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Partial Purification and Characterization of a Human 3-Methyladenine–DNA Glycosylase†

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ABSTRACT: A DNA glycosylase was purified about 30-fold from cultured human lymphoblasts (CCRF-CEM line) and was found to cleave 3-methyladenine from DNA alkylated with methyl methanesulfonate. The enzyme did not promote the release of 1-methyladenine, 7-methyladenine, or 7-methylguanine from DNA nor did it act on denatured methylated DNA. It produced apurinic sites in DNA alkylated with *N*-methyl-*N*-nitrosourea and ethyl methanesulfonate as well as methyl methanesulfonate but not in untreated DNA or in DNA alkylated with nitrogen mustard or irradiated with ultraviolet light or X-rays. The glycosylase was free of detectable endonuclease activity in experiments with untreated DNA or DNA exposed to ultraviolet light; low levels of endonuclease activity, obtained when X-irradiated, alkylated, or depurinated DNA was the substrate, were attributed to contaminant apurinic endonuclease activity. This 3-methyladenine–DNA glycosylase has an estimated molecular weight of 34 000, is not dependent on divalent metal ions, and shows optimal activity at pH 7.5–8.5.

The initial enzymatic step in the excision repair of damaged DNA was long believed to be mediated by an endonuclease. However, a recently discovered class of enzymes, termed DNA glycosylases, can recognize an abnormal base in DNA and cleave it from the deoxyribose moiety, leaving an apurinic or apyrimidinic (AP)[1] site. The glycosylases identified thus far act either on uracil in DNA or on alkylated DNA. Enzymes with the former activity have been isolated from *Escherichia coli* (Lindahl, 1974), *Bacillus subtilis* (Friedberg et al., 1975), and human cells (Sekiguchi et al., 1976; Teebor et al., 1978), while those with the latter have been isolated only from *E. coli* (Kirtikar & Goldthwait, 1974; Riazuddin & Lindahl, 1978) and *Micrococcus luteus* (Laval, 1977). Thus, the initial event in excision repair may be the recognition and cleavage of an abnormal base by DNA glycosylase, with incision of the DNA strand by an AP-endonuclease constituting the second step.

During efforts to characterize an endonuclease activity isolated from human lymphoblasts that acted on either ultraviolet (UV)- or X-irradiated DNA, a new activity specific for methylated DNA became apparent (Brent, 1977). The enzyme was evidently not endonucleolytic and, since it produced AP sites in methylated DNA, it was inferred to be a glycosylase. This paper describes the preliminary purification and characterization of this enzyme from cultured human lymphoblasts; the results confirm that it does indeed catalyze the release of methylated bases from DNA.

Materials and Methods

(*1*) *Cell Growth.* Human lymphoblasts of the CEM-CCRF line were grown in magnetically stirred suspensions in Eagle's minimum essential medium that contained Spinner salts and was supplemented with 10% fetal calf serum. The cultures were maintained in logarithmic growth phase until the time of harvest.

(*2*) *Enzyme Purification.* (a) Extract Preparation. About 1×10^{10} cells were harvested by low-speed centrifugation (200*g* for 5 min) before being washed with phosphate-buffered saline, pH 7.2, at 4 °C. All subsequent steps were carried out at 0 or 4 °C.

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[1] Abbreviations used: AP, apurinic or apyrimidinic; UV, ultraviolet; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; DEAE, diethylaminoethyl; MMS, methyl methanesulfonate; EMS, ethyl methanesulfonate; MNU, *N*-methyl-*N*-nitrosourea.

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The final cell pellet was resuspended in three volumes of 50 mM tris(hydroxymethyl)aminomethane (Tris) hydrochloride buffer (pH 7.5) containing 2 mM ethylenediaminetetraacetic acid (EDTA), 1 mM dithiothreitol, and 100 mM NaCl. The cells were disrupted with a Dounce homogenizer and forced through a 21-gauge needle. The homogenate was centrifuged in a Beckman Type 50 Ti rotor at 45 000 rpm for 45 min. The resulting supernatant was termed fraction I.

(b) Removal of Nucleic Acids. Fraction I was dialyzed against 0.2 M $(NH_4)_2SO_4$, 0.2 M potassium phosphate (pH 7.0), before being applied to a DEAE-cellulose column (Whatman DE-52, 25 × 1.7 cm diameter) that was equilibrated with the same buffer. Further elution with the same buffer yielded a peak of protein that was essentially free of nucleic acids. When pooled, the material constituted fraction II.

