). Such assay conditions, although convenient, are of inherently limited interpretability and are unsuited to studies concerned with the reconstruction of in vitro DNA replication systems in which the use of defined DNA primer-template molecules is essential (Schekman et al., 1974).

We have previously described the structure and some of the enzymatic properties of near homogeneous DNA polymerase $\beta$ obtained from cultured human KB cells (Sedwick et al., 1972; Wang et al., 1974, 1975). To obtain additional information about the catalytic capabilities of this enzyme, and to define a system suitable for the detection and assay of candidate accessory protein factors, we have attempted to extend these studies to the use of defined DNA primer-template molecules. In our initial investigation of exonuclease III gapped Col E1 DNA and the cohesive ends of bacteriophage $\phi$80 DNA, we were unable to detect reactivity of polymerase $\beta$ under standard assay conditions with $Mg^{2+}$ (Sedwick et al., 1972), but we observed that the enzyme was highly active with these molecules in the presence of $Mn^{2+}$. This observation prompted a more detailed analysis of this phenomenon which revealed that the replacement of $Mg^{2+}$ by $Mn^{2+}$ in the standard assay has a profound effect on the kinetic parameters of the polymerase $\beta$ reaction. Of particular importance is the demonstration that, under certain incubation conditions, specifically, with low concentrations of defined DNA primer-templates containing relatively few primer sites per molecule, $Mn^{2+}$ is the obligatory metal activator for this enzyme.

## Materials and Methods

Unlabeled deoxyribonucleotides were obtained from P-L Biochemicals, [$^3$H]deoxyribonucleotides from New England Nuclear, and [$\alpha$-$^{32}$P]dCTP (200 Ci/nmol) from Amersham/Searle. [$\gamma$-$^{32}$P]ATP was prepared according to Glynn and Chappell (1964) and was a gift from Dr. A. Gillum, Stanford. Polyethylenimine-impregnated cellulose thin-layer plates (polygram Cel 300 PEI) were purchased from Brinkmann Instruments; Whatman Chromedia DE-81, GF/C, DEAE-cellulose[1] (DE-52), and phosphocellulose (P11) were from Whatman; Sephacryl S-200 was from Pharmacia. DNA-cellulose was prepared from heat-denatured calf thymus DNA and Cellex-N-1 (Bio-Rad) according to Alberts and Herrick (1971). Salmon sperm DNA was obtained from Calbiochem. Ampholytes (BioLyte pH 3-10 and pH 8-10) were from Bio-Rad. Poly(dA) and $d(pT)_{\bar{4}}$ were from Collaborative Research. Oligonucleotides $(dT)_{\overline{16}}$, $(dT)_{\overline{59}}$, [$^3$H]$(dC)_{\overline{0.7}}$ (14 170 cpm/pmol terminal dCMP residue), $(dT)_{\overline{200}}$, and $(dT)_{\overline{200}}$-[$^3$H]$(dT)_{\bar{4}}$ (16 800 cpm/pmol terminal dTMP residue) were prepared as before (Wang et al., 1974) with terminal transferase, using $d(pT)_{\bar{4}}$ as initiator. The average chain lengths of the oligonucleotide products were estimated as described by Richardson (1966). [5′-$^{32}$P]$d(pT)_1$-$d(pT)_{\overline{200}}$ (27 600 cpm/pmol) was synthesized according to Lehman and Chien (1973). Homopolymer primer-templates were prepared as described (Wang et al., 1974). Micrococcal nuclease, spleen phosphodiesterase (SPH), bacterial alkaline phosphatase (BAPF), pancreatic DNase I (DPFF), and snake venom

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[1] Abbreviations used: Tris, tris(hydroxymethyl)aminomethane; PEI, polyethylenimine; EDTA, ethylenediaminetetraacetic acid; DEAE, diethylaminoethyl; $P_i$, inorganic phosphate.

phosphodiesterase (VPF) were purchased from Worthington. Specifically gapped (Uyemura and Lehman, 1976) Col E1 DNA and T4 polynucleotide kinase were gifts from Dr. I. R. Lehman (Stanford); $\phi$80 DNA was from Dr. R. Bambara (Stanford); PM2 [$^3$H]DNA (1800 cpm/nmol) was from Dr. D. A. Clayton, (Stanford); terminal deoxynucleotidyltransferase, purified from calf thymus, was from Dr. R. L. Ratliff (Los Alamos Scientific Laboratory); and T4 DNA polymerase, P11 fraction (Nossal, 1974), was from Dr. N. G. Nossal, (National Institutes of Health).

*Buffers* were as follows. Triton buffer: 0.3% Triton X-100; 1 mM $\beta$-mercaptoethanol; 2 mM $MgCl_2$; 1 mM $KPO_4$, pH 7.5. Sucrose buffer: 0.2 M sucrose; 2 mM $MgCl_2$; 1 mM $\beta$-mercaptoethanol. Extraction buffer: 0.2 M $KPO_4$, pH 8.5; 1 mM EDTA, 1 mM $\beta$-mercaptoethanol. Buffer A: $KPO_4$, pH 7.5; 1 mM EDTA; 1 mM $\beta$-mercaptoethanol. Buffer B: $KPO_4$, pH 8.5; 20% glycerol; 1 mM EDTA; 1 mM $\beta$-mercaptoethanol. Buffer C: $KPO_4$, pH 7.2; 20% glycerol; 1 mM EDTA; 1 mM $\beta$-mercaptoethanol. Buffer D: 10 mM $KPO_4$, pH 7.2; 0.2 M NaCl; 1 mM EDTA; 1 mM $\beta$-mercaptoethanol. Buffer E: 20 mM Tris-HCl, pH 7.0; 50% glycerol; 1 mM EDTA; 1 mM $\beta$-mercaptoethanol. Buffer F: 0.3 M sucrose; 4 mM $CaCl_2$; 2.5 mM Tris-HCl, pH 7.5. Buffer G: 2 M sucrose; 1 mM $CaCl_2$.

*Growth of KB Cells.* KB cells were grown, harvested, and fractionated for enzyme preparation as described (Fisher and Korn, 1977).

*Preparation of Nuclei from KB Cells.* The crude nuclear pellet was washed twice by suspension with a loose fitting Dounce homogenizer in Triton buffer (5 mL of buffer per g wet weight of original cell pellet) and collected by centrifugation for 5 min at 1200$g$. The resulting nuclear pellet was washed twice more with sucrose buffer (same buffer volume to pellet weight as above) by two strokes with a loose fitting Dounce homogenizer, again collected by sedimentation, and stored at −70 °C.

