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Purification of Nuclear and Mitochondrial Uracil–DNA Glycosylase from Rat Liver. Identification of Two Distinct Subcellular Forms†

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ABSTRACT: Rat liver uracil–DNA glycosylase has been purified from nuclear extracts over 3000-fold to apparent homogeneity as determined by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. The enzyme is a monomeric protein with a polypeptide molecular weight of approximately 35 000. It has a native molecular weight of 33 000 as determined by gel filtration chromatography and a sedimentation coefficient of 2.6 S in glycerol gradients. The nuclear enzyme has an alkaline pH optimum and a p*I* value of 9.3. Nuclear uracil–DNA glycosylase catalyzes the release of free uracil from both single-stranded and double-stranded DNA with the former being the preferred substrate. The enzyme is unable to recognize dUTP, dUMP, or poly(dA-dT) containing a 3′-terminal uracil residue as a substrate. However, internalization of terminal uracil residues by limited chain elongation produced a substrate for the glycosylase. Another species of uracil–DNA glycosylase has been partially purified from mitochondria. This activity differs from the nuclear enzyme in that it has (i) distinctive chromatographic properties, (ii) a lower native molecular weight of 20 000 as determined by molecular sieving, (iii) a distinct NaCl inhibition profile, and (iv) a longer half-life during thermal denaturation.

Uracil residues can be introduced into DNA during DNA synthesis or by chemical modification of existing cytosine in DNA. The incorporation of deoxyuridine monophosphate into DNA occurs efficiently by DNA polymerases, since no significant difference exists between the K_m values for dUTP and dTTP (Shlomai & Kornberg, 1978; Dube et al., 1979). Thus, dUMP incorporation, in place of dTMP, into nascent DNA is primarily dependent on the relative intracellular concentration of the two deoxyribonucleoside triphosphates. Under normal physiological conditions, the dUTP to dTTP ratio in mammalian cells is less than $1/10^5$ (Goulian et al., 1980a). Intracellular dUTP is normally limited by the action of deoxyuridinetriphosphatase which rapidly hydrolyzes dUTP to dUMP and PP_i (Williams & Cheng, 1979; Ingraham & Goulian, 1982). However, perturbations that increase the cellular dUTP/dTTP ratio result in increased incorporation of dUMP into DNA which contributes to cytotoxic effects (Goulian et al., 1980a). Uracil may also arise in nonreplicating DNA upon deamination of cytosine residues by hydrolytic reactions (Lindahl & Nyberg, 1974) or by mutagenic agents (Shapiro & Pohl, 1968; Shapiro et al., 1973). On the basis of the rate of spontaneous cytosine deamination of duplex DNA, the extent of uracil formed per genome during each mammalian cell generation could account for the conversion of at least 150 cytosine to uracil residues. However, the extent of cytosine deamination may actually be significantly higher since cytosine in single-stranded DNA deaminates at a rate 200–300 times faster than that in a double-stranded polymer (Lindahl, 1979). Deamination of cytosine in duplex DNA forms G·U mismatched base pairs which are premutagenic lesions. If such uracil residues remain in the genome during DNA replication, G·C → A·T transition mutations will occur (Duncan & Miller, 1980). Fortunately, uracil residues are normally introduced only as transient components of prokaryotic and eukaryotic DNA (Tye et al., 1977; Goulian et al., 1980b).

Uracil–DNA glycosylases catalyze the removal of uracil from DNA by cleaving the N-glycosylic bond between uracil and deoxyribose (Lindahl et al., 1977; Lindahl, 1982). This process leaves an apyrimidinic site in the phosphodiester chain

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and initiates base excision repair. In mammalian cells, apyrimidinic sites can be excised by the sequential action of some class I and II apurinic/apyrimidinic (AP)[1] endonucleases which cleave on the 3′ and 5′ sides of the deoxyribose residue, respectively, releasing a 5′ baseless sugar phosphate (Mosbaugh & Linn, 1980). The incised DNA product contains a one-nucleotide gap with a 3′-hydroxyl and a 5′-phosphomonoester terminus that can be filled by DNA polymerase β and covalently closed by DNA ligase (Mosbaugh & Linn, 1983). Alternatively, the baseless site can be removed by the combined action of an AP endonuclease and exonuclease (Bose et al., 1978; Goffin & Verly, 1982; Mosbaugh & Linn, 1983). Under this condition, depending on the size of the excision tract, DNA polymerase β alone or in combination with DNA polymerase α can fill the gap prior to ligation (Wang & Korn, 1980; Mosbaugh & Linn, 1984).

Uracil–DNA glycosylase was first purified to homogeneity from *Escherichia coli* (Lindahl et al., 1977) but has been identified in partially purified fractions of several eukaryotic cells (Kuhnlein et al., 1978; Wist et al., 1978; Sirover, 1979; Gupta & Sirover, 1981; Krokan & Wittwer, 1981; Talpaert-Borle et al., 1982; Arenaz & Sirover, 1983) and plant tissues (Talpaert-Borle & Liuzzi, 1982; Blaisdell & Warner, 1983). In addition, human uracil–DNA glycosylase has been extensively purified and characterized from blast cells of acute myelocytic leukemia patients (Caradonna & Cheng, 1980). Multiple forms of eukaryotic uracil–DNA glycosylase have been reported with molecular weights ranging from 18 000 to 50 000 (Wist et al., 1978; Sirover, 1979; Krokan, 1981). However, in most systems, the subcellular location of the enzyme(s) has not been established, although nuclear, mitochondrial, and cytoplasmic forms have been described (Anderson & Friedberg, 1980; Gupta & Sirover, 1981).

In this report, we (i) describe the purification to apparent homogeneity of uracil–DNA glycosylase isolated from rat liver nuclei, (ii) characterize the physical and catalytic properties of the enzyme preparation, and (iii) demonstrate that the nuclear enzyme exhibits distinctly different properties from those of the mitochondrial-associated uracil–DNA glycosylase.