(c) Ammonium Sulfate Fractionation. A saturated solution of ammonium sulfate was slowly added to fraction II at 0 °C with continuous stirring. The material that precipitated between 20 and 40% saturated ammonium sulate was centrifuged at 10000*g* for 20 min and redissolved in 10 mM Tris-HCl (pH 7.5), 2 mM EDTA, 1 mM dithiothreitol, 150 mM NaCl, 10% glycerol, and 0.02% sodium azide (buffer A). This product, after extensive dialysis against buffer A, became fraction III.

(d) DNA–Agarose Chromatography. DNA–agarose was prepared according to the procedure of Schaller et al. (1972). Calf-thymus DNA (Sigma V) dissolved in 0.02 M NaOH was irradiated with ultraviolet light (10 000 J/m^2) before mixing with agarose (Sigma; electrophoretic grade).

A column measuring 8 × 2.5 cm in diameter and packed with DNA–agarose was equilibrated with buffer A. Four milliliters of fraction III were applied to the column and washed slowly with about 50 mL of buffer A. The column was then eluted with a linear NaCl gradient (0–1.0 M) in 200 mL of buffer A. Fractions (2 mL) were collected and assayed for enzyme activity. The bulk of activity eluted as a peak between 0.4 and 0.5 M NaCl. The peak fractions were pooled, dialyzed against buffer A, and concentrated about fivefold under pressure in an Amicon ultrafiltration unit with an UM-2 membrane filter. This material, designated fraction IV, was stored at −70 °C and was stable for several months.

(*3*) *Preparation of DNA and Polydeoxynucleotide Substrates.* PM2 DNA labeled with [^{3}H]dThd was isolated from bacteriophage by the methods of Espejo et al. (1968, 1969). The DNA was alkylated with methyl methanesulfonate (MMS; 5 × 10^{-3} M) for 20 min at 37 °C and isolated again by G-50 Sephadex chromatography as previously described (Brent, 1977). A similar procedure was used to alkylate PM2 DNA with ethyl methanesulfonate (EMS, 5 × 10^{-2} M for 40 min), *N*-methyl-*N*-nitrosourea (10^{-3} M for 20 min), or nitrogen mustard (10^{-4} M for 20 min). The labeled PM2 DNA was partially depurinated by heating to 70 °C at pH 5 for 5 min or was irradiated with ultraviolet light (1000 J/m^2) or X-rays (3000 rad) as described earlier (Brent, 1975, 1976).

^{14}C-Labeled MMS (250 μCi; sp act. 52 mCi/mmol) was added to 1 mg of calf-thymus DNA in 200 μL of 20 mM Tris-HCl (pH 7.5), 1 mM EDTA, 0.1 M NaCl, and 0.02% sodium azide (buffer B) to a final concentration of 2.4 × 10^{-2} M. The solution was then incubated for 5 h at 37 °C. Poly(dA-dT), 500 μg, was similarly alkylated in 500 μL of buffer B with 200 μCi of ^{14}C-labeled MMS (final concentration 7.7 × 10^{-3} M). Poly(dG-dC), 500 μg, was alkylated in 200 μL of buffer B with 50 μCi of ^{14}C-labeled MMS (final concentration 4.8 × 10^{-3} M). In each instance the ^{14}C-labeled methylated polynucleotide was reisolated by gel filtration on Sephadex G-50.

Calf-thymus DNA alkylated with ^{14}C-labeled MMS was denatured by adjusting the pH to 12.65 at 0 °C for 1 min and then neutralizing it (pH 7.5).

(*4*) *Assays for Methylated Base Release.* (a) Gel Filtration. One hundred microliters or less of solution containing methylated polynucleotides was applied to a column (28 × 0.7 cm diameter) of Sephadex G-10. The column was eluted at about 10 mL/h with buffer B, and 0.5-mL fractions were collected and assayed for radioactivity by liquid scintillation spectrometry. Elution volumes were established for different methylated base markers: 1-methyladenine, 3-methyladenine, 7-methylguanine, and 7-methyladenine. The first three markers were purchased from Tridon-Fluka, New York; the fourth was a gift from Dr. R. Cox, VA Hospital, Memphis, TN.