*Preparation of Nuclei from Human Liver.* Intact human livers were removed under sterile precautions in the operating theater from cardiac transplant donors, usually within 60 to 90 min of cardiectomy. The following procedures were performed at 0 to 4 °C. The liver was perfused via the portal vein with ~6 L of 4 mM $CaCl_2$, 7% glycerol, trimmed of connective tissue and capsule, and cut into ~0.5-in. cubes. The tissue was passed through a meat grinder, suspended in 1.5 L of buffer F, and then macerated with a "food mill" to remove remaining fragments of connective tissue. The resulting tissue paste was suspended in buffer F in a final volume of 4 L and passed twice through a fine mesh stainless steel strainer. The fragments retained by the strainer were rinsed with an additional 500 mL of buffer F. The final tissue suspension (~3600 mL) was centrifuged in a Sorvall GSA rotor for 10 min at 4000 rpm. The sediment was taken up in 1 L of buffer F, suspended (in aliquots) by three strokes in a Dounce homogenizer fitted with the B pestle, diluted to 2.5 L with buffer F, and again centrifuged as above. The pellets were suspended in 1.2 L of buffer F with the Dounce homogenizer, an equal volume of buffer G was added, and the suspension was centrifuged in the GSA rotor for 30 min at 10 000 rpm. The pellets were suspended in 2.5 L of buffer G, homogenized in a Waring blender for 10 s at low speed and again centrifuged as above. The resulting pellet contains nuclei, and the supernatant contains a coagulum of connective tissue, debris, and trapped nuclei. Therefore, the supernatant was again treated in the Waring blender and resedimented. The two pellets were combined and taken up in 1.2 L of buffer G, dispersed with the blender, and centrifuged for 16 h at 8000 rpm. The pellet was washed twice with 200 mL of Triton buffer, as described above for KB cell nuclei, and stored at −70 °C. The final preparation generally yielded ~8 × $10^{10}$ nuclei from a 2-kg liver, and based on DNA determinations, this represented a recovery of ~30%. The purified nuclei were free of significant contamination as revealed by examination of stained thin sections with the electron microscope.

*Purification of DNA Polymerase $\beta$.* Initial efforts to apply our previously published procedure (Sedwick et al., 1972; Wang et al., 1974) to the purification of the hepatic enzyme were unsuccessful because polymerase $\beta$ was found to chromatograph anomalously on DEAE-cellulose. Specifically, a substantial fraction of this enzyme activity (≥50%) adsorbed to the resin and eluted coincidentally with DNA polymerase $\alpha$ in a 0.3 M $KPO_4$ (pH 8.2) step.[2] This splitting of DNA polymerase $\beta$ activity was disadvantageous to its further purification, and thus we developed a new protocol that is modified from those earlier described by ourselves (Wang et al., 1974) and Chang (1973a).

*Preparation of Nuclear Crude Extract.* Purified KB or hepatic nuclei were extracted with extraction buffer (70 mL per $10^9$ nuclei) for 2 h at 4 °C, and the suspension was centrifuged at 10 000$g$ for 15 min. The residue was extracted two more times and all of the supernatants were combined (nuclear crude extract, fraction I).

*First Phosphocellulose Column Chromatography.* The nuclear crude extract from 1.3 × $10^{10}$ nuclei was diluted in 0.1 M buffer A and loaded onto a column (5 × 15 cm) of P11 equilibrated in the same buffer. The column was washed with this buffer until all unadsorbed protein and nuclei acids were removed, and polymerase activity was then eluted in a single step with 0.4 M buffer A. Active fractions were pooled and dialyzed into 0.025 M buffer B (fraction II).

*DEAE-Cellulose Column Chromatography.* The dialyzed enzyme fraction was applied to a DEAE column (2.5 × 21 cm) in 0.025 M buffer B, and the column was washed with 0.25 M buffer B. The polymerase activity that did not adsorb to the resin was collected and designated as DNA polymerase $\beta$ (fraction III).

*Second Phosphocellulose Column Chromatography.* Fraction III was layered directly onto a 7-mL P11 column equilibrated with 0.1 M buffer C. The column was washed first with 20 mL of 0.1 M buffer C, then with 30 mL of 0.15 M buffer C, and was then eluted with a single step in 0.4 M buffer C. The active fractions were pooled (fraction IV).

*Sephacryl S-200 Gel Filtration.* Fraction IV was applied immediately to a column (1.5 × 66 cm; $V_0$ = 44 mL) of Sephacryl S-200 equilibrated in buffer D containing 10% glycerol. Fractions, 1 mL, were collected at a flow rate of 9.5 mL/h. DNA polymerase $\beta$ was eluted ($V_e$ = 60 mL) at the position of the ovalbumin marker (fraction V).

*DNA-Cellulose Column Chromatography.* Fraction V was loaded directly onto a column (1 × 1.5 cm) of DNA-cellulose equilibrated in buffer D containing 20% glycerol. The column was washed with 10 mL of this buffer and was developed with a 30-mL linear gradient of 0.2 M to 0.75 M NaCl in the same buffer. DNA polymerase $\beta$ activity was eluted between 0.35 M and 0.45 M NaCl. In contradiction to an earlier report with the calf thymus polymerase (Chang, 1973a), but in agreement with the finding of Stalker et al. (1976) with the Novikoff

[2] In our experience with these histologically normal, young adult human livers, we have repeatedly observed that ~50% of the polymerase activity in the purified nuclei, and ≥70% of the activity in whole liver homogenates, appears to be polymerase $\alpha$. This result is at odds with reports that the normal liver (rat) lacks significant polymerase $\alpha$ (Baril et al., 1973; Lynch et al., 1975, 1976).

hepatoma enzyme, we found the DNA-cellulose eluate to be extremely unstable (loss of ~50% of activity/h), and thus this fraction was collected directly into 100 $\mu$g/mL of bovine serum albumin. Active fractions were pooled, dialyzed, and concentrated in buffer E (fraction VI). The yield of DNA polymerase $\beta$ activity from both KB and liver nuclei was typically from 15 to 20% taking the DEAE activity (fraction III, where $\alpha$ and $\beta$ are first separated) as 100%. The presence of albumin prevented us from obtaining an estimate of enzyme protein in this fraction.

