Two DNA Glycosylases in *Escherichia coli* Which Release Primarily 3-Methyladenine†

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ABSTRACT: Two enzymes have been partially purified from *Escherichia coli* and designated 3-methyladenine DNA glycosylases I and II. The glycosylase I is that described by Riazuddin & Lindahl [Riazuddin, S., & Lindahl, T. (1978) *Biochemistry 17*, 2110–2118]. The apparent molecular weight of glycosylase I is 20000, and that of II is 27000. Glycosylase I releases 3-methyladenine (3-MeA) while II releases 3-MeA, 3-methylguanine (3-MeG), 7-methylguanine (7-MeG), and 7-methyladenine (7-MeA). The rate of release of 3-MeA by glycosylase II is 30 times that of 7-MeG. Glycosylase I is missing in mutants tag 1 and tag 2 [Karran, P., Lindahl, T., Ofsteng, I., Evenson, G. B., & Seeberg, E. (1980) *J. Mol. Biol. 140*, 101–127]. In crude extracts, the 3-MeA activity of II is approximately 10% of the total 3-MeA activity. A 50% inactivation at 48 °C required 5 min for I and 65 min for II. The apparent K_m for 3-MeA residues for glycosylase I was 1.4×10^{-8} M. The enzyme was inhibited noncompetitively by 3-MeA with an average apparent K_i of 1.6 mM. The apparent K_m for 3-MeA, for glycosylase II, was 9.2×10^{-9} M, and it was not inhibited by 3-MeA. The 3-MeA and 7-MeG activities of the glycosylase II preparation could not be separated by isoelectric focusing, by chromatography on DEAE, Sephadex G-100, phosphocellulose, DNA–cellulose, or carboxymethylcellulose, or by heating at 50 °C. The apparent K_m for 7-MeG was 1.1×10^{-8} M. Glycosylase II released N^1-(carboxyethyl)adenine and N^7-(carboxymethyl)guanine from DNA treated with β-[^{3}H]propiolactone but did not release the aflatoxin B-1 adduct at N-7 of guanine.

The first evidence for an enzyme that recognizes alkylated DNA was presented by Strauss (1962), who demonstrated that extracts of *Micrococcus lysodeikticus* and *Bacillus subtilis* inactivated transforming DNA treated with methyl methanesulfonate (MMS). Reiter et al. (1967) demonstrated that an endonuclease is present in extracts of *B. subtilis* that recognizes DNA treated with MMS and that there is repair in vivo of the bacterial DNA after treatment with the alkylating agent. An enzyme fraction from *Escherichia coli* active on MMS-treated DNA and designated endonuclease II was purified 300-fold (Friedberg & Goldthwait, 1969), and some of its properties were reported (Friedberg et al., 1969). Later, a fraction active on MMS-treated DNA was purified 1600-fold from *E. coli* in this laboratory and was found to recognize apurinic sites (Hadi & Goldthwait, 1971). At the same time, Verly's group (Verly & Paquette, 1972; Paquette et al., 1972) obtained a partially purified fraction from *E. coli* that recognized apurinic sites, but not MMS-treated DNA. Weiss (1976) then showed that exonuclease III also acted as an apurinic endonuclease, and this was confirmed by Ljungquist & Lindahl (1977). The activity for apurinic sites in *E. coli* was purified further and designated endonuclease VI (Gossard & Verly, 1978).

The 1600-fold-purified preparation from *E. coli*, prepared in this laboratory on the basis of endonuclease activity toward MMS-treated DNA, was reported to release free 3-methyladenine (3-MeA)[1] and O^6-methylguanine (Kirtikar & Goldthwait, 1974), and it was hypothesized that the depurination was an intermediate step in the cleavage of phosphodiester bonds. However, subsequent difficulties in repeating these experiments,[2] and the recent demonstration of a different mechanism for removal of O^6-methylguanine (Karran et al., 1979), make the report of the O^6-methylguanine glycosylase activity suspect. With regard to 3-methyladenine, both Lindahl (Lindahl, 1976; Riazuddin & Lindahl, 1979) and Laval (1977) demonstrated glycosylase activities for 3-methyladenine DNA in partially purified preparations from *E. coli* and from *Micrococcus luteus*, respectively. In searching for various glycosylase activities in fractions of *E. coli*, we have accumulated evidence for the existence of two different glycosylase enzymes that release primarily 3-methyladenine. One of these also releases 7-methylguanine. The separation of these enzymes as well as some of their properties is the subject of this paper.

Materials and Methods

7-Methylguanine (7-MeG) and 3-methyladenine (3-MeA) were purchased from Cyclo Chemicals, 3-methylguanine (3-MeG) was from Vega Biochemicals, and 7-methyladenine (7-MeA) was from Heterocyclic Chemical Co. *N*-[^{3}H]-Methyl-*N*-nitrosourea (MNU) (1.6 Ci/mmol) and *N*-[^{3}H]-ethyl-*N*-nitrosourea (ENU) (123 mCi/mmol) and [^{3}H]thymidine (6.7 Ci/mmol) were purchased from New England Nuclear. β-[^{3}H]Propiolactone was obtained from Amersham Corp.; DEAE-cellulose (DE-52) and phosphocellulose (P-11) were purchased from Whatman Inc. Sephadex G-75 and G-100 and the ampholyte solutions for isoelectric focusing were from Pharmacia. Bio-Lyte electrofocusing gel was purchased from Bio-Rad. DNA–cellulose containing roe DNA (Sigma) was prepared by the method of Alberts & Herrick (1971). Glass-distilled methanol for high-performance liquid chromatography (HPLC) was from MCB reagents. Ultrapure

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[1] Abbreviations: 3-MeA, 3-methyladenine; 7-MeG, 7-methylguanine; 3-MeG, 3-methylguanine; 7-MeA, 7-methyladenine; O^6-MeG, O^6-methylguanine; 3-EtA, 3-ethyladenine; 7-EtG, 7-ethylguanine; 3-EtG, 3-ethylguanine; 7-EtA, 7-ethyladenine; MNU, methylnitrosourea; ENU, ethylnitrosourea; AP-DNA, DNA with apurinic sites; PMSF, phenylmethanesulfonyl fluoride; EDTA, ethylenediaminetetraacetic acid; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid.

[2] See correction in *Biochemistry* (1978) *17*, 4578.

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monobasic potassium phosphate was from J. T. Baker. Calf thymus DNA was from Calbiochem.

Substrates. Calf thymus DNA was reacted with *N*-[^{3}H]-methyl-*N*-nitrosourea as described previously (Cathcart & Goldthwait, 1981) to yield material with a specific activity of 2500–2700 cpm/nmol.