(b) Thin-Layer Chromatography. About 10 μg of each methylated base marker was added to each 100 μL of reaction mixture containing ^{14}C-labeled methylated polynucleotides. Aliquots of the reaction mixture were applied directly to cellulose thin-layer plates (20 × 20 cm). Ascending chromatography made use of the solvent system described by Kirtikar & Goldthwait (1974): 2-methyl-1-propanol–0.8 M boric acid–14.8 M NH_4OH (100:14:0.4 by volume). An alternative solvent system consisted of 2-methy-1-propanol–NH_4OH–H_2O (7:1:2 by volume). Radioactivity was determined either by autoradiography or by cutting out the UV-absorbing marker spots, extracting them with 1.0 mL of 0.01 N HCl, and assaying the radioactivity by liquid scintillation spectrometry.

(*5*) *DNA Glycosylase Assay.* In the assay used during purification and preliminary characterization of the enzyme, AP-endonuclease was included in the reaction mixture so that base release mediated by glycosylase would be followed immediately by DNA strand scission. The assay mixture (115 μL) consisted of about 0.1 μg of ^{3}H-labeled PM2 DNA alkylated with MMS (5 × 10^{-3} M; 30 min; 37 °C) together with 0.05 M *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (Hepes) potassium hydroxide (pH 8.0), 0.5 mM $MgCl_2$, and 5 μL of AP-endonuclease [purified from CEM-CCRF cells as previously described (Brent, 1976)]. To this was added 10 μL of enzyme in buffer A prior to incubation for 30 min at 37 °C. The reaction was stopped by addition of 50 μL of 10 mM EDTA with 1 mg of Proteinase K/mL and continuation of incubation at 37 °C for a further 5 min. The fraction of nicked (Form II) PM2 DNA was determined by use of the filter-binding assay described earlier (Brent, 1976). To ensure that AP-endonuclease activity was not limiting in this coupled assay, 5 μL of the AP-endonuclease was required to induce two breaks per molecule in less than 5 min at 37 °C in heat-depurinated PM2 DNA that contained two AP sites per molecule (i.e., 24 units of activity, where a unit induces one break per PM2 DNA molecule per hour).

A unit of DNA glycosylase in the coupled assay was defined as that activity producing one AP site per molecule of methylated PM2 DNA per hour. This value was calculated as the difference between strand breaks produced by glycosylase in combination with AP-endonuclease and strand breaks produced by AP-endonuclease alone.

(*6*) *Other Methods.* Assays for protein were done with a commercially available modification (Bio-Rad Laboratories) of the method of Bradford (1976). DNA concentrations were determined from absorbance at 260 nm, by assumption of an

Table I: Purification of DNA Glycosylase from CCRF-CEM Cells

fraction	vol (mL)	protein (mg)	units	sp act. (units/mg of protein)
I. high-speed supernatant	74.0	762	103×10^3	136
II. DEAE-cellulose	80.0	536	59×10^3	111
III. ammonium sulfate	4.5	180	45×10^3	250
IV. DNA-agarose	9.9	1.5	5×10^3	3333

absorption coefficient of 0.02 mL μg^{-1} cm^{-1}. Molecular weight was estimated by gel filtration with Sephadex G-100 (superfine) equilibrated with buffer A in a column measuring 60 × 0.9 cm in diameter. The column was calibrated with aldolase, ovalbumin, chymotrypsin A, and ribonuclease A.

Results

Enzyme Purification. The results of a typical purification of the enzyme are summarized in Table I. DEAE-cellulose chromatography, which removed the nucleic acids, did not produce any purification; rather the specific activity of fraction II was reduced, an indication that the enzyme could be bound to nucleic acids. Ammonium sulfate fractionation removed most of the AP-endonuclease activity, which does not precipitate at sulfate concentrations below 45% saturation. DNA-agarose chromatography (Figure 1) separated the enzyme from the AP-endonuclease remaining in fraction III and from endonuclease activities for UV- and X-irradiated DNA as well. The peak of glycosylase (fraction IV), eluting at 0.4–0.5 M NaCl, bound more strongly to the DNA-agarose than did the endonucleases. The overall purification was typically 25- to 50-fold with about 5% recovery of activity.

The molecular weight of the enzyme, as estimated by gel filtration with Sephadex G-100, was 34 000.