*Isoelectric Focusing.* Portions of fraction VI were introduced into 12 mL of a 5 to 50% glycerol gradient containing 2% BioLytes in the pH 3–10 range and 0.4% BioLytes in the pH 8–10 range. Isoelectric focusing was carried out for 5 days in a J tube as described (Wang et al., 1974), using either of two methods. To prepare enzyme for enzymological studies, method A was used, where the cathode solution was in 65% glycerol and the anode solution in 5% glycerol. For structural analysis, to avoid contamination of the enzyme fraction by the huge excess of albumin in the loaded sample, method B was used, where the densities of the anode and cathode solutions were reversed. Fractions of the gradient in the pH 9–9.5 range that contained polymerase activity were pooled and stored at − 2 °C for at least 6 months without loss of activity (fraction VII). Recovery of loaded activity by method A was generally 80–100%; by method B, ~50%. It is not possible to measure the protein content of this fraction directly because of the very low concentration of protein and the presence of interfering ampholytes and glycerol. From the results of Coomassie blue staining of denaturing polyacrylamide gel electropherograms (see below), we estimate the specific activity of fraction VII to be ~8000 units per mg of protein, a value similar to those previously reported by us for the KB enzyme (Wang et al., 1974) and by Chang (1974) for a polymerase $\beta$ fraction obtained from "human liver chromatin".

*Assay of DNA Polymerase $\beta$.* The standard assay with activated salmon sperm DNA has been described (Sedwick et al., 1972). For kinetic studies with activated DNA, the reaction mixture contained: Tris-HCl, pH 8.9, 50 mM; KCl, 100 mM; $\beta$-mercaptoethanol, 1 mM; a mixture of the four dNTPs, each at 100 $\mu$M, with [$^3$H]dTTP at 2.5 $\mu$Ci/nmol; $MnCl_2$, 5 mM, or $MgCl_2$, 20 mM; the appropriate amount of activated DNA; and enzyme. A zero-time sample was removed within 2 s after addition of enzyme to the incubation mixture at 0 °C. Reactions were performed at 37 °C and samples taken at various intervals into 200 $\mu$L of "stop solution" containing: 40 mM $NaPP_i$; 10 mM EDTA; 0.53 mM (nucleotide) denatured calf thymus DNA; 0.2 mg/mL of bovine serum albumin. Cold 15% trichloroacetic acid, 200 $\mu$L, was added, and the samples were maintained at 0 °C for 15 min. The acid precipitates were collected at 10 000*g* for 10 min, and the pellets were dissolved in 200 $\mu$L of 0.1 M $NaPP_i$, 0.2 M NaOH. Equal volumes of 15% trichloroacetic acid were added, and the precipitates were processed on GF/C filter discs (Sedwick et al., 1972).

For assays with synthetic primer-templates, the reaction mixture contained: Tris-HCl, pH 7.5, 50 mM; $\beta$-mercaptoethanol, 1 mM; KCl, 100 mM; $MgCl_2$, 5 mM or $MnCl_2$, 0.5 to 1 mM; poly(dA), 25 $\mu$M; oligo(dT), 2.5 $\mu$M; [$^3$H]dTTP, 100 $\mu$M (2.5 $\mu$Ci/nmol). For assays with poly(A)·oligo(dT), the incubation mixture was identical, except that it contained $MnCl_2$, 1.5 mM; poly(A), 100 $\mu$M; oligo(dT), 100 $\mu$M. Reactions were carried out at 35 °C. Samples were removed at various times and analyzed on DE-81 paper discs, as described (Wang et al., 1975). Radioactivity was measured by liquid scintillation spectrometry and quantitated as before (Wang et al., 1974). Reaction velocity is expressed as pmol of [$^3$H]-dTMP incorporated per min. One unit of polymerase activity is defined as the amount catalyzing the incorporation of 1 nmol of labeled dTMP in 60 min with activated DNA primer-template.

*Assay of Nuclease Activities. 3′→5′-Exonuclease.* Assays were performed as described (Wang et al., 1974), using $(dT)_{\overline{59}}$-[$^3$H](dC)$_{\overline{0.7}}$ or $(dT)_{\overline{200}}$-[$^3$H]$(dT)_{\overline{4}}$ as substrates.

*5′→3′-Exonuclease.* The assay measures the generation of acid-soluble [$^{32}$P]dTMP from [$^{32}$P]d$(pT)_1$-$(dT)_{\overline{200}}$ (plus or minus poly(dA)) and was carried out under optimal homopolymer conditions in the absence of dTTP but in the presence of 100 $\mu$g/mL bovine serum albumin and 0.1 mM $KPO_4$, pH 7.0. Incubation was at 37 °C for 10 min. T4 DNA polymerase and *E. coli* DNA polymerase I served as positive controls for the 3′→5′- and 5′→3′-exonuclease assays, respectively.

*Endonuclease.* The assay was performed with PM2 [$^3$H]-DNA under standard polymerization conditions, with and without dNTPs. The reaction was stopped by addition of EDTA to a final concentration of 10 mM, and the sample was brought to 2.5 mL in CsCl (final density 1.60 g/cm$^3$) and 0.1 mg/mL ethidium bromide. The preparations were centrifuged in an SW 56 rotor at 38 000 rpm for 24 h at 20 °C. Fractions (~50 $\mu$L) were collected and precipitated after the addition of 0.25 mL "stop solution" with 0.3 mL of 15% trichloracetic acid at 0 °C. The precipitates were then processed on GF/C filters as above.