Experimental Procedures

Materials

Unlabeled deoxyribonucleotide triphosphates and monophosphates, calf thymus DNA, and uracil were purchased from Sigma. Ultrapure sucrose and ammonium sulfate came from Bethesda Research Laboratories and ampholines from LKB. Poly(dA-dT), DEAE-Sephadex A-50, and Sephadex G-75 were purchased from Pharmacia; DE-52 cellulose, P-11 phosphocellulose, and 3MM chromatographic paper were from Whatman; and AG 1-X8 (chloride form) resin was from Bio-Rad. [*methyl*-^{3}H]Thymidine, [^{3}H]dTTP, and [^{32}P]dATP were obtained from New England Nuclear, and [^{3}H]dUTP came from Amersham.

Escherichia coli DNA polymerase I was obtained from New England Biolabs; 1 unit is defined as the amount required to convert 10 nmol of deoxyribonucleotides to an acid-insoluble form in 30 min at 37 °C. Homogeneous Novikoff hepatoma DNA polymerase β was purified by the procedure of Stalker et al. (1976). One unit of DNA polymerase β catalyzes the incorporation of 1 nmol of total nucleotide into DNA in 1 h at 37 °C.

Methods

Preparation of DNA Substrates. PM2 DNA (>90% form I) was isolated from phage grown on a wild-type strain of *Alteromonas espajiana* Bal 31, as described by Espejo & Canelo (1968) and modified by Kuhnlein et al. (1976). T7 [^{3}H]DNA (22 000 cpm/nmol) was prepared by the method of Richardson (1966).

Activated calf thymus DNA was prepared according to Stalker et al. (1976) and nick-translated with *E. coli* DNA polymerase I to produce [*uracil*-^{3}H]DNA as described by Mosbaugh & Linn (1980). Reaction mixtures contained 70 mM potassium phosphate buffer (pH 7.5), 1 mM 2-mercaptoethanol, 10 mM $MgCl_2$, 90 μM each of dATP, dGTP, dCTP, and [^{3}H]dUTP (215–6400 cpm/pmol), 450 μg/mL activated DNA, and 1.5 unit/mL *E. coli* DNA polymerase I. After incubation for 30 min at 37 °C, the reaction was adjusted to 500 mM NaCl and the enzyme inactivated by heating for 5 min at 70 °C. The product was then extracted with phenol and dialyzed against 10 mM Tris-HCl (pH 7.5), 1 mM EDTA, and 500 mM NaCl, then against 10 mM Tris-HCl (pH 7.5) and 1 mM EDTA, and finally against 10 mM Tris-HCl (pH 7.5). The product contained approximately 28% uracil residues within the newly synthesized region and about 3.4% of total residues as dUMP.

[*uracil*-^{3}H]Poly(dA-dT) containing a 3′-terminal uracil residue was synthesized in a reaction mixture (7.5 mL) containing 67 mM potassium phosphate buffer (pH 7.5), 6.7 mM $MgCl_2$, 1 mM 2-mercaptoethanol, 160 μM poly(dA-dT), 13 μM [^{3}H]dUTP (950–1430 cpm/pmol), and 37.5 units of *E. coli* DNA polymerase I. After incubation at 30 °C for various times, 50-μL aliquots were removed, and 100 μL of 1 mg/mL bovine serum albumin in 0.1 M sodium pyrophosphate was added on ice prior to precipitation with 7.7% trichloroacetic acid. Precipitates were collected on Whatman GF/C filters, washed with 18 mL of 1 N HCl in 0.1 M sodium pyrophosphate, and dried with 95% ethanol. Acid-insoluble radioactivity was measured in a Beckman LS 6800 liquid scintillation spectrometer with 0.4% 2,5-bis(5-*tert*-butyl-2-benzoxazolyl)thiophene in toluene. After the incorporation of dUMP plateaued, the reaction was terminated by heating for 15 min at 100 °C. Unincorporated deoxyuridine triphosphate and mononucleotides were removed from the reaction by chromatography on a NACS PREPAC (BRL) column. The sample (7.3 mL) was adjusted to 200 mM NaCl, diluted with 2 volumes of 10 mM Tris-HCl (pH 7.2), 1 mM EDTA, and 200 mM NaCl, and applied to the column, equilibrated in the dilution buffer. After the column was washed with 4 mL of buffer, [*uracil*-^{3}H]poly(dA-dT) was eluted with 10 mM Tris-HCl (pH 7.2), 1 mM EDTA, and 1 M NaCl and dialyzed against the same buffer minus NaCl.

[^{32}P,*uracil*-^{3}H]Poly(dA-dT) containing an internal uracil residue was synthesized in a reaction mixture (750 μL) containing 25 mM Tris-HCl (pH 8.0), 10 mM $MgCl_2$, 5 mM 2-mercaptoethanol, 1 mM EDTA, 50 mM NaCl, 15% (w/v) glycerol, 25 μM dTTP, 25 μM [^{32}P]dATP (3150 cpm/pmol), 133 μM [*uracil*-^{3}H]poly(dA-dT) containing a 3′-terminal uracil residue (1430 cpm/pmol of uracil), and 3.6 units of Novikoff hepatoma DNA polymerase β. In some cases, dideoxythymidine triphosphate (ddTTP) was added to the reaction as indicated in the table legend. Incubation was at 30 °C for 1 h after which time no further incorporation was

[1] Abbreviations: AP, apurinic/apyrimidinic; form I, covalently closed supercoiled duplex DNA; form II, relaxed duplex DNA containing one or more phosphodiester bond discontinuities; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetate; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; NTA, nitrilotriacetic acid; SDS, sodium dodecyl sulfate; BSA, bovine serum albumin; PMSF, phenylmethanesulfonyl fluoride.

observed. The reaction mixture was then adjusted to 200 mM NaCl and the [^{32}P,*uracil*-^{3}H]poly(dA-dT) purified by chromatography on a NACS PREPAC column as described above.