Substrate Specificity. Figure 2A shows the kinetics of fraction IV reacting with MMS-treated PM2 DNA in the presence or absence of added AP-endonuclease. As determined from the action of AP-endonuclease alone, the alkylated DNA contained about 0.2 apurinic site per molecule. By itself, fraction IV produced only slight endonucleolytic activity, but when its reaction was coupled with AP-endonuclease the rate of strand breakage increased tenfold, reaching a maximum of 1.5 breaks per molecule in 20 min. Thus the increment of 1.3 AP sites represents DNA glycosylase activity. When the same experiment was performed with heat-depurinated DNA containing about one AP site per molecule, fraction IV again showed a low level of endonuclease activity and its strand-breaking activity in combination with AP-endonuclease was no greater than observed with the endonuclease alone (Figure 2B). Thus, the nuclease activity in fraction IV for MMS-treated DNA appears to be specific for AP sites and is probably a contaminant.

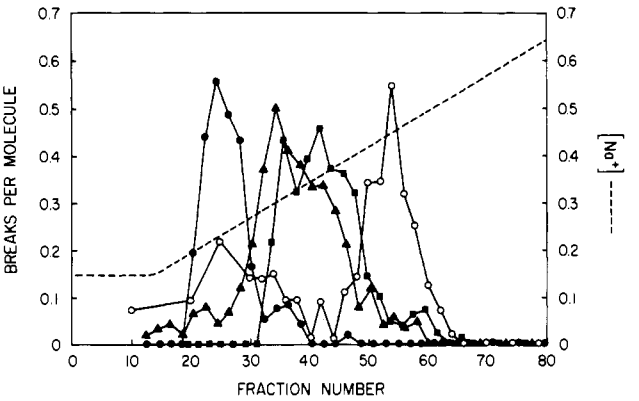

FIGURE 1: DNA-agarose chromatography of fraction III. DNA glycosylase activity (○) was assayed with MMS-treated PM2 DNA in the presence of Mg^{2+} (0.5 mM) with the addition of nonlimiting amounts of AP-endonuclease (25 units). Endonuclease activity specific for UV-irradiated (▲) or X-irradiated (■) PM2 DNA was assayed in the presence of EDTA (2 mM). (●) AP-endonuclease activity was assayed with partially depurinated PM2 DNA (Brent, 1976). The number of breaks per molecule in DNA incubated without enzyme was subtracted for each point.

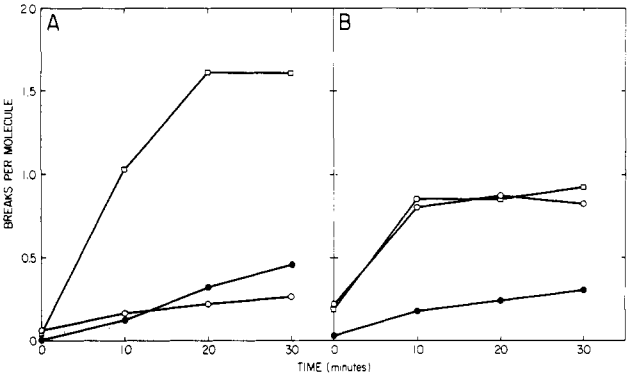

FIGURE 2: Kinetics of the enzyme reaction with (A) methylated PM2 DNA or (B) heat-depurinated PM2 DNA containing about 1 AP site per molecule. The DNA was incubated under the standard DNA glycosylase assay conditions with either 25 units of AP-endonuclease (○) or 5 units of glycosylase (●) or both enzymes (□). The average number of strand breaks in DNA incubated without enzyme has been substracted from each value.

Table II: Substrate Specificity[a]

DNA treatment	DNA strand breakage with: fraction IV (5 units) (1)	AP-endonuclease (25 units) (2)	fraction IV coupled with AP-endonuclease (3)	AP-site production by DNA glycosylase [(3) − (2)]
none	0.00	0.00	0.00	0.00
UV	0.00	0.10	0.14	0.04
X-rays	0.10	0.40	0.44	0.04
heat depurination	0.24	0.87	0.85	−0.02
MMS	0.32	0.22	1.61	1.39
MNU	–	0.15	0.60	0.45
EMS	–	0.13	0.48	0.35
nitrogen mustard	–	0.40	0.35	−0.05

[a] Reactions were carried out for 20 min under standard conditions for the glycosylase assay. PM2 DNA substrates were prepared as described under Materials and Methods. Each value represents the average number of strand breaks per molecule of PM2 DNA. Columns 1 and 3 were taken from the linear portion of the time course and represent reaction rates. Column 2 is the plateau value reached and represents the number of AP sites per molecule.