*Assay of Fidelity of DNA Polymerase $\beta$.* Assays were performed by measuring the misincorporation of [$^{32}$P]dCMP with poly(dA)·$(dT)_{\overline{16}}$. The purity of the [$\alpha$-$^{32}$P]dCTP substrate was analyzed by two-dimensional thin-layer chromatography on PEI plates (Randerath and Randerath, 1967). Contamination by dATP was <0.2%; by dTTP, <0.3%. The incubations were performed under standard homopolymer assay conditions with either $Mn^{2+}$ or $Mg^{2+}$ as divalent cation. Three incubations were carried out in parallel. The first contained [$^3$H]-dTTP and served as control; the second contained [$^{32}$P]dCTP plus [$^3$H]dTTP; and the third contained [$^{32}$P]dCTP alone. At 0, 10, 30, and 60 min, 20-$\mu$L aliquots were removed and analyzed by the DE-81 paper disc procedure. To determine whether the trace levels of misincorporation that were observed in these experiments were due to contaminating dATP or dTTP in the [$^{32}$P]dCTP substrate, the 60-min reaction products were further analyzed. A portion (100 $\mu$L) of the incubation mixture was added to carrier T7 [$^3$H]DNA, precipitated with trichloroacetic acid, and collected by centrifugation. The pellets were washed twice with 100 $\mu$L of 90% ethanol (−20 °C) and then dissolved in 100 $\mu$L of a solution containing: sodium glycinate, pH 9.0, 50 mM; $CaCl_2$, 2 mM; $KPO_4$, pH 8.0, 1 mM; snake venom phosphodiesterase I, 100 $\mu$g/mL; pancreatic DNase I, 100 $\mu$g/mL. After 2 h at 37 °C the reaction was stopped by adding EDTA to 5 mM, boiled 2 min at 100 °C, and then chilled. The denatured enzyme proteins were removed by centrifugation, and the 5′-nucleotides in the supernatant were analyzed by chromatography on PEI plates (Wang et al., 1974).

The 60-min polymerization reaction product was also subjected to nearest neighbor analysis (Josse and Swartz, 1963), and the 3′-nucleotides were similarly resolved by PEI thin-layer chromatography.

*Other Methods.* Protein was determined by the method of Schaffner and Weissmann (1973) or, for the isoelectric focused fraction of polymerase $\beta$, by quantitation of Coomassie brilliant blue staining of denaturing polyacrylamide gels. Sodium dodecyl sulfate-polyacrylamide (12%) gels were formulated and run as described by Laemmli (1970). The stained gels were

scanned at 600 nm with a Transidyne RFT densitometer. The pH and ionic strength of buffers were measured at room temperature with a Corning digital pH meter and a Radiometer conductivity meter, respectively.

## Results

*Comparison of Properties of DNA Polymerase β from KB Cells and Human Liver.* The modified seven-step purification protocol that is described under Materials and Methods is equally applicable to KB cell and human hepatic nuclei, and it provides in the final fraction from either tissue an enzyme product in comparable yield and of comparable specific activity. The polymerase β activities from both sources behave identically at each purification step, and the fraction VII preparations are essentially indistinguishable in their structural and enzymatic properties. Thus, the polymerases have identical sedimentation values in low salt glycerol gradients (~3.5 S) and elution positions from Sephacryl S-200 gel columns, and they isoelectric focus similarly at a p*I* of ~9.2. The fraction VII polymerases appear to be 50–70% pure, as estimated from Coomassie blue staining of denaturing polyacrylamide gel electropherograms (Figures 1A and 1B). The hepatic fraction contains a low molecular weight contaminant that migrates slightly faster than the chymotrypsinogen marker, while the profile of the KB fraction indicates either some degree of electrophoretic heterogeneity of the polymerase itself or the presence of minor contaminating protein species of very similar size. In each instance, the mobility of the major protein band is the same within experimental error and is consistent with a size of 39 000 daltons.[3] Based on the size of the polymerase activity determined by gel filtration and velocity sedimentation, the specific activity of the fraction VII preparations, and the size of the polymerase β polypeptide previously documented for the KB cell (Wang et al., 1974) and calf thymus chromatin (Chang, 1973a) enzymes, we conclude that the major protein band is polymerase β. With respect to enzymatic properties, the two polymerases show identical primer-template preferences, pH optima, divalent cation optima, response to salt, and resistance to sulfhydryl-blocking reagents. Both preparations are similarly free of contaminating DNase activities.[4] By the highly sensitive assays described under Materials and Methods, 3′→5′-exonuclease activity was <0.4 pmol of 3′-terminal nucleotide released per unit of polymerase; 5′→3′-exonuclease activity was <6 fmol of 5′-terminal nucleotide released per unit of polymerase; and endonuclease activity was <0.1 fmol of phosphodiester bond (in the closed circular PM-2 DNA) nicked per unit of polymerase. On the basis of these analyses the two polymerase β preparations are indistinguishable, and, unless otherwise indicated, we have used both fraction VII enzymes interchangeably in the catalytic studies described below.

*Effect of Divalent Cation on Kinetic Parameters of the DNA Polymerase Reaction.* At the relatively high concentrations of activated DNA (>100 μM in nucleotide) that are typically used in the standard in vitro assay for this enzyme (Sedwick et al., 1972; Chang, 1973a; Stalker et al., 1976), $Mg^{2+}$ is the preferred cation (Figure 2). In contrast, at low DNA concentrations $Mn^{2+}$ is preferred, and at very low concentrations (≤10 μM), this preference is striking (Figure 2, insert). In the presence of $Mn^{2+}$ the apparent $K_m$ for polymerase β with activated DNA (Table I) is <10 μM, while with $Mg^{2+}$, this value is ~300 μM. Table I also shows that the two cations have comparable, although less dramatic, effects on

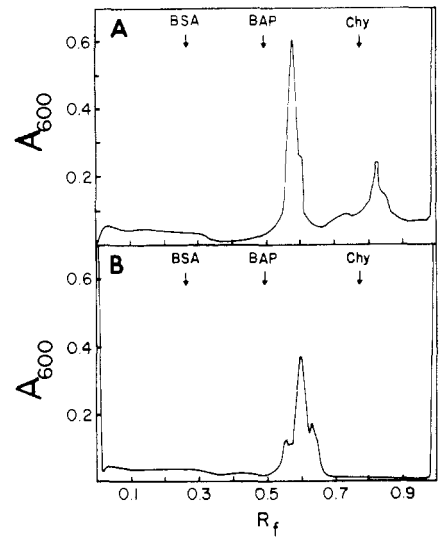


FIGURE 1: Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of human DNA polymerase β. Densitometric scan of Coomassie blue stained gels of hepatic fraction VII polymerase, 34 units (A), and of KB fraction VII polymerase, 26 units (B). Enzyme samples were dialyzed against 0.0625 M Tris-HCl (pH 6.8), 1 mM EDTA and Amberlite IRA-400, 5 mL/L of buffer, for 3 days to remove ampholytes, and then against 0.0625 M Tris-HCl (pH 8.6), 1 mM EDTA, 0.1% sodium dodecyl sulfate, 10% glycerol. The dialyzed samples were adjusted to 1% β-mercaptoethanol, heated for 5 min at 100 °C, and analyzed on 12% gels as described under Materials and Methods. The standard proteins run under identical conditions were bovine serum albumin (BSA, 67 000); bacterial alkaline phosphatase (BAP, 50 000); ovalbumin (ov, 43 000); pancreatic DNase (Panc DNase, 31 000); and chymotrypsinogen (Chy, 24 000).