The DNA was hydrolyzed at 70 °C for 30 min in 0.2 M HCl, neutralized, and run through a 1-mL DEAE-cellulose column (Cathcart & Goldthwait, 1981). The percentages of ^{3}H-labeled alkyl bases in the eluate were as follows: 76.5% 7-MeG, 9.0% O^6-MeG, 10% 3-MeA, 2.2% 7-MeA, and 0.9% 3-MeG. [^{3}H]Thymidine was used to label T7 DNA purified from phage grown on *E. coli* B-3 (Richardson et al., 1964; Richardson, 1966). DNA with apurinic sites was prepared by heating calf thymus DNA at 70 °C for 4 h in 0.1 M NaCl–0.01 M sodium citrate, pH 5.0. DNA reacted with β-[^{3}H]propiolactone was prepared as described by Chen et al. (1981).

The preferential depurination of 3-methyladenine compared to 7-methylguanine at acid pH was utilized to prepare a substrate with a low 3-methyladenine content. The [^{3}H]-MNU-treated DNA was incubated in 0.02 M sodium malate, pH 3.5, at 37 °C for 64 h which caused a loss of approximately 95% of the 3-MeA and 36% of the total radioactivity. The ratio of 3-MeA to 7-MeG was 0.005, the ratio of 7-MeA to 7-MeG was 0.004, and the ratio of O^6-MeG to 7-MeG was 0.21. This substrate when assayed on a hydroxylapatite column without and with 50% formamide in the buffer was found to be greater than 97% double-stranded DNA. This was confirmed by CsCl centrifugation of d(A-T) polymer either with double- plus single-stranded DNA or with the substrate.

High-Performance Liquid Chromatography (HPLC). The system used included two Model 600A solvent pumps, a 660 solvent programmer, a 440 UV detector (254 nm), a U6K injector, and a μBondapak fatty acid analysis column, all from Waters Associates.

The alkylated bases could be separated by two different programs. The program used most extensively (program A) was a linear gradient from 10 mM potassium phosphate buffer, pH 7.0, to 25% of an 80% methanol solution over a period of 15 min at 1.6 mL/min. The elution times in minutes for the alkylated bases were the following: 3-MeG, 7; 7-MeG, 11; 7-MeA, 12; O^6-MeG, 16; 3-MeA, 17.

Program B was a linear gradient from 10 mM monobasic potassium phosphate, pH 4.0, to 20% of an 80% methanol solution in 20 min at 1.3 mL/min. The elution times in minutes were the following: 3-MeG, 8; 3-MeA, 10; 7-MeG, 12; 7-MeA, 13; O^6-MeG, 17.

Enzyme Assays. The standard assay (assay A) used throughout the purification, and also for glycosylase I, contained 0.07 M Hepes–KOH, pH 7.8, 5% glycerol, 1 mg/mL bovine serum albumin, 1 mM 2-mercaptoethanol, 1 mM EDTA, and approximately 0.2 unit of enzyme in a reaction volume of 0.3 mL. [^{3}H]MNU-treated DNA (4–5 μg) (30 000–40 000 cpm) was added to start the reaction. After 30 min at 37 °C, the reaction was terminated by chilling and the addition of 50 μL of 2 M sodium acetate, pH 5.0, and 0.3 mg of roe DNA as a carrier. The DNA was precipitated by the addition of 1 mL of cold ethanol. After 10 min at 0 °C, the samples were centrifuged at 1300*g* for 15 min, and a 0.6-mL aliquot of the supernatant fraction was taken for counting. One unit of DNA glycosylase is defined as the amount of enzyme that releases 1 pmol of free 3-MeA per min under these conditions.

The standard assay for glycosylase II (assay B) was a reaction mixture containing 0.07 M Tris-HCl, pH 8.0, 5% glycerol, 1 mM EDTA, 1 mM spermidine, 1 mM 2-mercaptoethanol, and 1 mg/mL bovine serum albumin, plus enzyme and DNA in a reaction volume of 0.3 mL.

The conditions for a third assay (assay C) were as follows: 0.02 M Tris-HCl, pH 8.0, 5% glycerol, 1 mM EDTA, 1 mM spermidine, and 1 mM 2-mercaptoethanol, plus enzyme and DNA in a reaction mixture of 0.3 mL.

When the ethanol supernatant fraction was further analyzed by HPLC, the DNA was precipitated as in assay A, but by the addition of 2 mL of cold ethanol. Then 1.9 mL of the supernatant fraction was evaporated to dryness in a Savant Speed Vaconcentrator and redissolved in 1.3 mL of water prior to HPLC. The peaks for the methylated bases were collected and counted in a Packard Tri-Carb scintillation counter.

Tests for Endo- and Exonuclease Activity. T7 [^{3}H]DNA (628 cpm/nmol) was made single stranded by heating at 100 °C for 3 min and then quickly cooling. Apurinic T7 [^{3}H]DNA was made by incubating the DNA for 2 h at 70 °C in 0.01 M sodium citrate, pH 5, and 0.1 M NaCl and dialyzing against 10 mM Tris-HCl, pH 8.0. Double-stranded or partially apurinic T7 [^{3}H]DNA (14 μg of each) was incubated with 0.09 or 0.27 unit of glycosylase I and with 0.06 or 0.18 unit of glycosylase II for 1 h at 37 ° C in the presence of 1 mM $MgCl_2$. The double-stranded and apurinic DNA reaction mixtures were cooled to 0 °C and dialyzed overnight against 50 mM Tris, pH 7.6. Sodium dodecyl sulfate was added to a final concentration of 2%, and 200 μL was layered on top of a 4.2-mL 70–100% formamide gradient. The single-stranded DNA reaction mixtures were adjusted to 0.9 M NaCl, 0.1 M NaOH, and 3 mM EDTA; 200 μL was layered on top of a 4.2-mL 5–20% sucrose gradient, made in 0.9 M NaCl, 0.1 M NaOH, and 1 mM EDTA. Sucrose gradients were centrifuged in a TV865 vertical rotor (Sorvall) for 1 h at 40 000 rpm, at 20 °C in an OTD 65 ultracentrifuge (Sorvall). Ten-drop fractions were collected from the botton of the centrifuge tube and counted.

For the exonuclease assay, 14 μg of T7 [^{3}H]DNA that had been sonicated for 1 min was incubated with 0.42 unit of glycosylase I or 0.16 unit of glycosylase II for 1 h at 37 °C in the presence of 1 mM $MgCl_2$. The DNA was precipitated, and the ethanol supernatant fraction was counted.

Enzyme Purification. The method followed was basiclly that of Riazuddin & Lindahl (1978), with some variations. All operations were carried out at 0.5 °C, and centrifugations were for 30 min at 20000*g*.