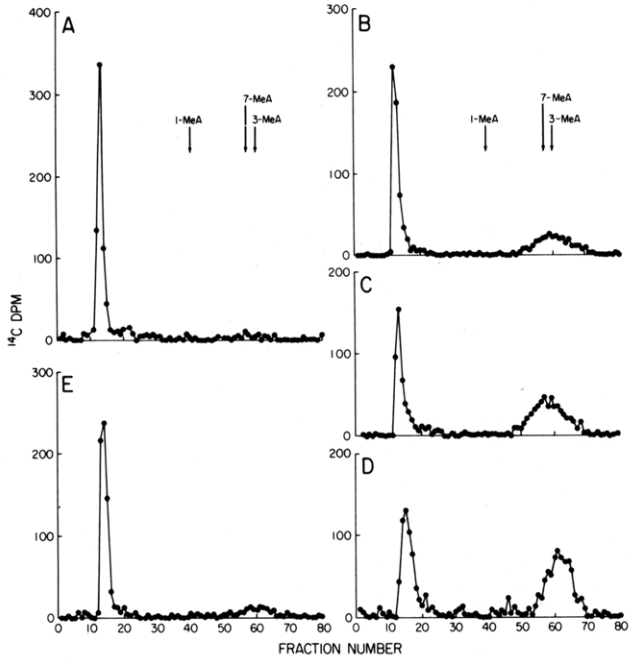

FIGURE 3: Elution profiles from Sephadex G-10 chromatography of methylated poly(dA-dT). Before the samples were chromatographed, about 20 μg of ^{14}C-labeled methylated poly(dA-dT) in 100 μL of buffer B was incubated at 37 °C with glycosylase (50 units in 100 μL of buffer A) for 0 (A), 15 (B), 30 (C), or 100 min (D). The control sample was incubated with 100 μL of buffer A for 100 min (E). The arrows indicate the elution positions of markers: 1-methyladenine (1-MeA), 3-methyladenine (3-MeA), and 7-methyladenine (7-MeA). The peak of radioactivity eluting at about fraction 13 is poly(dA-dT).

Similar experiments were performed with DNA that was either untreated or damaged by a variety of agents (Table II). DNA alkylated by MMS, EMS, or MNU served as a substrate for the DNA glycosylase activity, whereas DNA alkylated by nitrogen mustard, X-irradiated, UV-irradiated, depurinated, or untreated DNA did not. Fraction IV contained no detectable endonuclease activity for either untreated or UV-irradiated DNA. X-irradiated DNA elicited slight endonucleolytic activity, probably because of the contaminant AP-endonuclease, which would act on the AP sites known to be present in such DNA (Brent, 1976). Endonuclease activity was higher with depurinated or MMS-treated DNA, both containing more AP sites than found in X-irradiated DNA.

Specific Base Release. Further experiments were needed to demonstrate that specific bases were in fact being cleaved from methylated DNA by the apparent glycosylase residing in fraction IV. Also needed, as a means of determining optimal reaction conditions, was as assay that did not depend on a second enzyme (AP-endonuclease). Earlier experiments (Brent, 1977) had shown that the glycosylase acted on only a small fraction of the alkylation products in MMS-treated DNA, suggesting that the substrate was methylated adenine rather than the predominant 7-methylguanine. If so, spontaneous depurination of 7-methylguanine would cause an appreciable background of nonenzymatic base release; hence, initial experiments were done with poly(dA-dT) alkylated with ^{14}C-labeled MMS. The methylated substrate was incubated with glycosylase or with buffer for various times before chromatographic analysis of the reaction mixture on Sephadex G-10. Figure 3 shows that the enzyme mediates the release of ^{14}C-labeled material eluting in the region corresponding to 3- and 7-methyladenine and hence acts as a DNA glycosylase. 1-Methyladenine was not released, although about 15% of the alkylation product in the polymer did occur in this form. When a similar experiment was done with methylated poly(dG-dC),