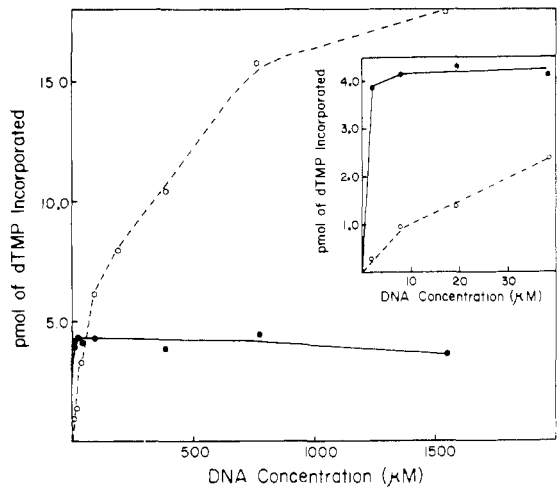


FIGURE 2: The effect of divalent cation on utilization of activated DNA by human polymerase β. Reaction mixtures (0.1 mL) contained: Tris-HCl, pH 8.9, 50 mM; KCl, 100 mM; β-mercaptoethanol, 1 mM; dATP, dGTP, dCTP, and [$^3$H]dTTP (752 cpm/pmol), 100 μM each; activated salmon sperm DNA at the indicated concentrations (as nucleotide); DNA polymerase β, 3 units/mL, and either $MgCl_2$, 20 mM, (O---O), or $MnCl_2$, 5 mM (●—●). Incubations were at 37 °C for 10 min. The insert shows the results obtained at very low DNA concentrations.

[3] This size value is smaller than that of 43 000–45 000 previously determined for the KB (Wang et al., 1974) and calf thymus chromatin (Chang, 1973a) polymerases. As in both of the earlier studies, polymerase β is found to migrate close to the ovalbumin marker in denaturing acrylamide gels. We suggest that these small differences in size are not significant and are due to the gel methodology and the behavior of the particular standard proteins that are used to generate calibration curves. The principal conclusion we wish to establish here is that the KB and hepatic enzymes behave similarly in our hands.

[4] In contrast to the results of Chang (1973a) with the calf thymus polymerase, we found that the DNA-cellulose enzymes (fraction VI) contained significant levels of contaminating 3′→5′-exonuclease activity which was removed completely by the final isoelectric focusing step.

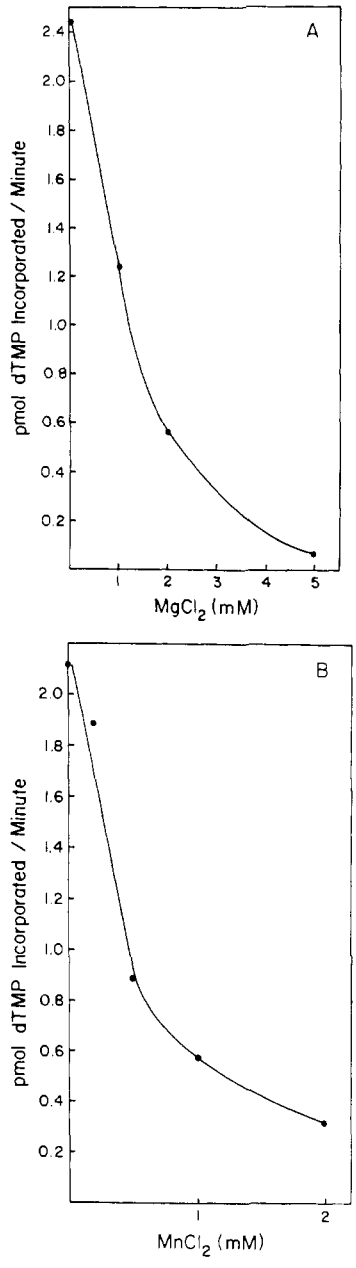


FIGURE 3: Inhibition of the polymerization reaction by one divalent cation when the other cation is present at optimal concentration. The reaction mixtures (0.1 mL) contained Tris-HCl, pH 8.9, 50 mM; $\beta$-mercaptoethanol, 1 mM; KCl, 100 mM; dATP, dGTP, dCTP, and [$^3$H]dTTP (230 cpm/pmol), 100 $\mu$M each; $MnCl_2$, 1 mM, plus activated salmon sperm DNA, 50 $\mu$M in A; or $MgCl_2$, 20 mM, plus activated DNA, 500 $\mu$M in B; and DNA polymerase $\beta$ (fraction VI), 3 units/mL. The concentration of the other divalent cation was varied as indicated. Incubations were at 37 °C for 20 min.

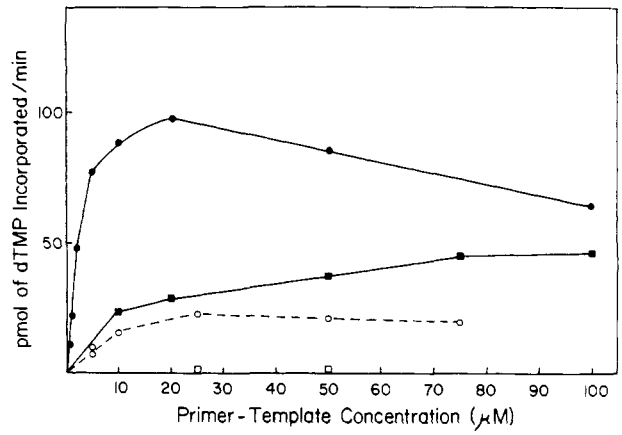


FIGURE 4: The effect of divalent cation on utilization of synthetic primer-template by human polymerase $\beta$. Reaction mixtures (0.1 mL) contained Tris-HCl, pH 7.5, 50 mM; KCl, 100 mM; $\beta$-mercaptoethanol, 1 mM; [$^3$H[dTTP, 100 $\mu$M (2.5 $\mu$Ci/nmol); DNA polymerase $\beta$, 3.7 units/mL; poly(dA)·oligo(dT)$_{\overline{16}}$ at the indicated concentrations (as nucleotide); and either $MnCl_2$, 0.5 mM (●—●), or $MgCl_2$, 5 mM (○----○). For oligodeoxynucleotide-initiated polyribopolymer assays, the incubation mixtures were identical with the above, except that 1.5 mM $MnCl_2$, 2.1 units/mL of DNA polymerase $\beta$, and poly(A)·oligo(dT)$_{\overline{16}}$ were used. Incubations were at 35 °C for 10 min. $MnCl_2$ (■—■); $MgCl_2$ (□----□).