Enzyme Assays. Uracil–DNA glycosylase was assayed by a modified procedure of that described previously by Duncan et al. (1978). Standard reaction mixtures (100 μL) contained 70 mM Hepes–KOH (pH 7.5), 1 mM dithiothreitol, 1 mM EDTA, 9.2 nmol of calf thymus DNA containing [^{3}H]uracil (215–415 cpm/pmol of uracil), and 0.002–0.2 unit of enzyme. After incubation for 30 min at 37 °C, the reaction was terminated on ice with 250 μL of 10 mM ammonium formate (pH 4.2). A portion of the reaction mixture (300 μL) was then applied to a Bio-Rad AG 1-X8 (formate form) column (0.2 cm^2 × 2 cm) equilibrated with 10 mM ammonium formate (pH 4.2) and then washed with 2.2 mL of the same buffer. Under these conditions, DNA absorbed to the resin, but free [^{3}H]uracil passed through the column. After four 500-μL fractions were collected into mini-scintillation vials, 5 mL of Ready-Solv HP/b (Beckman) scintillation counting mixture was added, and the radioactivity was measured. The blank, which contained standard reaction components, minus enzyme, generally accounted for less than 150 cpm. One unit of uracil–DNA glycosylase is defined as the amount of enzyme that catalyzes the release of 1 nmol of uracil per hour under standard reaction conditions. Changes from the standard conditions are noted in the legends to the tables and figures.

Exonuclease activity was measured by using standard uracil–DNA glycosylase reaction conditions except 8.5 nmol of nondenatured T7 [^{3}H]DNA or heat-denatured T7 [^{3}H]DNA was substituted for the uracil–DNA in order to measure double-stranded or single-stranded exonuclease activity, respectively. In addition, some reactions contained 10 mM $MgCl_2$. After 2 h at 37 °C, the reactions were chilled on ice and adjusted to 0.6 mg/mL calf thymus DNA, and the DNA was precipitated with 170 mM perchloric acid. After 30 min at 0 °C, the samples were centrifuged for 5 min in an Eppendorf Model 544 centrifuge. The supernatant fraction (150 μL) was added to 350 μL of water and counted for radioactivity in 5 mL of Ready-Solv HP/b fluor. Assays were performed in duplicate. The acid-soluble radioactivity of the untreated sample (generally 0.2% of the total) was subtracted from that of the samples containing uracil–DNA glycosylase.

Apurinic or nonspecific endonuclease activity was detected by using 10 nmol of PM2 DNA (native or depurinated) which was substituted for uracil–DNA in the standard reaction. Partial depurination of form I PM2 DNA was carried out at 70 °C for 15 min to generate about two apurinic sites per genome as described by Mosbaugh & Linn (1980). Reactions containing 0.05 unit of nuclear uracil–DNA glycosylase (fraction VI) were incubated for various times up to 1 h at 37 °C and form I and II DNAs resolved by electrophoresis on an 0.8% agarose gel in 40 mM Tris–acetate and 2 mM EDTA buffer, pH 8.2.

Molecular Weight Determination. Sodium dodecyl sulfate–polyacrylamide slab gel electrophoresis was performed as described previously (Laemmli & Favre, 1973) using 12.5% acrylamide with 0.24% *N,N*-methylenebis(acrylamide). The stacking gel contained 3% acrylamide with 0.24% *N,N*-methylenebis(acrylamide). In order to prepare nuclear uracil–DNA glycosylase for electrophoresis, it was necessary to remove the added bovine serum albumin from the samples as described in the legends. Samples (75 μL) were heated to 100 °C for 10 min in 25 mM Tris-HCl (pH 6.8), 73 mM 2-mercaptoethanol, 0.5% sodium dodecyl sulfate, 5% (w/v) glycerol, and 0.04% bromophenol blue prior to being loaded onto the stacking gel. Electrophoresis was carried out at 200 V until the bromophenol blue tracking dye migrated about 90% the length of the gel. Protein bands were detected by using the silver-staining technique (Merril et al., 1981).

Gel filtration chromatography was performed by using a Sephadex G-75 column (1.8 cm^2 × 57 cm) equilibrated with 50 mM Tris-HCl (pH 8.0), 1 mM dithiothreitol, 1 mM EDTA, 100 mM NaCl, and 10% (w/v) glycerol. Protein standards bovine serum albumin, ovalbumin, soybean trypsin inhibitor, and cytochrome *c* were run separately to calibrate the column. Blue dextran 2000 was used to determine the void volume.

The sedimentation coefficient of uracil–DNA glycosylase was determined by centrifugation through a linear 10–30% (w/v) glycerol gradient containing 20 mM Tris-HCl (pH 7.5), 1 mM dithiothreitol, 1 mM EDTA, and 100 mM NaCl. Samples (200 μL) were layered onto the top of the gradient and centrifuged in a Spinco SW 50.1 rotor at 48 000 rpm for 20 h at 4 °C. Fractions (185 μL) were collected from the top of the gradients by using an ISCO Model 185 density gradient fractionator at a flow rate of 750 μL/min. The sedimentation coefficient and molecular weight of the enzyme were determined by the method of Martin & Ames (1961) using bovine serum albumin, ovalbumin, and cytochrome *c* as standards.

Isoelectric Focusing. The isoelectric point of uracil–DNA glycosylase was determined by using a flat-bed electrofocusing gel (LKB 2117 Multiphor). An initial 5% (w/v) Sephadex G-75 gel slurry containing 5% (v/v) ampholines (pH 7–10) was poured onto the electrofocusing tray (24.5 × 11 cm) and evaporated to 71% of the initial weight. The sample was dialyzed against 1% glycine, and 3 mL was applied to the middle of the gel. Electrofocusing was carried out at 8 W for 16 h at 4 °C with an initial voltage and current of 390 V and 20.5 mA, respectively. After being focused, the gel was fractionated, the resin removed by centrifugation, and the pH of each aliquot measured. After the fractions were dialyzed against 20 mM Tris-HCl (pH 7.5), 1 mM dithiothreitol, 1 mM EDTA, and 10% (w/v) glycerol (TDEG buffer), 15-μL aliquots were assayed for uracil–DNA glycosylase.

Other Methods. Protein concentrations were determined by using the Coomassie blue G-250 dye binding procedure (Bradford, 1976) and by the fluorescamine technique (Bohlen et al., 1973).

RESULTS

Purification of Uracil–DNA Glycosylase from Nuclei

All procedures were performed at 0–4 °C unless otherwise indicated. Generally, 300 g (wet weight) of liver was processed from 20 male Sprague–Dawley rats (~250 g) in a single batch.