Crude Extract. E. coli BW 9062 cells (xth$^-$) (61 g) were suspended in 375 mL of the extraction buffer [0.06 M Hepes–KOH, pH 7.8, 5% glycerol, 1 mM EDTA, 1 mM 2-mercaptoethanol, and 0.1 mM phenylmethanesulfonyl fluoride (PMSF)]. The PMSF was dissolved in 2-propanol (17.4 mg per 2 mL of 2-propanol per L of buffer). The cells were broken by sonication (6 times for 1-min intervals), and the debris was removed by centrifugation (fraction I, 395 mL).

Streptomycin and Ammonium Sulfate Treatment. Streptomycin sulfate (395 mL of 1.6%) in the extraction buffer was added to fraction I with gentle stirring over a 1-h period. The suspension was stirred for 30 min more and then centrifuged. Solid ammonium sulfate was added to the supernatant fraction during 1 h, to a final concentration of 40% saturation (at 0 °C). After being stirred for another hour, the precipitate was removed by centrifugation. More ammonium sulfate was added to reach 67% saturation, and after an hour of being stirred and subsequent centrifugation, the precipitate was

redissolved in buffer A (0.01 M Hepes–KOH, pH 7.4, 1 M NaCl, 0.1 mM PMSF, 5% glycerol, 1 mM EDTA, and 1 mM 2-mercaptoethanol) and dialyzed overnight against 2 L of this buffer (fraction II).

Sephadex G-75 Column. Fraction II (80 mL) was chromatographed on a Sephadex G-75 column (5 × 108 cm) equilibrated with buffer A. The 3-MeA glycosylase activity eluted as a broad peak. All the active fractions were pooled (fraction III) and dialyzed twice against 6 L of buffer B (0.03 M potassium phosphate, pH 6.8, 0.1 mM PMSF, 5% glycerol, 1 mM EDTA, and 1 mM 2-mercaptoethanol).

Phosphocellulose Column. Fraction III (325 mL) was applied to a phosphocellulose (P-11) column (1.4 × 10.5 cm) which had been equilibrated with buffer B. The column was washed with 40 mL of buffer B, and then the enzyme was eluted with a linear gradient (2 × 200 mL) of 0–0.3 M NaCl in buffer B. The 3-MeA glycosylase activity eluted in two overlapping peaks, the first, a broad peak at 0.05 M NaCl (3-MeA I), and the second, a sharper peak at 0.10 M NaCl (3-MeA II). The overlapping peaks were pooled (fraction IV) and dialyzed twice against 4 L of buffer C (0.01 M Tris, pH 7.6, 5% glycerol, 1 mM EDTA, and 1 mM 2-mercaptoethanol).

DNA–Cellulose Column. Fraction IV (193 mL) was applied to a DNA–cellulose column (1.1 × 6 cm) equilibrated with buffer C. The column was washed with 10 mL of buffer C and the enzyme eluted with a linear gradient (2 × 20 mL) of 0–1.0 M NaCl in buffer C. The glycosylase activity eluted as a sharp peak at 0.25 M NaCl. The active fractions were combined (fraction V) and dialyzed twice against 2 L of buffer D (0.02 M Tris, pH 8.5, 5% glycerol, 1 mM EDTA, and 1 mM 2-mercaptoethanol).

DEAE-cellulose Column. Fraction V (16.6 mL) was applied to a DEAE column (1.1 × 10.5 cm) which had been equilibrated with buffer D. The column was washed with 10 mL of buffer, and the activity eluted with a linear gradient (2 × 25 mL) of 0–0.2 M NaCl solution. Bovine serum albumin was added to each tube so that the final concentration in the collected fraction was 1 mg/mL. This helped to stabilize the eluted enzymes. The activity eluted in two peaks, a sharp peak at 0.025 M NaCl (3-MeA II) and a broad peak at 0.075–0.1 M NaCl (3-MeA I). The fractions within each peak were pooled and dialyzed against 0.02 M Tris-HCl, pH 8.0, 5% glycerol, 1 mM EDTA, and 0.2 mM dithiothreitol. Fractions VIA and VIB (3-MeA I, 23 mL, and 3-MeA II, 11 mL) were stored in 1-mL aliquots at −70 °C. This material was stable for months.

Isoelectric Focusing of Glycosylases I and II. A Model 1415 horizontal electrophoresis cell (Bio-Rad) was used for the isoelectric focusing. Swollen Bio-Lyte electrofocusing gel (100 mL) was mixed with 2.5 mL of pH 5–8 Pharmalyte ampholytes and 2.5 mL of pH 6.5–9 Pharmalyte ampholytes and poured into a flat bed, 15 × 11 × 0.2 cm. The enzymes were added slowly to the middle of the bed which was cooled to 6 °C. Focusing was for 17 h at a constant 20 W. Starting voltage was 700 V which increased to 1800 V at the end of the run. A grid was pressed into the gel bed to divide it into fractions, and each fraction was scraped off and put into a mini column. The enzyme was eluted from the gel with 2 mL of 0.02 M Tris, pH 8.0%, 5% glycerol, 1 mM EDTA, 1 mM 2-mercaptoethanol, and 1 mg/mL bovine serum albumin. Water, 0.75 mL, was added to one-third of each gel fraction, and the pH was measured with a standard electrode.

Results

Separation of Two 3-Methyladenine DNA Glycosylases.

Table I: Purification of 3-Methyladenine DNA Glycosylase Activities[a]

fraction	protein (mg)	total activity (units)	sp. act (units/mg)	% yield
I, crude extract	4350	3332	0.77	100
II, ammonium sulfate	2144	2066	0.96	62
III, Sephadex G-75	218	1933	8.9	58
IV, phosphocellulose	7.9	1025	130	24
V, DNA-cellulose	0.9	481	537	14
VIA, DEAE-cellulose		488		14.6
VIB, DEAE-cellulose		91		2.7

[a] One unit of enzyme activity equals 1 pmol of 3-MeA released per min at 37 °C in assay buffer A.

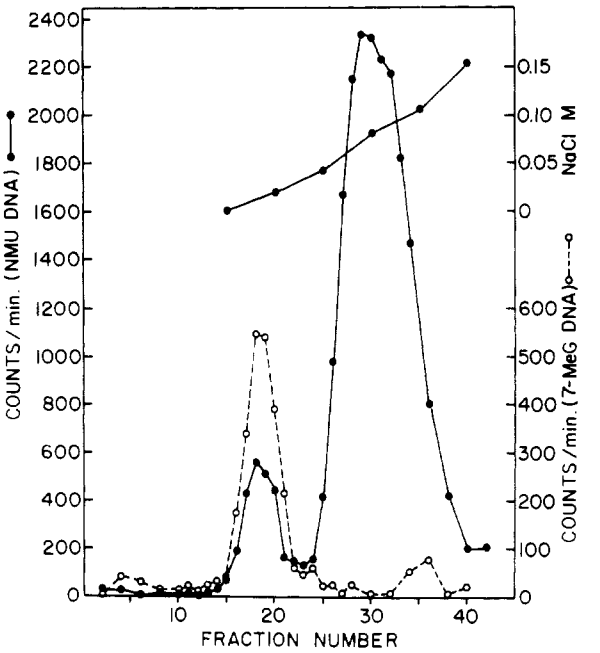

FIGURE 1: Separation of 3-methyladenine DNA glycosylases I and II by DEAE chromatography. Fraction V, 118 μg, was applied and eluted as described under Materials and Methods. Fractions (1.5 mL) were collected. (●) Counts released from [^{3}H]MNU-treated DNA in a 70-min incubation; (○) counts released from the 7-methylguanine substrate after a 3.5-h incubation.