TABLE I: Effect of Divalent Cation on Michaelis-Menten Constants.

| Primer-template | dNTP | Divalent cation | $K_m$ Primer-template[c] | $K_m$ dNTP[d] |
|---|---|---|---|---|
| "Activated" salmon sperm DNA | 4dNTP | $Mg^{2+}$ | 300 | 160 |
| | | $Mn^{2+}$ | <10 | 20 |
| $(dA)_n$·$(dT)_{\overline{16}}$ | dTTP[a] | $Mg^{2+}$ | 7 | (*a*) |
| | | $Mn^{2+}$ | 2 | 10 |
| $(A)_n$·$(dT)_{\overline{16}}$ | dTTP | $Mg^{2+}$ | *b* | *b* |
| | | $Mn^{2+}$ | 53 | 500 |

[a] In the presence of $Mg^{2+}$ and poly(dA)·oligo(dT)$_{\overline{16}}$, saturating levels of dTTP are not reached up to 500 $\mu$M of the triphosphate. [b] Human DNA polymerase $\beta$ can not copy poly(A)·oligo(dT)$_{\overline{16}}$ in the presence of $Mg^{2+}$. [c] $\mu$M of nucleotide. [d] $\mu$M of each.

the affinity of this enzyme for substrate deoxynucleoside triphosphates. The results of mixing experiments, in which one divalent cation was added to a polymerization reaction "optimized" for the other, are presented in Figure 3. Under either circumstance, i.e., whether the incubation is initially optimized for $Mg^{2+}$ or $Mn^{2+}$, the addition of increasing amounts of the second cation leads to profound inhibition of the reaction. Of note is the fact that in Figure 3A, 50% inhibition is effected by the introduction of 1 mM $Mg^{2+}$ to a reaction containing 1 mM $Mn^{2+}$, while in Figure 3B, comparable inhibition is achieved by the addition of 0.5 mM $Mn^{2+}$ to an incubation containing 20 mM $Mg^{2+}$.

It has been generally recognized (Bollum, 1974) that, in reactions of polymerase $\beta$ with synthetic homopolymer primer-templates, $Mn^{2+}$ is the preferred cation. As demonstrated in Figure 4, this preference is maintained over a wide range of concentration of the deoxyhomopolymer template, although the relative advantage of $Mn^{2+}$ is substantially decreased at very high primer-template concentrations. In the presence of $Mn^{2+}$, the apparent $K_m$ of the polymerase for the primer-template is about threefold lower than the value determined with $Mg^{2+}$ (Table I). We note without explanation that in reactions containing $(dA)_n$·$(dT)_{\overline{16}}$ a $K_m$ value for dTTP is readily measured in the presence of $Mn^{2+}$, while with $Mg^{2+}$ as cation, saturating levels of dTTP are not attained at substrate levels up to 500 $\mu$M.

In reactions with the initiated homoribopolymer, $(A)_n$·$(dT)_{\overline{16}}$, $Mn^{2+}$ is absolutely required; polymerase $\beta$ is completely unreactive with this primer-template in the presence of $Mg^{2+}$ (Figure 4). As shown in Table I, in the reaction with the ribopolymer and $Mn^{2+}$ the apparent affinity of the enzyme for both primer-template and substrate dTTP is lower by 25- to 50-fold than that measured with the homologous deoxypolymer. Whether this reflects an intrinsic difference in the manner in which the polymerase protein interacts with these

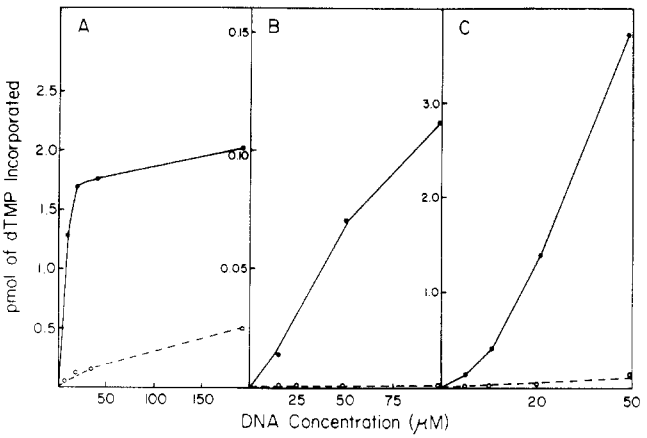


FIGURE 5: The effect of divalent cation on utilization of specific "gapped" DNA primer-templates by human polymerase β. The incubation conditions were as described in Figure 2, except 3 μM each of dATP, dGTP, dCTP, and [$^3$H]dTTP (14 600 cpm/pmol) were used. DNA concentrations (as nucleotide) were varied at shown. (A) activated DNA and 3 units/mL of polymerase β; (B) φ80 DNA and 3 units/mL of polymerase β; (C) "gapped" Col E1 DNA and 5.6 units/mL of polymerase β. $MnCl_2$ (●—●); $MgCl_2$ (○----○).

two synthetic primer-templates, or whether it is artifactual and explicable, for example, by differences in the conformation of the two polymers under incubation conditions (Wells et al., 1972; Tamblyn and Wells, 1975) is not clear.

The effect of divalent cation on the reactivity of polymerase β with DNA is most dramatically observed in assays employing defined DNA primer-templates that contain only a few primer sites per molecule (Figure 5). The experiment illustrated in Figure 5A is a control with activated salmon sperm DNA and demonstrates the strong preference of polymerase β for $Mn^{2+}$ at low DNA concentrations. Assays using defined primer-template molecules are shown in Figures 5B and 5C. With low concentrations of φ80 DNA (Figure 5B), in which the template consists of the two 12-nucleotide long cohesive ends per molecule (Bambara and Wu, 1975), or of exonuclease III gapped Col E1 DNA (Figure 5C), the polymerase was inactive with $Mg^{2+}$ but fully capable of promoting DNA synthesis in the presence of $Mn^{2+}$.