Preparation of Nuclear Extract. Rat livers were suspended in a final volume of 1.2 L of homogenization buffer [50 mM Tris-HCl (pH 8.0), 25 mM KCl, 5 mM $MgCl_2$, 1 mM dithiothreitol, 1 mM EDTA, and 250 mM sucrose] to obtain a 25% (w/v) suspension which was minced and homogenized. The homogenate was then filtered through four layers of cheesecloth before being centrifuged at 800*g* for 10 min. The resulting postnuclear supernatant fraction was saved for isolating mitochondria, and the crude nuclear pellet was resuspended in an equal volume of homogenization buffer. This resuspension and centrifugation step was repeated twice before the final nuclear pellet was resuspended with 270 mL of homogenization buffer. Then 540 mL of homogenization buffer containing 2.3 M sucrose was mixed with the suspension. Approximately 28 mL of the suspension was overlayed

onto a 10-mL cushion of the buffer containing 2.3 M sucrose and centrifuged in a Spinco SW 28 rotor at 24 000 rpm for 2 h. Nuclear pellets were resuspended with 60 mL of buffer [50 mM Tris-HCl (pH 8.0), 25 mM KCl, 5 mM $MgCl_2$, 1 mM dithiothreitol, 1 mM EDTA, 250 mM sucrose, and 25% (w/v) glycerol]. The nuclei were disrupted by the addition of 2 volumes of lysis buffer [50 mM Tris-HCl (pH 8.0), 1 mM dithiothreitol, 1 mM EDTA, and 3 M NaCl]. Extraction was carried out for 12 h with constant stirring and was followed by centrifugation in a Spinco 50.2 Ti rotor at 49 000 rpm for 65 min. The supernatant fraction was then dialyzed against buffer containing 50 mM Tris-HCl (pH 8.0), 1 mM dithiothreitol, 1 mM EDTA, and 150 mM NaCl, and the white precipitate that formed was removed by centrifugation at 15000*g* for 10 min. The resulting nuclear extract constituted fraction I.

Ammonium Sulfate Precipitation. Fraction I was brought to 35% saturation with ammonium sulfate by slowly adding salt to the solution over a 30-min period while stirring on ice. Stirring was continued for an additional 30 min, and the precipitate was collected by centrifugation at 15000*g* for 15 min. The supernatant fraction was then brought to 65% saturation with ammonium sulfate, and the proteins precipitating at this concentration were collected by centrifugation. The precipitate was suspended in 10 mL of 50 mM Tris-HCl (pH 8.0), 1 mM dithiothreitol, 1 mM EDTA, 150 mM NaCl, and 10% (w/v) glycerol and dialyzed against the same buffer. Additional dialysis against 20 mM Tris-HCl (pH 7.5), 1 mM dithiothreitol, 1 mM EDTA, and 10% (w/v) glycerol (TDEG buffer) was carried out, and the precipitate which formed was removed by centrifugation at 15000*g* for 10 min. This supernatant fraction constituted fraction II.

DEAE-Sephadex Chromatography. A DEAE-Sephadex A-50 column (1.8 cm^2 × 9.5 cm) was equilbrated with TDEG buffer. Fraction II was loaded and the column washed with 45 mL of equilibration buffer at a flow rate of approximately 20 mL/h. Uracil–DNA glycosylase activity was not absorbed but eluted in the flow-through fractions prior to the bulk of the rat liver protein. Peak fractions were pooled and represented fraction III.

DNA–Agarose Chromatography. Fraction III was loaded directly onto a single-stranded DNA–agarose column (2.5 cm^2 × 7.5 cm) equilibrated in TDEG buffer. After the column was washed with 40 mL of equilibration buffer, a 150-mL linear gradient was applied from 0 to 400 mM NaCl in TDEG buffer at a flow rate of about 10 mL/h. The glycosylase activity eluted as a single peak at approximately 70 mM NaCl. Active fractions were pooled and concentrated about 10-fold in an Amicon ultrafiltration cell containing a Diaflo PM10 membrane using 30–50 psi of N_2 gas. Greater than 90% of the applied enzyme activity was recovered following ultrafiltration, with about 25% of the protein being lost during the concentration step. The concentrated enzyme fraction (~3 mL) was designated fraction IV.

Sephadex G-75 Chromatography. Fraction IV was adjusted to a final concentration of 100 mM NaCl and 15% (w/v) glycerol before being applied to a Sephadex G-75 column (1.8 cm^2 × 57 cm), equilibrated in 50 mM Tris-HCl (pH 8.0), 1 mM dithiothreitol, 1 mM EDTA, 100 mM NaCl, and 10% (w/v) glycerol. The enzyme was eluted with a constant hydrostatic head of the same buffer at a flow rate of 14 mL/h. Fractions containing uracil–DNA glycosylase activity were pooled and identified as fraction V.

Phosphocellulose Chromatography. Fraction V was adjusted to 0.5 mg/mL bovine serum albumin in order to stabilize the enzyme activity. After the enzyme was dialyzed against 20 mM potassium phosphate (pH 7.5), 1 mM dithiothreitol, 1 mM EDTA, and 10% (w/v) glycerol (PDEG buffer), the sample was applied to a phosphocellulose column (1.8 cm^2 × 11 cm) equilibrated in the same buffer but containing 0.5 mg/mL bovine serum albumin. The column was then washed with 25 mL of the equilibration buffer and eluted with a 150-mL linear gradient of 0–600 mM potassium phosphate (pH 7.5) in the equilibration buffer at a flow rate of about 15 mL/h. The peak of uracil–DNA glycosylase activity eluted at approximately 240 mM potassium phosphate. Fractions containing enzyme activity were pooled and designated as fraction VI. The purification scheme is summarized in Table I. Fraction VI was used in all studies described below unless otherwise indicated.