Table I shows the purification through fraction V. These steps are basically those described by Riazuddin & Lindahl (1978), whose preparation did not contain glycosylase II probably because of exclusion in the phosphocellulose fractionation. They also obtained a 4-fold higher purification. When fraction V was passed through a DEAE-cellulose column, two peaks of activity were observed as shown in Figure 1. The activity which eluted first was designated 3-methyladenine DNA glycosylase II (3-MeA II) while the larger amount of activity which eluted later was called 3-methyladenine DNA glycosylase I (3-MeA I). When aliquots of each of the enzymes were collected in the absence of bovine serum albumin, precipitated, and then examined on a sodium dodecyl sulfate–polyacrylamide gel, several bands were observed in each fraction.

Isoelectric focusing in a bed of polyacrylamide beads also resulted in separation of the two glycosylase activities. When focused in separate lanes, 3-MeA I appeared as a sharp peak at pH 6.0 while 3-MeA II formed a more broad peak at pH 7.3 (Figure 2). A similar pattern was obtained when fraction V was the starting material. The yields from isoelectric focusing vary and were generally below 25%.

The differences in charge also allow separation of glycosylases I from II in fraction III on a phosphocellulose column

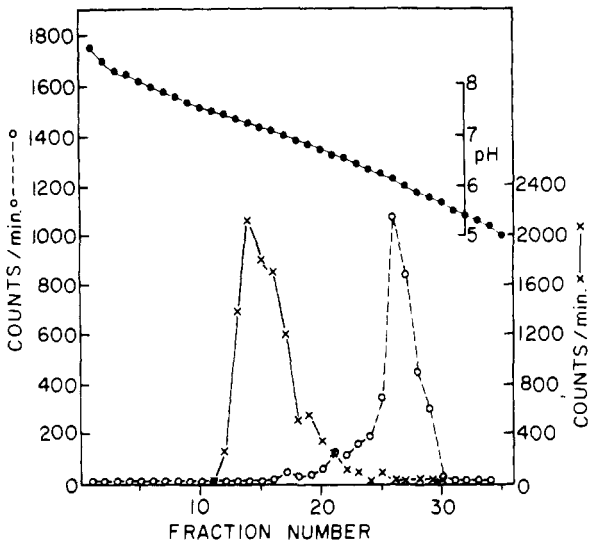

FIGURE 2: Behavior of 3-methyladenine DNA glycosylases I and II on isoelectric focusing. The granulated gel bed was divided into three channels for glycosylase I, glycosylase II, and pH measurement. Release was determined by assay A. (O) Glycosylase I, 6 units of fraction VIA; (×) glycosylase II, 3 units of fraction VIB.

as shown in the top panel of Figure 3. In this case, a shallow gradient was used. The first peak was 3-MeA I and eluted at 0.06 M NaCl while the second was 3-MeA II and eluted with 0.11 M NaCl. Karran et al. (1980) obtained two mutants of *E. coli* (tag 1 and tag 2) that lack most of the 3-methyladenine DNA glycosylase activity. These were selected because they were more sensitive than wild-type cells in growth on agar-containing methyl methanesulfonate. The tag 1 mutant is thermosensitive when grown in the presence of MMS, and its 3-methyladenine DNA glycosylase is also thermosensitive. When this organism was grown in this laboratory at elevated temperature and the 3-methyladenine glycosylase activity purified on the phosphocellulose column, the pattern shown in the middle panel of Figure 3 was found. The first peak was missing, and a single peak was eluted with 0.10 M NaCl. The enzyme pattern in the tag 2 mutant was similar to that in the tag 1 mutant (bottom panel of Figure 3), and the peak was eluted with 0.11 M NaCl. This mutant is not thermosensitive. Thus, in both mutant strains, the 3-methyladenine DNA glycosylase I is absent, but the 3-methyladenine DNA glycosylase II is present. The relative amounts of 3-methyladenine DNA glycosylases I and II are difficult to assess. When the release of the free base was measured in the original extracts by HPLC analysis, the levels of activity in the tag 1 and tag 2 mutants were 11 and 13%, respectively, of the levels in the crude extracts of the wild-type cells. Because of the losses of activity on purification, the levels of the two different enzymes in wild-type cells observed after various column fractionation procedures are not an accurate reflection of the original units.

Assay Conditions. Both enzymes have a broad pH optimum between 7.0 and 9.0. Glycosylase I is more active in 0.07 M Hepes–KOH buffer than in 0.07 M Tris-HCl buffer while the opposite is observed with glycosylase II. Both enzymes are less active at lower ionic strength. Spermidine (1 mM) inhibits glycosylase I and stimulates glycosylase II. Both glycosylases I and II are stimulated by 1 mM Mg^{2+}, glycosylase II even in the presence of 1 mM spermidine. Finally, NaCl concentrations have an inhibitory effect on both enzymes at levels between 0.05 and 0.1 M. Both glycosylases I and II are inhibited by 1 mM *N*-ethylmaleimide, 29% and 24%, respectively. The rate of release of 3-MeA by glycosylase II from

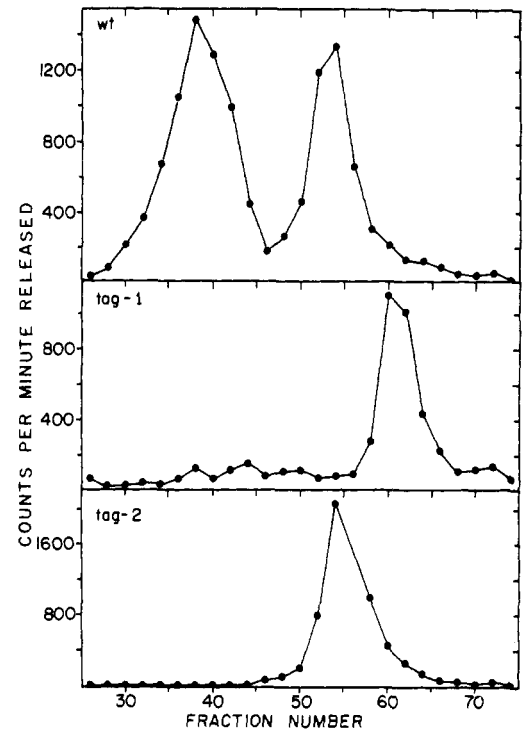

FIGURE 3: Separation of 3-methyladenine DNA glycosylases I and II by phosphocellulose chromatography analysis of wild-type and mutant strains. Fraction III of the wild-type and mutant cells (98–331 mg) was applied to a P-11 column (3.1 cm × 16 cm) and eluted with a linear gradient (2 × 200 mL) of 0–0.2 M NaCl in 0.03 M potassium phosphate, pH 6.8, 5% glycerol, 1 mM EDTA, 1 mM 2-mercaptoethanol, and 0.1 mM PMSF. Fraction volume was 6 mL.

single-stranded DNA was 9% of the rate from double-stranded DNA.