Since there are several reports in the literature demonstrating that the replacement of $Mg^{2+}$ by $Mn^{2+}$ in reactions with prokaryotic and oncornavirus DNA polymerases can lead to the insertion of complementary ribonucleotides and the enhanced misincorporation of noncomplementary deoxynucleotides (Berg et al., 1962; Hall and Lehman, 1968; Dube and Loeb, 1975), we evaluated the fidelity of synthesis carried out by DNA polymerase β with poly(dA)·oligo(dT)$_{\overline{16}}$ in the presence of $Mg^{2+}$ and $Mn^{2+}$. The results indicated that the amount of misincorporation of dCMP in these reactions was on the order of 1 in $10^4$ nucleotides and was unaffected by the particular divalent cation that was present. By the product analyses described under Materials and Methods, we established that the misincorporated α-$^{32}$P was in 5′-dCMP (<1% of the label was associated with incorporated dAMP and dTMP that derive from the trace amounts of dATP and dTTP in the [α-$^{32}$P]dCTP substrate). Moreover, the nearest neighbor analysis showed that >90% of the dCMP residues had been inserted into the product adjacent to dTMP; <10% of the dCMP was next to dCMP, a position that would have suggested clustering of the mismatched monomers, or to dAMP, the position to be expected if significant end-addition to the 3′-termini of poly(dA) template chains had occurred. The error rate estimated from these experiments is in reasonable agreement with the values reported for polymerase β by Chang (1973b, 1974).[5]

## Discussion

In our initial efforts to study the capacity of highly purified human DNA polymerase β to use defined DNA primer-templates, with which it is simply not practicable to attain the high concentrations of 3′-hydroxyl termini that are present in the conventional assay, we were puzzled by the inactivity of the enzyme in a reaction that had been optimized for all constituents except DNA. Our observation that the replacement of $Mg^{2+}$ by $Mn^{2+}$ led to a dramatic restoration of enzyme activity stimulated a closer examination of this phenomenon. As documented in this report, the presence of $Mn^{2+}$ as divalent cation results in a marked increase in the apparent affinity of polymerase β[6] for DNA primer-templates and a smaller but still substantial increase in its affinity for deoxynucleoside triphosphates (Table I). These effects are clearly seen with "activated" salmon sperm DNA (Figure 2) and synthetic homopolymers (Figure 4), and they are particularly striking with such primer-templates as φ80 DNA and gapped Col E1 DNA (Figure 5), with which, at DNA concentrations <100 μM (nucleotide), polymerase activity can not be detected with $Mg^{2+}$. Within the limits of interpretability (Chang, 1973b) and sensitivity of fidelity assays, we find that the polymerization carried out by polymerase β in the presence of $Mn^{2+}$ is about as accurate as that performed with $Mg^{2+}$. Thus, we have no evidence that the $Mn^{2+}$-promoted reaction is in any obvious way "aberrent".

On the basis of current models of the role of divalent cations in the DNA polymerization reaction, a satisfactory explanation for our observations is not readily apparent. Two different metals are recognized as obligatory participants in this enzymatic reaction. Zinc, which has been found to be tightly bound to a number of highly purified DNA polymerases (Slater et al., 1971; Loeb, 1974), is thought to function in the binding of polymerase to the 3′-terminus of the primer chain (Slater et al., 1971, 1972). The requirement for the second divalent cation, sometimes designated a "metal activator" (Loeb, 1974; Sirover and Loeb, 1976), is most effectively met by $Mg^{2+}$ or $Mn^{2+}$ which appears to coordinate the binding, alignment and conformational alteration of the incoming deoxynucleoside triphosphate within the active site of the polymerase molecule (Englund et al., 1969; Slater et al., 1972; Sloan et al., 1975). A recent magnetic resonance study (Slater et al., 1972) of the interaction of $Mn^{2+}$ with *E. coli* DNA polymerase I demonstrated the presence of multiple metal binding sites (1 tight, 4 ± 1 intermediate), all of which were postulated to be involved with the binding of deoxynucleotides but not with the binding of the polymerase to the primer-template. An additional class of weak metal binding sites (20 ± 4 per molecule) were considered to be possible sites of metal inhibition.

Despite the important insights that have been gained from

[5] In similar experiments reported by Chang (1973b) with calf thymus DNA polymerase β and poly(dA)·oligo(dT), $Mn^{2+}$ was shown to promote a greater rate of misincorporation of dCMP than $Mg^{2+}$. However, the differential was due almost entirely to the insertion of dCMP residues next to dCMP, a result interpreted by Chang as due to a limited addition reaction at the ends of primer or template chains. By contrast, the frequency of insertion of dCMP next to dTMP in that study was almost the same whether $Mg^{2+}$ or $Mn^{2+}$ was present, a finding in agreement with the present results.

[6] In experiments to date we have been unable to demonstrate a similar effect of $Mn^{2+}$ on the reactivity of KB cell DNA polymerase α or of a highly purified DNA polymerase that we have isolated from *Mycoplasma orale*. No other DNA polymerases have been examined.

those studies, they do not provide a complete explanation for the several effects of divalent cations on the complex DNA polymerase reaction. In particular, they do not address the important role of these metals in determining the ability of different polymerases to respond to a variety of natural and synthetic primer-templates. Many studies with eukaryotic DNA polymerases have been concerned with differential primer-template utilization (Bollum, 1974; Weissbach, 1975), and this parameter has been incorporated into the current system of nomenclature of these enzymes (Weissbach et al., 1975a, b). It is generally accepted that $Mg^{2+}$ is the preferred cation with DNA, while $Mn^{2+}$ may be preferred or even required for reactivity with different homoribo- and homodeoxyribopolymers (Chang, 1973b; Bollum, 1974). However, the designation of $Mg^{2+}$ as the cation of choice in DNA templated reactions is based entirely on the results of assays that employ "activated" (gapped) DNA, in which the concentration of DNA nucleotides is very high and the actual number and disposition of functional primer sites are undefined (Wickner et al., 1972; Sedwick et al., 1972). Although the mechanisms that are at play in the phenomena described in this paper are obscure, our results are of considerable practical significance. They specify for the first time feasible assay systems that will permit the use of defined DNA primer-template molecules for more sophisticated catalytic studies with polymerase $\beta$.