Table I: Purification of Rat Liver Nuclear Uracil–DNA Glycosylase[a]

fraction	total protein (mg)[b]	total act. (units)	sp act. (units/mg)	purification (*x*-fold)
(I) nuclear extract	825	1950	2.4	1.0
(II) ammonium sulfate	75.6	492	6.5	2.7
(III) DEAE-Sephadex	6.3	215	34.1	14.2
(IV) DNA–agarose	1.9	325	171	71.3
(V) Sephadex G-75	0.15	179	1190	496
(VI) phosphocellulose	0.01[c]	73.6	7360	3070

[a] Standard uracil–DNA glycosylase assays were described under Experimental Procedures. [b] Protein concentrations were determined by the method of Bradford (1976) with bovine serum albumin as a standard. [c] In order to determine the protein concentration of fraction VI, it was necessary to remove the bovine serum albumin which was added to stabilize the uracil–DNA glycosylase. Fraction VI enzyme was diluted with an equal volume of 20 mM potassium phosphate (pH 7.5), 1 mM dithiothreitol, 1 mM EDTA, and 10% (w/v) glycerol and applied to a second phosphocellulose column (0.8 cm^2 × 1.3 cm) equilibrated in the same buffer. After the bovine serum albumin was washed from the column with 30 mL of equilibration buffer, uracil–DNA glycosylase was then eluted with 5 mL of 300 mM potassium phosphate (pH 7.5), 1 mM dithiothreitol, 1 mM EDTA, and 10% (w/v) glycerol. Fractions (1 mL) were collected and assayed for glycosylase, and protein concentration was determined by the fluorescamine technique using a 390-nm excitation and a 475-nm emission wavelength.

Associated Enzyme Activities

The purified enzyme preparation specifically catalyzed the release of free uracil from DNA containing dUMP residues. Under standard reaction conditions, the ^3H label released from the DNA migrated on a paper chromatogram (solvent system 86% 1-butanol) with a mobility (R_f = 0.46) identical with that of legimate uracil. Under similar reaction conditions, the purified preparation contained no detectable single- or double-stranded DNA exonuclease activity. In addition, no exonuclease activity was detected in reactions containing 10 mM $MgCl_2$. The limit of exonuclease detection, using 0.1 unit of uracil–DNA glycosylase, was less than 2% of the glycosylase activity. Similarly, the enzyme preparation lacked nonspecific and apurinic endonuclease activity. The endonuclease assay allowed for detection of nonspecific or apurinic endonuclease at a level greater than 0.2% that of the glycosylase activity.

Enzyme Purity and Molecular Weight

Fraction VI uracil–DNA glycosylase was purified 3070-fold from the nuclear extract, with a 3.8% yield (Table I). To determine the purity of the enzyme, samples across the activity peak from the phosphocellulose column were electrophoresed on a 12.5% SDS–polyacrylamide gel (Figure 1). In each sample, a single silver-stainable band was observed which

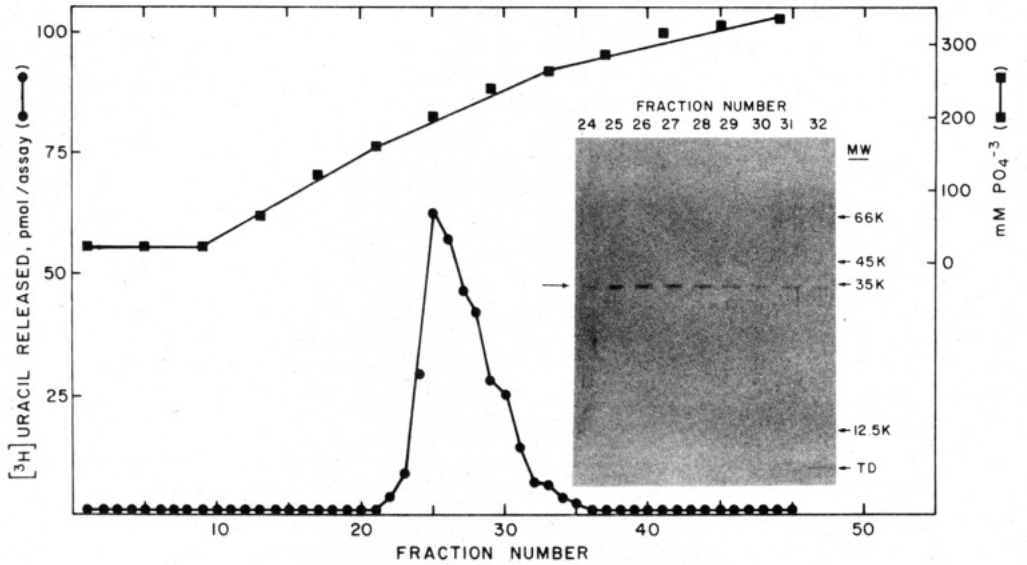

FIGURE 1: Sodium dodecyl sulfate–polyacrylamide gel electrophoresis of nuclear uracil–DNA glycosylase fractions from phosphocellulose chromatography. Uracil–DNA glycosylase (fraction V) was chromatographed on a phosphocellulose column as described under Results. Fractions were assayed for uracil–DNA glycosylase activity as shown. Equal volumes (4 mL) of fractions (24–32) across the peak were prepared for 12.5% SDS–polyacrylamide slab gel electrophoresis. Bovine serum albumin was removed from each sample by chromatography on a second phosphocellulose column as described in Table I. Column fractions (1 mL) were collected, assayed for enzyme activity, and concentrated 7.5-fold. Generally, 90% of the activity that eluted was recovered in a single fraction. Samples (75 μL) were denatured and electrophoresed, and the gel was stained for protein by using the silver-staining technique as described under Experimental Procedures. A polypeptide (R_f = 0.5) with band intensity which correlated with the glycosylase activity present in fractions from the phosphocellulose column, indicated along the *top* of the *insert*, is designated by the *arrow* on the *left side* of the gel. The molecular weights of standards are indicated by the *arrows* on the *right side* of the gel and correspond to bovine serum albumin, ovalbumin, lactic acid dehydrogenase, and cytochrome *c* from *top* to *bottom*.