Both double-stranded DNA and DNA with apurinic sites (AP-DNA) inhibit glycosylases I and II. When the inhibiting DNA was present at 10-fold the concentration of the substrate, DNA glycosylase I was inhibited 66% by double-stranded DNA and 90% by AP-DNA. Glycosylase II was inhibited 82% by double-stranded DNA and 92% by AP-DNA.

Based on experiments described under Materials and Methods, it was concluded that neither glycosylase I nor glycosylase II was contaminated with exonuclease or with an endonuclease which recognized double-stranded DNA, or single-stranded DNA, or DNA with apurinic sites.

Apparent Molecular Weights of 3-Methyladenine DNA Glycosylases I and II. After separation of the two activities in fraction V by DEAE, they were applied separately to a Sephadex G-100 column calibrated with bovine serum albumin, ovalbumin, chymotrypsinogen, and ribonuclease A. By this method, the apparent molecular weight calculated for glycosylase I was 20 000, in agreement with that published by Riazuddin & Lindahl (1978), and the apparent molecular weight of glycosylase II was 27 000.

Heat Inactivation of 3-Methyladenine DNA Glycosylases I and II. The 3-methyladenine DNA glycosylase I was much more sensitive to heat inactivation than the glycosylase II as shown in Figure 4. When heated at 48 °C, the glycosylase I activity was reduced 50% in 5 min while a similar reduction of glycosylase II activity required approximately 65 min.

Specificity of Glycosylases I and II. Although both 3-methyladenine DNA glycosylase enzyme preparations liberated 3-methyladenine, one recognized other substrates. The release of various methylated bases from MNU-treated DNA as a function of time is shown in Figure 5. The 3-methyladenine DNA glycosylase I (top panel) was very active in the

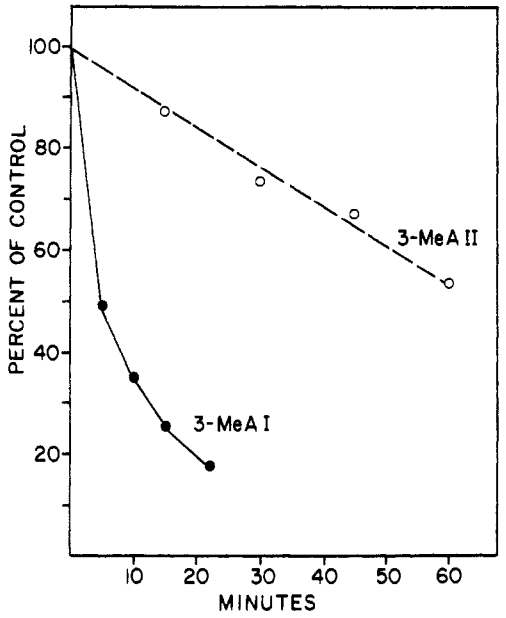

FIGURE 4: Inactivation of 3-methyladenine DNA glycosylases I and II by heating at 48 °C. Enzymes were incubated in 50 mM Tris, pH 8.0, 5% glycerol, 1 mM EDTA, and 0.2 mM dithiothreitol (DTT) and were tested by using assay C: (●) glycosylase I; (○) glycosylase II.

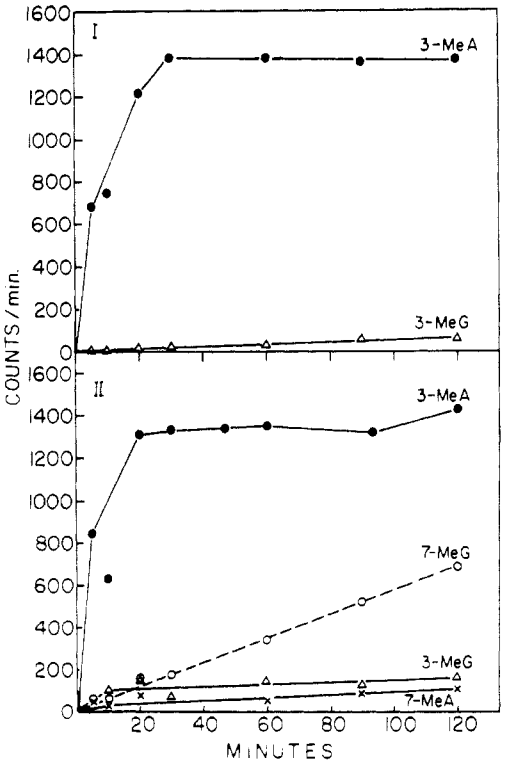

FIGURE 5: Release of purines by 3-methyladenine DNA glycosylases I and II from DNA treated with methylnitrosourea. The reaction mixtures contained either 0.09 unit of glycosylase I or 0.05 unit of glycosylase II plus 0.05 M Tris-HCl, pH 8.0, 5% glycerol, 1 mM $MgCl_2$, 1 mM spermidine, 1 mM 2-mercaptoethanol, 1 mg/mL bovine serum albumin and 2 μg of [^{3}H]DNA (19 000 dpm). Incubation was at 37 °C. Analysis of the alcohol-soluble fractions was by HPLC. The blank value (not shown) obtained in the glycoylase II experiment at 30 min for 3-MeA was 4% of the amount released with the enzyme and at 120 min for 3-MeG was 6%; 7-MeG was 18%, and 7-MeA was 60%. (●) 3-MeA; (△) 3-MeG; (×) 7-MeA; (○) 7-MeG.

release of 3-methyladenine. The preparation also released a minimal amount of 3-MeG. This was shown by a heat-inactivation study, as noted later, to be from the glycosylase II. After being heated to 48 °C for 10 min 90% of the 3-MeA