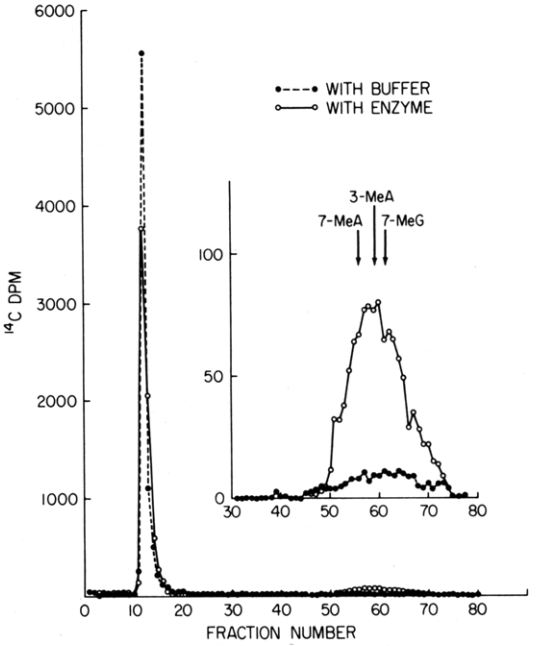

FIGURE 4: Elution profiles from chromatography of methylated calf-thymus DNA on Sephadex G-10. ^{14}C-labeled methylated calf-thymus DNA (20 μg in 100 μL of buffer B) was incubated for 30 min at 37 °C with 50 units of glycosylyase (O) or with buffer A (●) before chromatography. The arrows indicate the elution positions of 3-methyladenine (3-MeA), 7-methyladenine (7-MeA), and 7-methylguanine (7-MeG).

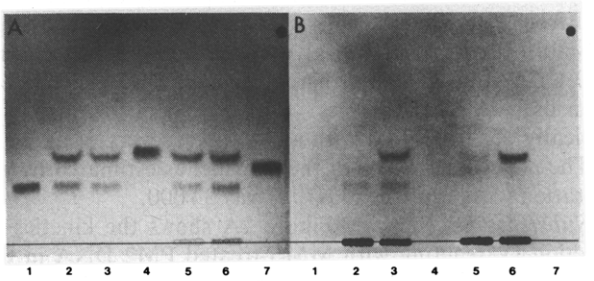

FIGURE 5: DNA glycosylase reaction products analyzed by thin-layer chromatography in 2-methyl-1-propanol–boric acid–NH_4OH. Panel A is a photograph of thin-layer plate illuminated with short-wave ultraviolet light. Panel B is an autoradiograph of the same thin-layer plate. The samples consisted of: (1) 7-methylguanine marker; (2) ^{14}C-labeled methylated calf-thymus DNA (5 μg in 25 μL of buffer B) after incubation at 37 °C for 1 h with 10 μL of buffer A; (3) the same DNA after similar incubation with 12.5 units of glycosylase; (4) 3-methyladenine marker; (5) ^{14}C-labeled methylated poly(dA-dT) (5 μg in 25 μL of buffer B) after incubation at 37 °C for 1 h with 10 μL of buffer A; (6) poly(dA-dT) as in (5) after incubation with 12.5 units of glycosylase; and (7) 1-methyladenine plus 7-methyladenine markers. 3-Methyladenine and 7-methylguanine markers were included with samples 2, 3, 5, and 6.

with 7-methylguanine comprising virtually all of the methylation product, no enzyme-catalyzed base release could be detected. Figure 4 shows the results of the same type of experiment in which methylated calf-thymus DNA was used as a substrate. Most of the alkylation product was resistant to the enzyme, with only a small proportion of methylated base being released enzymatically. Since G-10 Sephadex chromatography did not resolve the identity of the base, whether 3-methyladenine, 7-methyladenine, or 7-methylguanine, thin-layer chromatography was used in subsequent analyses. Figure 5 shows an autoradiographic analysis of ^{14}C-labeled methylated poly(dA-dT) and ^{14}C-labeled methylated calf-thymus DNA, incubated with or without enzyme. In each instance the polymer remained at the origin. Incubation of poly(dA-dT) with glycosylase resulted in the release of a single

Table III: Enzyme-Mediated Specific Base Release[a]

polynucleotide	base	amount of base released (pmol) buffer	fraction IV
poly(dA-dT)	3-methyladenine	1.2	7.4
	1-methyladenine + 7-methyladenine	1.0	1.1
DNA	3-methyladenine	2.6	12.8
	7-methylguanine	3.0	4.1
denatured DNA	3-methyladenine	1.2	1.8
	7-methylguanine	2.8	3.4

[a] ^{14}C-Labeled methylated poly(dA-dT) (10 μg) was incubated for 60 min with 20 units of fraction IV or buffer A. ^{14}C-Labeled methylated DNA (native or denatured) (8 μg) was incubated for 60 min with 30 units of enzyme or with buffer A. After thin-layer chromatography of the reaction products, as in Figure 5, spots identified by UV absorption of markers were cut out, eluted, and assayed for radioactivity. The amount of methylated base was calculated by use of the specific radioactivity of the labeled MMS.

prominant radioactive base coinciding with the 3-methyladenine marker, whereas only faint traces of label corresponding to 3-methyladenine and 1-methyladenine plus 7-methyladenine were found in the buffer-treated control. For DNA, three faint radioactive spots, corresponding to 3-methyladenine, 7-methylguanine, and 1-methyladenine plus 7-methyladenine, appeared after incubation with buffer. Incubation with glycosylase resulted in a prominent radioactive spot corresponding to 3-methyladenine with no obvious increase in the other methylated bases. The same results were obtained with a second chromatography solvent system (see Materials and Methods).