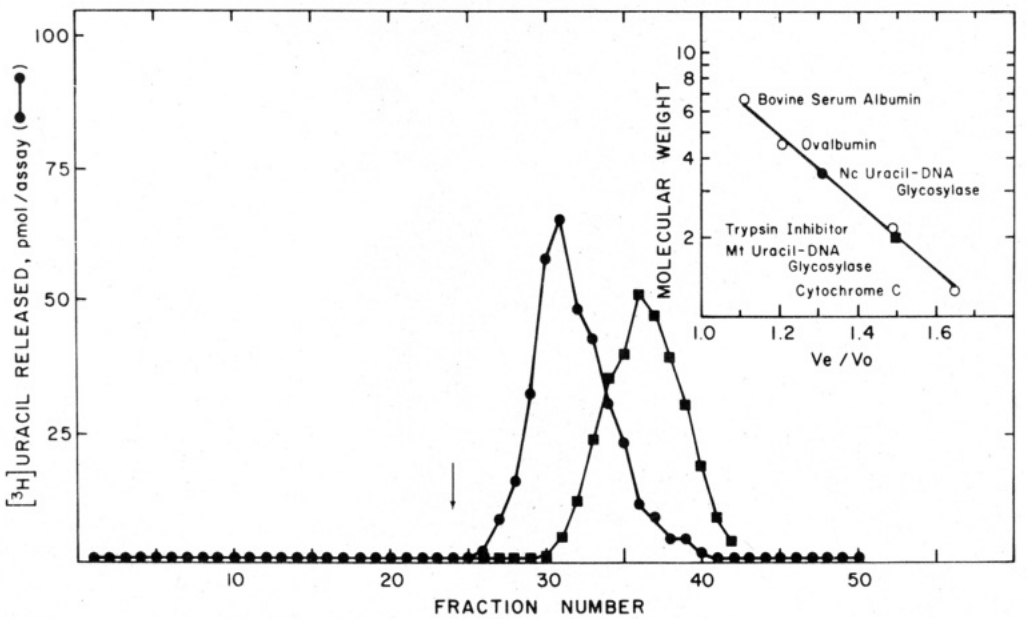

FIGURE 2: Molecular weight determination of nuclear and mitochondrial uracil–DNA glycosylase by gel filtration chromatography. Fraction IV nuclear uracil–DNA glycosylase (77 units) or fraction VI mitochondrial uracil–DNA glycosylase (56 units) was chromatographed on a Sephadex G-75 column and assayed for activity as described under Experimental Procedures. The *arrow* indicates the location of the void column (V_0 = 53 mL). The column was calibrated by using the protein standards bovine serum albumin (M_r 66 000), ovalbumin (M_r 45 000), soybean trypsin inhibitor (M_r 21 000), and cytochrome *c* (M_r 12 500). A plot of molecular weight vs. V_e/V_0 ratio for the standards (○) is shown in the *insert* along with nuclear (●) and mitochondrial (■) uracil–DNA glycosylase.

migrated with a polypeptide molecular weight of 35 000. The intensity of this band appeared to directly correlate with the uracil–DNA glycosylase activity in respective phosphocellulose fractions. During purification on a Sephadex G-75 column, the enzyme eluted as a symmetrical peak corresponding to an apparent molecular weight of 33 000 (Figure 2). Hence, the native molecular weight appears to be in good agreement with the apparent polypeptide molecular weight of the enzyme. Taken together, the data suggest that the band observed on the SDS–polyacrylamide gel represents the glycosylase. We estimate that the enzyme purification procedure yields a preparation that is at least 90% homogeneous.

The sedimentation coefficient of uracil–DNA glycosylase was determined in glycerol gradients to be 2.6 S relative to several standard proteins. Assuming that the enzyme has a partial specific volume of approximately 0.725 cm^3/g, the molecular weight was calculated to be about 29 500. This estimate of the native molecular weight is slightly lower but is within experimental error of the value obtained by gel filtration chromography.

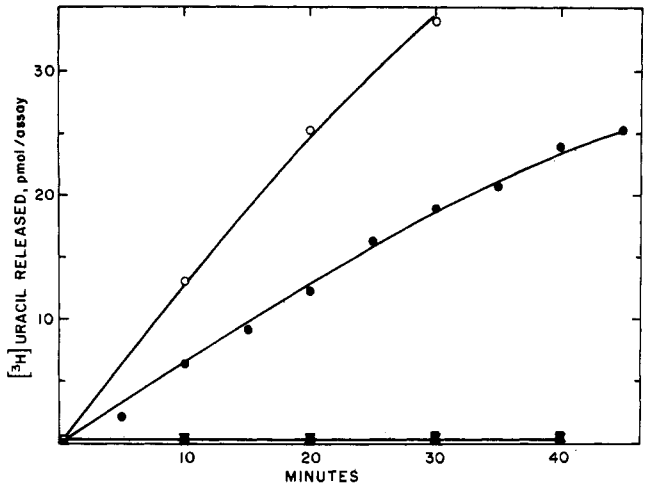

FIGURE 3: Substrate specificity of nuclear uracil-DNA glycosylase. Five reaction mixtures (500 μL) each containing 0.38 unit of uracil-DNA glycosylase were prepared as described under Experimental Procedures, except that in some cases the standard substrate, 92 nmol of double-stranded calf thymus [*uracil*-^{3}H]DNA (●), was replaced with 92 nmol of single-stranded calf thymus [*uracil*-^{3}H]DNA (215 cpm/pmol) (○), 400 nmol of [*uracil*-^{3}H]poly(dA-dT) containing a 3′-terminal uracil residue (950 cpm/pmol) (■), 25 μM [^{3}H]dUTP (950 cpm/pmol) (▼), or 25 μM [^{3}H]dUMP (510 cpm/pmol) (▲). After incubation at 37 °C, 50-μL aliquots were removed at the times indicated, and glycosylase activity was measured. Single-stranded [*uracil*-^{3}H]DNA was prepared by heating the standard substrate at 100 °C for 10 min followed by rapid chilling on ice.

General Properties of the Nuclear Uracil-DNA Glycosylase

Temperature Optimum and Stability. The initial velocity of the enzyme increased with incubation temperature to 37 °C. The release of uracil at 25, 30, and 37 °C was proportional with time for at least 45 min. The enzyme was stable at 4 °C throughout the purification procedure; however, the addition of 0.5 mg/mL bovine serum albumin to fraction V was required to stabilize the activity. In the absence of added protein, >90% of the activity was lost after 24 h or upon further purification. On the other hand, fraction VI uracil-DNA glycosylase containing bovine serum albumin showed no significant loss of activity when stored at 4 °C for at least 3 months.

pH Optimum. The enzyme was active over a broad pH range with significant activity detected between pH 6.8 and 9.0. Maximal activity was obtained with Hepes-KOH at pH 7.5 or 8.0 in Tris-HCl; the former buffer was slightly more effective. Reactions carried out in phosphate buffer gave significantly lower activity, with 52% and 45% of the maximal activity being observed at pH 7.0 and 8.0, respectively.