As a final point we note that we have developed a method for the preparation of highly purified nuclei from adult human liver, and a modified protocol for the purification of DNA polymerase $\beta$ that is equally applicable to hepatic and KB cell nuclei. We have been able to isolate this enzyme, at comparable specific activity, from both of these tissue sources by a common procedure and to demonstrate that the hepatic and KB enzymes are essentially identical in their structural and enzymatic properties. In a recent study of the phylogeny of the DNA polymerases, Chang (1976) found that enzymes of the polymerase $\alpha$ class were ubiquitous among eukaryotes, but activity of the $\beta$ class appeared to have evolved only at the time of development of the metazoa. Although the in vivo functions of the eukaryotic DNA polymerases remain to be established, considerable circumstantial evidence suggests that polymerase $\alpha$ is importantly involved in DNA replication, while polymerase $\beta$ has been proposed to be a "repair" enzyme (Bollum, 1974; Loeb, 1974; Weissbach, 1975). These observations are consistent with the hypothesis that there may have been less evolutionary pressure for the stringent conservation of polymerase $\beta$ than of polymerase $\alpha$ and raised the possibility that the enzyme we had characterized in detail from a highly aneuploid, transformed (KB) cell line (Wang et al., 1974, 1975) might not be prototypic of the "normal" human enzyme, which had not heretofore been examined in comparable detail. The results of this study indicate that polymerase $\beta$ has in fact been highly conserved in a cell line that has been in continuous culture for over 20 years.

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# Repair of Nitrous Acid Damage to DNA in *Escherichia coli*†

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ABSTRACT: A number of mutant strains of *Escherichia coli* have been examined for their sensitivity to nitrous acid and in some instances to methylmethanesulfonate. All *ung*$^-$ mutants tested are abnormally sensitive to nitrous acid. Since the *ung* mutation is phenotypically expressed as a defect in uracil DNA glycosidase, this observation supports the contention that treatment of cells with nitrous acid causes deamination of cytosine to uracil. In addition the observed sentitivity indicates that the *ung* gene is involved in the repair of uracil in DNA. Studies with other mutants suggest that both exonuclease III and DNA polymerase I of *E. coli* are involved in the repair of nitrous acid damage in vivo.

In recent years a number of laboratories have demonstrated enzyme activities that catalyze the hydrolysis of *N*-glycosidic bonds linking nitrogenous bases to the sugar-phosphate backbone in DNA (Kirtikar and Goldthwait, 1974; Lindahl, 1974, 1976; Friedberg, et al., 1975; Duncan et al., 1976a,b; Katz et al., 1976; Lindahl et al., 1977; Cone et al., 1977). These enzymes (termed DNA *N*-glycosidases (Lindahl et al., 1977) appear to be specific for unusual or modified bases in DNA and may function in initiating DNA repair by releasing the free base from the DNA and creating a site susceptible to attack by apurinic endonucleases.

A uracil DNA glycosidase has been shown in extracts of *E. coli* to attack DNA containing uracil derived by the deamination of cytosine (Lindahl, 1974, 1976; Lindahl et al., 1977). The copolymer (dC, [$^3$H]dU) is also a substrate for this activity (Lindahl, 1974, 1976; Lindahl et al., 1977). A similar (if not identical) activity in extracts of *B. subtilis* has been shown to attack phage PBS2 DNA which naturally contains uracil instead of thymine, as well as the polymer [$^3$H]poly(dU) (Friedberg et al., 1975; Duncan et al., 1976b; Katz et al., 1976). Following infection of *B. subtilis* with phage PBS2, the uracil DNA glycosidase activity disappears due to the induction of a presumably phage-coded inhibitor (Tomita and Takahashi, 1975; Friedberg et al., 1975; Duncan et al., 1976b; Katz et al., 1976). Inhibition of RNA or protein synthesis during phage infection prevents the synthesis of the inhibitor and the phage DNA is rapidly degraded (Duncan and Warner, 1977). When chloramphenicol is added after the inhibitor has been synthesized, the phage DNA remains stable (Duncan and Warner, 1977). These observations suggest that the uracil DNA glycosidase functions in vivo to remove uracil residues from DNA and support a hypothetical biological role for this enzyme in the excision repair of uracil when it occurs in DNA.

It has been reported that treatment of nucleic acids with nitrous acid results in a number of chemical modifications of the DNA, including deamination of cytosine to uracil (Schuster and Schramm, 1958; Schuster, 1960a,b; Litman, 1962; Shapiro and Pohl, 1968; Shapiro and Yamaguchi, 1972). The isolation of mutants of *E. coli* defective in uracil DNA glycosidase (*ung*$^-$) (B. K. Duncan and H. R. Warner, manuscript in preparation) provides an experimental system to test this directly and in the present studies we show that such mutants are abnormally sensitive to nitrous acid treatment. In an effort to gain insights into the molecular mechanisms of subsequent steps in the uracil excision repair pathway(s) in *E. coli*, we have also examined the nitrous acid sensitivity of a number of mutants defective in enzyme activities that might be involved in repair pathways initiated by removal of uracil as the free base (Lindahl, 1976). The sensitivity of a number of these mutants to methylmethanesulfonate has also been tested by ourselves and by Kirtikar et al. (1977).

## Material and Methods

1. *Bacterial Strains* (*See Table II*). The wild-type strains AB1157 and W3110 are maintained in our laboratory stocks. The BD series of strains were isolated by us. Strains BD10 and BD13 were independent isolates from a stock of *E. coli* W3110 mutagenized with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine. Strain BD1119 is isogenic (by phage $P_1$ transduction) with an *ung*$^+$ strain BD1117 maintained in the laboratory of H. R. W. A full description of the isolation of the BD strains will be presented elsewhere (B. K. Duncan and H. R. Warner, manuscript in preparation). The BW series of mutants were originally isolated in Dr. B. Weiss's laboratory (Milcarek and Weiss, 1972; Yajko and Weiss, 1975) and were obtained from Dr. David A. Goldthwait, as was strain NH5016, originally isolated by Ljungquist et al. (1976). Strains JG138, JG139, KS463, and RS5069 were obtained from Dr. Priscilla Cooper, Stanford University. All strains were maintained on agar plates containing 1% tryptone (Difco), 0.5% yeast extract (Difco), 1% sodium chloride, and 1.5% agar. Strains KS463 and

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