Spots corresponding to 3-methyladenine, 1-methyladenine plus 7-methyladenine, and 7-methylguanine were identified by UV absorbance of markers and then cut from the thin-layer plates, eluted, and assayed for radioactivity. Table III shows the amounts of methylated base that were removed from poly(dA-dT), native DNA, or denatured DNA by the glycosylase or were lost spontaneously in buffer. The amount of 3-methyladenine released by the enzyme from double-stranded substrates was about five times greater than that released spontaneously, whereas the release of other methylated bases was not appreciably altered. Denatured methylated DNA did not serve as a substrate for the enzyme.

General Properties of Enzyme. For subsequent characterization of the enzyme, the assay employed ^{14}C-labeled methylated poly(dA-dT) or ^{14}C-methylated calf-thymus DNA as substrate. After incubation of the substrate with the enzyme, 3-methyladenine was added as a marker, the reaction products were separated by thin-layer chromatography, as described earlier, and the UV-absorbing spot corresponding to 3-methyladenine was cut out and eluted. The radioactivity thus recovered was taken as a measure of 3-methyladenine–DNA glycosylase activity.

When 20 units of enzyme was incubated with 10 μg of methylated poly(dA-dT), the release of 3-methyladenine was linear with time up to 90 min. Linearity was also observed with increasing amounts of enzyme up to 10 μg of protein per assay (30 units of enzyme) when incubation was for 1 h with 10 μg of methylated poly(dA-dT). The pH response of enzymatic 3-methyladenine release showed a broad optimum between pH 7.5 and 8.5 in 55 mM Hepes–KOH. At pH 6.5, enzyme activity was 55%, and at pH 9.5 enzyme activity was 70% of that at pH 8.0. The enzyme was active in 1 mM EDTA and was not stimulated by Mg^{2+}. Its activity was completely abolished by heating to 50 °C for 30 min. At 4 °C in buffer A the enzyme lost activity rapidly over a period of days. After being frozen in buffer A at −70 °C, it remained stable for several months.

Discussion

The human enzyme described in this report produces AP sites in methylated or ethylated DNA, characteristic of DNA glycosylases. When incubated with MMS-treated deoxyribonucleotide polymers, it causes the release of 3-methyladenine specifically but not 1-methyladenine, 7-methyladenine, or 7-methylguanine. It does not act on DNA either alkylated with nitrogen mustard or irradiated with UV or X-rays nor does it act on single-stranded MMS-treated DNA. By itself, the enzyme produces low endonucleolytic activity with methylated DNA, but when coupled with nonlimiting amounts of AP-endonuclease, its rate of DNA strand breakage increases strikingly. These properties are similar in many respects to the DNA glycosylases purified from *E. coli* (Riazuddin & Lindahl, 1978) and *M. luteus* (Laval, 1977). Each of these bacterial enzymes catalyzes the release of 3-methyladenine, but not 7-methylguanine, from DNA. The human and bacterial enzymes also resemble each other in that they bind tightly to DNA–agarose and are active in EDTA independent of Mg^{2+}.

The functional significance of a 3-methyladenine–DNA glycosylase is not yet clear. 3-Methyladenine is actively released in vivo from *E. coli* DNA at a much higher rate than observed in vitro under physiological conditions, whereas 7-methyladenine and 7-methylguanine are released at comparable rates in vivo and in vitro (Lawley & Orr, 1970; Lawley & Warren, 1976). The modes of release for ethylation and methylation products in *E. coli* DNA are essentially the same (Lawley & Warren, 1975). Observations of alkylated base release from bacterial DNA in vivo are entirely consistent with the properties of the *E. coli* 3-methyladenine–DNA glycosylase described by Riazuddin & Lindahl (1978). 3-Methyladenine but not 7-methylguanine is also rapidly removed from DNA in mammalian cells (Walker & Ewert, 1973; Margison & O'Connor, 1973). It seems likely, therefore, that the enzyme described in this report is the mammalian counterpart of the bacterial enzyme and, together with AP-endonuclease, is involved in the excision repair of 3-alkyladenine lesions that occur in human DNA.