Isoelectric Point. The isoelectric point of nuclear uracil-DNA glycosylase was determined on a flat-bed electrofocusing gel. The enzyme focused in a symmetrical peak as a basic protein with a p*I* value of 9.3.

Substrate Specificity. The nuclear enzyme removed uracil from single-stranded and double-stranded uracil-DNA (Figure 3). However, the initial velocity of the reaction on duplex uracil-DNA was about 50% that of heat-denatured DNA. The apparent K_m of the enzyme was 2×10^{-7} M uracil residues, as determined by a double-reciprocal plot of the duplex uracil-DNA concentration vs. initial velocity. The enzyme specifically recognized uracil located in DNA; neither dUMP nor dUTP was a substrate. Similarly, [*uracil*-^{3}H]poly(dA-dT) containing a 3′-terminal uracil residue was not efficiently hydrolyzed by the glycosylase. The rate of uracil release from [*uracil*-^{3}H]poly(dA-dT) containing a single 3′-terminal dUMP residue was less than 3% that of duplex uracil-DNA. When

Table II: Requirement for an Internal dUMP Residue for Nuclear Uracil-DNA Glycosylase Activity[a]

DNA polymerase	[dTTP] (μM)	[ddTTP] (μM)	[^{3}H]uracil released (pmol)
−			0.6
+	25		102
+	22.5	2.5	49
+	20	5	29

[a] The 3′-terminal uracil residue of [*uracil*-^{3}H]poly(dA-dT) was internalized by chain elongation using Novikoff hepatoma DNA polymerase β as described under Experimental Procedures. Four (750 μL) reaction mixtures, each containing 25 mM Tris-HCl (pH 8.0), 10 mM $MgCl_2$, 5 mM 2-mercaptoethanol, 1 mM EDTA, 50 mM NaCl, 15% (w/v) glycerol, 25 μM [^{32}P]dATP, and 133 μM [*uracil*-^{3}H]poly(dA-dT) (1430 cpm/pmol of uracil), were prepared, and 3.6 units of DNA polymerase β, dTTP, and ddTTP were added as indicated in the table. After incubation at 30 °C for 1 h, the poly(dA-dT) products were isolated and incubated under standard conditions with 0.09 unit of uracil-DNA glycosylase at 37 °C for 1 h. Each reaction contained approximately 120 pmol of uracil residues. The [^{3}H]uracil released was measured as described for the standard reaction.

terminal dUMP residues were internalized by incorporating [^{32}P]dAMP and dTMP onto the 3′ end, using DNA polymerase β, the [^{32}P,*uracil*-^{3}H]poly(dA-dT) became a more efficient substrate for the glycosylase (Table II). However, the ability of the glycosylase to release uracil from [^{32}P,*uracil*-^{3}H]poly(dA-dT) was dependent on the extent of chain elongation. When 2600 pmol of dAMP plus dTMP was incorporated onto [*uracil*-^{3}H]poly(dA-dT) containing 120 pmol of dUMP, about 85% of the [^{3}H]uracil residues were hydrolyzed by the glycosylase. Under this condition, an average of 21 bases would have been expected to be synthesized on each terminus. In contrast, less than 0.5% of the uracil was released in the absence of DNA synthesis. To limit the extent of chain elongation from terminal dUMP residues, DNA synthesis was carried out in the presence of various concentrations of ddTTP. When 2.5 μM ddTTP was added, the polymerase incorporated approximately 1500 pmol of nucleotides, and on the average, each chain contained about 12 additional bases. In this case, about 41% of the uracil was released by the glycosylase. With the addition of 5 μM ddTTP, each polynucleotide chain was elongated with approximately 7.5 bases, and only 24% of the uracil was susceptible to hydrolysis. The inability of the glycosylase to act on some of the elongated polymers was not due to the presence of the ddTMP residues since adding the standard uracil-DNA substrate to these reactions did not significantly inhibit the activity. Thus, the data suggest that uracil-DNA glycosylase does not efficiently recognize uracil residues which are located on or close to the terminus of a polynucleotide chain.

Purification of Uracil-DNA Glycosylase from Mitochondria

Preparation of Mitochondrial Extract. Mitochondria were harvested from the postnuclear supernatant fraction by centrifugation at 14500*g* for 10 min. The mitochondria were then washed 3 times in homogenization buffer. In some preparations, mitochondria were subsequently isolated by sedimentation in a linear sucrose gradient (1.0–1.9 M) as described by Anderson & Friedberg (1980), except that centrifugation was for 3 h at 21 000 rpm in an SW 28 rotor. After the gradient was fractionated, a mitochondrial band was identified which contained both uracil-DNA glycosylase and DNA polymerase γ activities. Mitochondria were then frozen in liquid nitrogen and disrupted by grinding with levigated alumina (2 parts of alumina to 1 part of mitochondria). The paste was suspended in 50 mL of extraction buffer [20 mM Tris-HCl

(pH 8.0), 5 mM 2-mercaptoethanol, 1 mM EDTA, and 1 M NaCl] and centrifuged at 500*g* for 15 min. The resulting supernatant was then centrifuged for 1 h at 125000*g* in a Spinco 50Ti rotor at 41 000 rpm and dialyzed against the extraction buffer but containing 150 mM NaCl. This extract constituted fraction I.

Ammonium Sulfate Precipitation. Mitochondrial proteins which precipitated between 35% and 65% ammonium sulfate were isolated as described for nuclear uracil–DNA glycosylase. The pellets remaining after the 65% ammonium sulfate extraction were resuspended and dialyzed against TDEG buffer. The dialyzed material represented fraction II.

DEAE-Sephadex Chromatography. Fraction II was loaded onto a column (4.9 cm^2 × 23 cm) of DEAE-Sephadex A-50 equilibrated with TDEG buffer. The column was then washed with equilibration buffer. Uracil–DNA glycosylase activity which eluted in the flow-through fractions was pooled and dialyzed against PDEG buffer. This constituted fraction III.