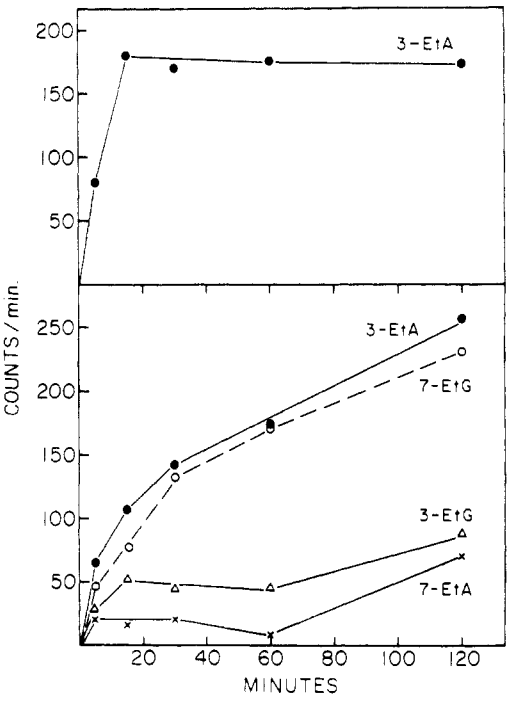

FIGURE 6: Release of purines by 3-methyladenine DNA glycosylases I and II from DNA treated with ethylnitrosourea. The reaction mixtures contained either 0.4 unit of glycosylase I or 0.16 unit of glycosylase II plus 0.07 M Tris-HCl, pH 8.0, 5% glycerol, 1 mM EDTA, 1 mM spermidine, 1 mM 2-mercaptoethanol, 1 mg/mL bovine serum albumin, and 44 μg of [^{3}H]DNA (4500 cpm). Incubation was at 37 °C. Analysis of the alcohol-soluble fractions was by HPLC. Designations were similar to those in Figure 5.

activity was lost in contrast to 42% of the 3-MeG activity. Thus, glycosylase I appears specific for 3-MeA and does not recognize 3-MeG, 7-MeA, or 7-MeG.

The bases released by 3-methyladenine DNA glycosylase II are shown in the lower panel of Figure 5. The major activity was again with 3-methyladenine, but this preparation also liberated 3-MeG, 7-MeG, and 7-MeA. The release of 3-MeG was rapid and similar to 3-MeA, while the 7-MeG was released at a slower but linear rate over the 2-h period. Control values without enzyme were determined for the data for each point in Figure 5.

The release of purine bases from DNA alkylated with [^{3}H]ethylnitrosourea by glycosylases I and II was also examined, and the results are shown in Figure 6. Glycosylase I released 3-EtA but had no significant activity for 3-EtG, 7-EtA, 7-EtG, or O^6-EtG. In contrast to glycosylase I, glycosylase II liberated 3-EtA, 3-EtG, 7-EtA, and 7-EtG (again blanks were run for each time point). A major difference from the methylnitrosourea-treated DNA was the similarity in the rates of release of 3-EtA and 7-EtG. This is apparently due to a lower rate of release of 3-EtA compared to 3-MeA rather than an increased rate of release of 7-EtG compared to 7-MeG.

Neither glycosylase I nor II will release 2,6-diamino-4-hydroxy-5-(*N*-methylformamido)pyrimidine, the alkali-induced derivative of 7-methylguanine in which the imidazole ring is opened (Chetsanga & Lindahl, 1979). The glycosylase II, which recognizes 7-methylguanine, does not recognize the aflatoxin B-1 adduct on the N-7 position of guanine. The [^{3}H]aflatoxin-treated DNA was kindly provided by Dr. G. Wogan. Glycosylase II will not release 7-MeG from the alkylated polymer (dG:dC). Glycosylase II released both N^1-(carboxyethyl)adenine (CEA) and N^7-(carboxyethyl)-guanine (CEG) from DNA treated with β-[^{3}H]propiolactone. The ratio of CEA to CEG released was 1 to 8 while the ratio in the original substrate was 1 to 28. The rate of release of

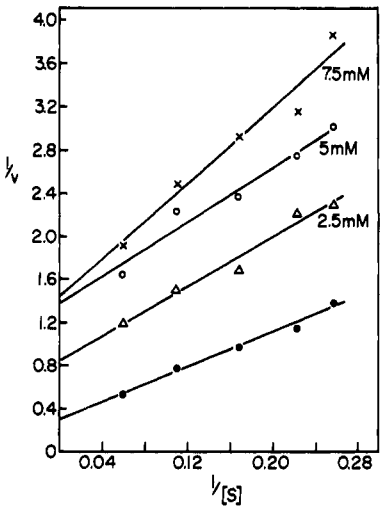

FIGURE 7: Double-reciprocal plots of velocity vs. substrate concentration with glycosylase I. The conditions for assay C were used. Incubation was for 30 min; 0.04 unit of fraction VIA was used. 3-Methyladenine was added in the concentrations noted.

CEG was similar to that of 7-MeG.

Apparent K_m Values for 3-Methyladenine DNA Glycosylases I and II. The apparent K_m for 3-methyladenine DNA glycosylase I was 1.4×10^{-8} M. The enzyme was inhibited by 3-methyladenine as originally demonstrated by Riazuddin & Lindahl (1978). The kinetics of inhibition were complicated as shown in Figure 7. At 2.3 and 5 mM concentrations of 3-methyladenine, the lines determined by least-squares analysis suggested a noncompetetive type of inhibition. With the assumption of uni-bi-bi kinetics and product inhibition, the apparent K_i determined at 2.5 and 5 mM concentrations of 3-methyladenine was 1.4 mM, and at 7.5 mM, it was 1.9 mM. An average of these values is 1.6 mM. A model, based on the uni-bi-bi mechanism in which 3-MeA is released from the enzyme before release of the second product, the DNA with the apurinic site, could explain these findings.

The apparent K_m for 3-methyladenine DNA glycosylase II was found to be 9.2×10^{-9} M. There was no inhibition of this enzyme by 5 mM 3-methyladenine. It is recognized that these values are approximate. All assays were done at points where the release was linear with time.

7-Methylguanine DNA Glycosylase Activity. The substrate with 3-MeA removed was used to demonstrate the presence of the 7-MeG DNA glycosylase activity in Figure 1. Here, this activity followed the 3-MeA glycosylase II activity.

The rate of release of 7-MeG from acid-treated substrate measured by HPLC was slower than that from the untreated substrate, and the ratio was 1:4.2. The slower release from the acid-treated substrate is probably due to enzyme binding to apurinic sites. No increase in the rate of release of 7-MeG was observed when the aldehyde groups of this substrate were reduced with $NaBH_4$ (Hadi & Goldthwait, 1971) or when a saturating level of the *E. coli* DNA binding protein (kindly provided by Dr. J. Hurwitz) was added. Because of the slower rate of release from the acid-treated DNA, this substrate was not used when careful comparisons of 3-MeA and 7-MeG DNA glycosylase activities were made.