O^6-Methylguanine is actively released from DNA in vivo to both bacteria (Lawley & Orr, 1970) and human cells (Goth-Goldstein, 1977) but does not serve as a substrate for the *E. coli* glycosylase (Riazuddin & Lindahl, 1978). Similarly, the results of preliminary experiments with DNA treated with *N*-methyl-*N*-nitrosourea (T. Brent, unpublished experiments) indicate that the human enzyme does not excise this methylation product either. Xeroderma pigmentosum cells appear to be defective in excision of O^6-methylguanine (Goth-Goldstein, 1977), suggesting that the lesion is repaired via the UV-dimer (nucleotide) excision pathway, which presumably is initiated by an endonuclease rather than DNA glycosylase, as in the base-excision pathway.

The physiological importance of a persistent 3-alkyladenine lesion in DNA should become apparent with the isolation of bacterial mutants defective in 3-methyladenine–DNA glycosylase (Riazuddin & Lindahl, 1978). Cerutti (1975) has suggested that the presence of 3-alkylpurines in DNA would cause a major distortion of the helix in contrast to 7-alkylguanine, for example, which would cause negligible distortion. Thus one might expect this type of lesion to be both mutagenic and carcinogenic. If 3-alkyladenine, as a component of DNA,

proves to have no great biological significance, then other substrates might be sought to clarify the function of this glycosylase.

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Analysis of the Message-Sequence Content of the Pulse-Labeled Poly(A)+ Heterogeneous Nuclear RNA from HeLa Cells by cDNA-Excess Hybridizations†

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ABSTRACT: The message-sequence content of pulse-labeled poly(A)+ HeLa heterogeneous nuclear RNA (hnRNA) has been examined by hybridizations to an excess of message cDNA. Control experiments show that the message cDNA accurately reflects the sequence distribution of the complex mixture of poly(A)+ messages present in the HeLa cytoplasm. Pulse-labeled poly(A)+ molecules in both the lamina-associated and shnRNA fractions contain message sequences, and approximately 65% of the poly(A)-adjacent hnRNA sequences are homologous to the 3′ ends of mRNA. The majority of the pulse-labeled hnRNA molecules contain abundant message sequences. By use of these techniques it is also shown that some pulse-labeled polyadenylated message sequences are still synthesized in the presence of the adenosine analogue 5,6-dichloro-β-D-ribofuranosylbenzimidazole under conditions where little or no new cytoplasmic mRNA is produced.

The synthesis of heterogeneous nuclear RNA (hnRNA)[1] and its relationship to cytoplasmic messenger RNA (mRNA) have been studied for many years (for reviews see Lewin, 1975a,b; Perry, 1976). While the metabolism of hnRNA is quite complex and only a small portion is exported to the cytoplasm (Perry et al., 1974; Herman & Penman, 1977), several lines of evidence suggest that mRNA is derived from the nuclear transcripts. Various experiments have shown that hnRNA is posttranscriptionally modified at both the 5′ and 3′ termini and these modified structures can be found in cytoplasmic mRNA (reviewed by Perry, 1976). Hybridization experiments have shown that steady-state poly(A)+ hnRNA contains the sequences for poly(A)+ mRNA (Herman et al., 1976; Levy et al., 1976; Jacquet et al., 1978). In addition, nuclear precursors, larger than the mature products, have been identified for several specific cellular messages (Ross et al., 1976; Curtis & Weissmann, 1976; Kwan et al., 1977; Bastos & Aviv, 1977; Gilmore-Herbert & Wall, 1978).

In HeLa cells two distinct subfractions of hnRNA have been observed. When isolated HeLa nuclei are exposed to high ionic strength ammonium sulfate, 10% of the mass of the pulse-labeled hnRNA is eluted (shnRNA) while the other 90% remains firmly attached to the nuclear lamina (Price et al., 1974). These two fractions differ both in their sedimentation profile and in their metabolic stability (Price et al., 1974;

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[1] Abbreviations used: hnRNA, heterogeneous nuclear RNA; shnRNA, small heterogeneous nuclear RNA subfraction; C_0t, the product of the concentration times the time of incubation; cDNA, complementary DNA; EDTA, ethylenediaminetetraacetate; $NaDodSO_4$, sodium dodecyl sulfate; DRB, 5,6-dichloro-β-D-ribofuranosylbenzimidazole.

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