A broad pH optimum for the 7-MeG release between pH 7.0 and 9.0 was observed with the acid-treated substrate. This was comparable to the 3-MeA activity. Similar degrees of inhibition of 7-MeG and 3-MeA release were observed with 0.07 M Hepes–KOH, pH 8.0, compared to 0.07 M Tris-HCl. The inhibitory effect of DNA with apurinic sites on the release of 3-MeA and 7-MeG by glycosylase II was similar. With

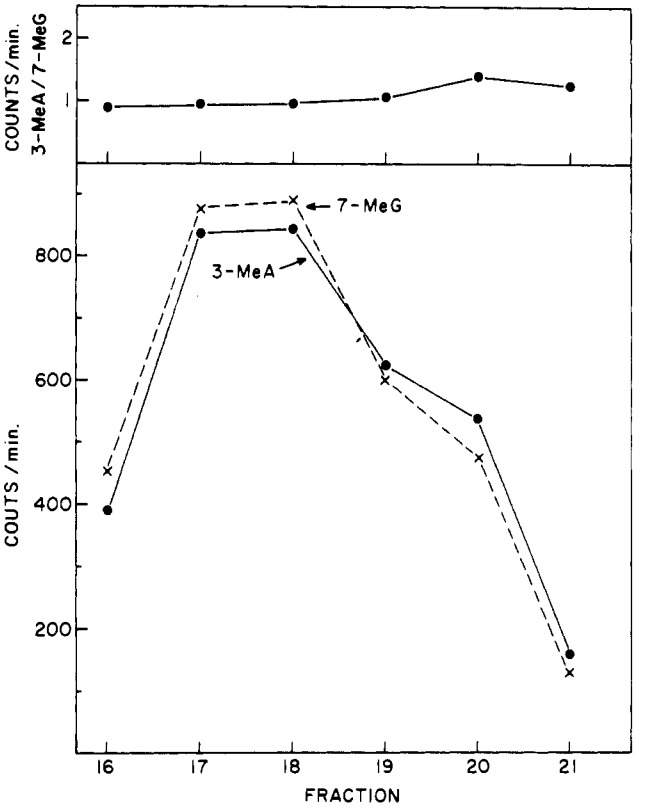

FIGURE 8: Comparison of 3-methyladenine and 7-methylguanine DNA glycosylase activities isolated by isoelectric focusing. The procedure with fraction VIB was similar to that in Figure 2. The 3-MeA release was determined after 10 min and the 7-MeG release after 180 min by using assay B. (●) 3-MeA; (×) 7-MeG release.

5, 10, and 15 μg of this DNA, the 3-MeA release was inhibited 24%, 48%, and 60% while the 7-MeG release was inhibited 32%, 50%, and 63%.

Apparent K_m for 7-Methylguanine DNA. The apparent K_m for 7-MeG, observed with [^{3}H]MNU-treated DNA and HPLC analysis, was 1.1×10^{-8} M. There was no inhibition by 3-MeA at concentrations up to 5 mM.

Attempts To Separate 7-Methylguanine DNA Glycosylase from the 3-Methyladenine DNA Glycosylase II. Many attempts were made to separate the 3-MeA and 7-MeG glycosylase activities, and none were successful. The two activities remained together on isoelectric focusing as shown in Figure 8. The enzyme fractions were incubated with [^{3}H]MNU-treated DNA to make the conditions as comparable as possible, and HPLC analysis was done to measure release of 3-MeA and 7-MeG. Because the relative rates of release of 3-MeA to 7-MeG are approximately 30 to 1, it was necessary to measure the 3-MeA release in 10 min and the 7-MeG release in 180 min in order to be on the linear portion of the curves. As shown in Figure 8, the activities parallel each other. The activities for 3-MeG and 7-MeA also parallel the 3-MeA activity. Comparable results with 3-MeA and 7-MeG were obtained when fraction VIB was applied to Sephadex G-100. Furthermore, these two glycosylase activities followed each other in peak fractions isolated from DEAE-cellulose, phosphocellulose, DNA–cellulose, and carboxymethylcellulose columns. In these cases, the 7-MeG activity was measured by using the acid-treated substrate.

Heat Inactivation of 3-MeG DNA Glycosylase Activities. Figure 9 shows the inactivation of the two glycosylase activities when the preparation was heated at 50 °C. Again analysis was by HPLC. The loss of the enzyme activity for 3-MeA was the same as the loss of the 7-MeG activity. Also the loss

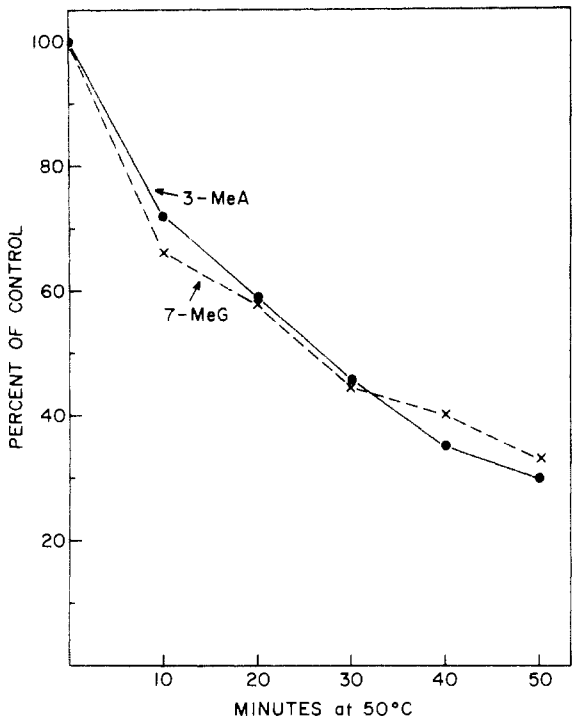

FIGURE 9: Heat inactivation of 3-methyladenine and 7-methylguanine DNA glycosylase activities at 50 °C. The enzyme, fraction VIB, was incubated in 0.02 M Tris-HCl, pH 8.0, 5% glycerol, 1 mM EDTA, and 0.2 mM DTT. A 0.24-unit sample of glycosylase II was tested under the conditions for assay C with 5 μg of [^{3}H]DNA (50000 cpm) for 5 min to determine 3-MeA and 7-MeG release and for 120 min to determine 7-MeG and 7-MeA release. Analysis was by HPLC as described. (●) 3-MeA and (×) 7-MeG release.

of activity for 3-MeG and 7-MeA was similar to that for 3-MeA and 7-MeG. These data provide strong support for the hypothesis that all these activities are present in one protein.

Inability To Demonstrate AP-DNA Adenine/Guanine Transferase Activity. Because of the inhibition of glycosylases I and II by DNA with apurinic sites and of glycosylase I by 3-MeA, it was possible that either glycosylase might also act to insert purine bases at apurinic sites (Deutsch & Linn, 1979). ^{3}H-Alkylated T7 DNA was treated to preferentially remove 3-MeA, and it was then incubated with either glycosylase I or glycosylase II and 1 mM adenine prior to alkaline sucrose gradient centrifugation. No evidence for an enzyme-catalyzed decrease in alkali-labile sites was observed.

Discussion

The isolation of two separate glycosylase activities from *E. coli*, both capable of releasing 3-methyladenine residues from DNA, evolved from attempts to separate the 3-methyladenine DNA glycosylase activity from the 7-methylguanine DNA glycosylase activity. A review of the properties of the two activities for 3-methyladenine is given in Table II.

Karran et al. (1980) isolated mutants of *E. coli*, the growth of which was more sensitive to methyl methanesulfonate than the wild-type strain, and these mutants were reported to be lacking the major 3-methyladenine DNA glycosylase. Our demonstration that 3-methyladenine DNA glycosylase I was absent in the tag 1 and 2 mutants and that the residual 3-methyladenine DNA glycosylase activity in the mutants was approximately 10% of that of the wild-type confirms the results of Karran et al. (1980).

The release of alkylated purine bases has been investigated in vivo in *E. coli* where, besides the rapid removal of 3-MeA (Lawley & Orr, 1970) and 3-EtA (Lawley & Warren, 1975), the removal of 3-MeG was observed (Lawley & Warren, 1976). However, no evidence for the enzymatic removal of 7-MeG (Lawley & Orr, 1970), 7-EtG (Lawley & Warren, 1975), or 7-MeA (Lawley & Warren, 1976) was obtained. The in vitro enzymatic release of 7-MeG has now been observed with the glycosylase II from *E. coli*, with a preparation from *M. luteus* (Laval et al., 1981) in which the activity for 7-MeG is inactivated by heat more readily than the activity for 3-MeA, and with several mammalian enzymes (Cathcart & Goldthwait, 1981; Singer & Brent, 1981; Margison & Pegg, 1981). A note of caution is in order regarding the use of crude extracts for the release of alkylated bases. Exonucleases can liberate 7-methylguanylic acid, which is hydrolyzed spontaneously to 7-methylguanine and deoxyribose phosphate with a $t_{1/2}$ at 37 °C of 16 h (Lawley & Brookes, 1963) as compared to the release of 7-MeG from DNA with a $t_{1/2}$ of 120 h (Lawley & Warren, 1976).

Table II: Comparison of 3-Methyladenine DNA Glycosylases I and II

	3-MeA I	3-MeA II
activity in crude extracts (%)	88	12
mol wt	20000	27000
substrates	3-MeA	3-MeA, 3-MeG, 7-MeA, 7-MeG
app K_m (M)	14×10^{-9}	9.2×10^{-9}
app K_i, 3-MeA (M)	2.4×10^{-3}	no inhibition
50% inactivation, 48 °C (min)	5	65
spermidine	inhibition	stimulation

The total activity of 7-MeG DNA glycosylase in *E. coli* is very low compared to the total 3-MeA DNA glycosylase activity. If the rate with the glycosylase II for 7-MeG is approximately one-thirtieth the rate for 3-MeA and if the level of 3-MeA DNA glycosylase II is only 10% of that of 3-MeA DNA glycosylase I, than the overall activity for 7-MeG may be approximately 0.003 of the activity for 3-MeA. This would explain the apparent absence of removal of 7-MeG in vivo.

The evidence that the 3-MeA DNA glycosylase and the 7-MeG DNA glycosylase are activities on the same protein is as follows: First, in a series of purification procedures including chromatography on DEAE-cellulose, phosphocellulose, DNA–cellulose, carboxymethylcellulose, and Sephadex G-100 and isoelectric focusing, the 3-MeA and 3-MeG activities followed each other. Second, when heated, both activities were lost at a comparable rate. Third, many of the properties of the two activities were the same such as pH optimum, effect of buffers, and inhibition by apurinic sites in DNA. Furthermore, the 3-MeG and 7-MeA activities of the glycosylase II preparation are also probably on the same protein, since these activities followed the 3-MeA and 7-MeG activities on isoelectric focusing and on heat inactivation. A thermosensitive mutant will be required to provide the most definitive proof for this single enzyme hypothesis.

Acknowledgments

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Mouse Kidney Nonpolysomal Messenger Ribonucleic Acid: Metabolism, Coding Function, and Translational Activity†

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ABSTRACT: To elucidate the distribution and function of mRNA in mouse kidney cytoplasm, we compared mRNA isolated from polysomal (>80S) and native postpolysomal (20–80S) ribonucleoproteins with respect to synthesis and lifetime, sequence content, and translational activity. The 20–25% of cytoplasmic mRNA recovered from postpolysomal ribonucleoprotein is similar to polysomal mRNA in size (20–22 S), in apparent half-life (11–13 h), in major products of cell-free translation, and in nucleotide complexity ($\sim 4 \times 10^7$ nucleotides). The labeling kinetics of polysomal and postpolysomal mRNA suggest these mRNA populations are in equilibrium. [^{3}H]cDNAs transcribed from polysomal and from postpolysomal poly(A)-containing mRNAs react with template mRNA and with the heterologous mRNA at the same rate ($C_0t_{1/2}$ ~6.3 mol·s/L) and to the same extent (95%). Therefore, these mRNAs are equally diverse and homologous and occur at similar relative frequencies. Postpolysomal mRNA directs cell-free protein synthesis at only ~30% of the rate of polysomal mRNA and to only 30% of the extent of mRNA from polysomes. Postpolysomal mRNA is ~3-fold less sensitive than polysomal mRNA to inhibition of translation by m^{7}GMP, suggesting postpolysomal mRNA contains a greater fraction of molecules deficient in 5′-terminal caps. Postpolysomal mRNA may derive from renal mRNAs that initiate translation inefficiently and thus accumulate as postpolysomal ribonucleoproteins.

In mammalian cells, newly synthesized messenger RNA exists both in polysomes and in native 20–80S postpolysomal ribonucleoprotein (RNP)[1] particles (Henshaw et al., 1965; Henshaw, 1968; Perry & Kelley, 1968; Spirin, 1969; Spohr et al., 1970). For example, 20–60% of newly synthesized cytoplasmic mRNA in cultured Vero cells (Lee & Engelhardt, 1978), HeLa cells (Spohr et al., 1970), Taper hepatoma cells (McMullen et al., 1979; Kinneburgh et al., 1979), and mouse sarcoma 180 cells (Geoghegan et al., 1979) is in the postpolysome region. The distribution of mRNA between the polysomal and postpolysomal cytoplasmic fractions can be modulated by the growth state of cells (Lee & Engelhardt, 1978), by starvation (Sonenshein & Brawerman, 1977), and

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[1] Abbreviations: poly(A+) mRNA, mRNA that contains poly(adenylate); RNP, ribonucleoprotein; mRNP, messenger ribonucleoprotein; C_0t, in hybridization studies initial concentration of RNA in moles of nucleotide per liter × time of reaction in seconds; EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane.

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