Phosphocellulose Chromatography. Fraction III was concentrated to about 10 mL by using an Amicon ultrafiltration cell as described above. The sample was then applied to a phosphocellulose column (1.8 cm^2 × 6 cm) equilibrated in PDEG buffer. After the column was washed, glycosylase was eluted with a 100-mL linear gradient from 0 to 400 mM potassium phosphate in the equilibration buffer. The enzyme eluted as a broad peak at approximately 130 mM potassium phosphate. Fractions containing peak activity were pooled and dialyzed against TDEG buffer. The dialyzed material constituted fraction IV.

DNA–Agarose Chromatography. A single-stranded DNA–agarose column (1.8 cm^2 × 6 cm) was equilibrated in TDEG buffer. Fraction IV was concentrated by ultrafiltration to approximately 10 mL and loaded onto the column. After the column was washed, the enzyme was eluted with a 100-mL linear gradient from 0 to 400 mM NaCl in the equlibration buffer. The enzyme eluted as a single peak at 105 mM NaCl. Active fractions were pooled and identified as fraction V.

Sephadex G-75 Chromatography. Fraction V was concentrated to ~3 mL by ultrafiltration and applied to the Sephadex G-75 column (1.8 cm^2 × 57 cm) as described under Experimental Procedures except that fractions were generally collected in 125 μg/mL BSA. Peak fractions containing enzyme activity were pooled and designated fraction VI. At this stage, fraction VI was purified approximately 1630-fold over the mitochondrial extract. However, 12.5% SDS–polyacrylamide gel electrophoresis revealed that the preparation still contained several protein bands.

Evidence for a Distinct Nuclear and Mitochondrial Form of Uracil–DNA Glycosylase

Several lines of evidence indicate that the mitochondrial-associated uracil–DNA glycosylase has distinctly different properties from the nuclear activity. (1) Mitochondrial uracil–DNA glycosylase exhibited different chromatographic behavior from the nuclear enzyme. The mitochondrial activity eluted from phosphocellulose at 130 mM potassium phosphate in contrast to a concentration of 240 mM which was required to elute the nuclear form. Similarly, the two enzymes recognized single-stranded DNA–agarose with a slightly different affinity. Nuclear uracil–DNA glycosylase eluted at 70 mM NaCl whereas the mitochondrial enzyme was disposed at 105 mM NaCl. (2) The native molecular weight, as determined by Sephadex G-75 gel filtration chromatography (Figure 2), of mitochondrial uracil–DNA glycosylase (M_r 20 000) is significantly lower than that of the nuclear activity (M_r 33 000).

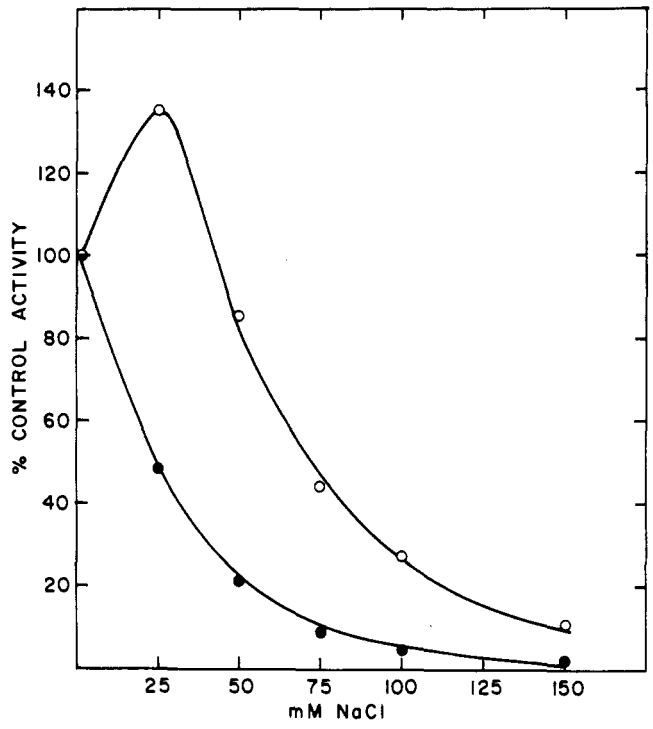

FIGURE 4: Effect of NaCl concentration on nuclear and mitochondrial uracil–DNA glycosylase. Standard reaction conditions were as described under Experimental Procedures except NaCl was added as indicated in the figure. Each reaction contained 0.06 unit of fraction VI nuclear uracil–DNA glycosylase (●) or 0.006 unit of fraction VI mitochondrial uracil–DNA glycosylase (○).

The addition of 1 mM PMSF during purification did not alter the molecular weight of either enzyme. (3) The mitochondrial enzyme was less sensitive to inhibition by NaCl than the nuclear enzyme (Figure 4). Unlike the nuclear activity, which was strongly inhibited by NaCl, the mitochondrial activity was stimulated by low concentrations (<25 mM) of NaCl. Even though higher concentrations were inhibitory, the extent of inhibition was consistently less for the mitochondrial enzyme. (4) Mitochondrial uracil–DNA glycosylase was significantly more heat-stable at 45 °C than the nuclear enzyme (Figure 5). The nuclear activity demonstrated a linear rate of heat inactivation with a half-life of 4 min. On the other hand, the mitochondrial enzyme had a half-life of 17.5 min on the basis of an extrapolation of the linear portion of the curve. The greater stability exhibited by the mitochondrial fraction was most likely not attributable to a difference in purity since 125 μg/mL BSA was added to both fractions to eliminate the possibility of nonspecific protein protection. In addition, the half-life of the nuclear enzyme was not significantly increased by the addition of DNA or uracil (data not shown). Taken together, these results suggest that two different forms of uracil–DNA glycosylase exist in rat liver.

DISCUSSION

Rat liver nuclear uracil–DNA glycosylase was purified to apparent homogeneity as a single polypeptide of 35 000 molecular weight. Many of the general properties of this basic protein are similar to those of uracil–DNA glycosylases isolated from other mammalian sources (Kuhnlein et al., 1978; Caradonna & Cheng, 1980; Krokan & Wittner, 1981). However, unlike the calf thymus nuclear uracil–DNA glycosylase which prefers a double-stranded uracil–DNA substrate (Talpaert-Borle et al., 1982), the corresponding rat liver enzyme has a 2-fold preference for single-stranded DNA. A similar substrate preference has also been described for the homogeneous uracil–DNA glycosylase purified from human

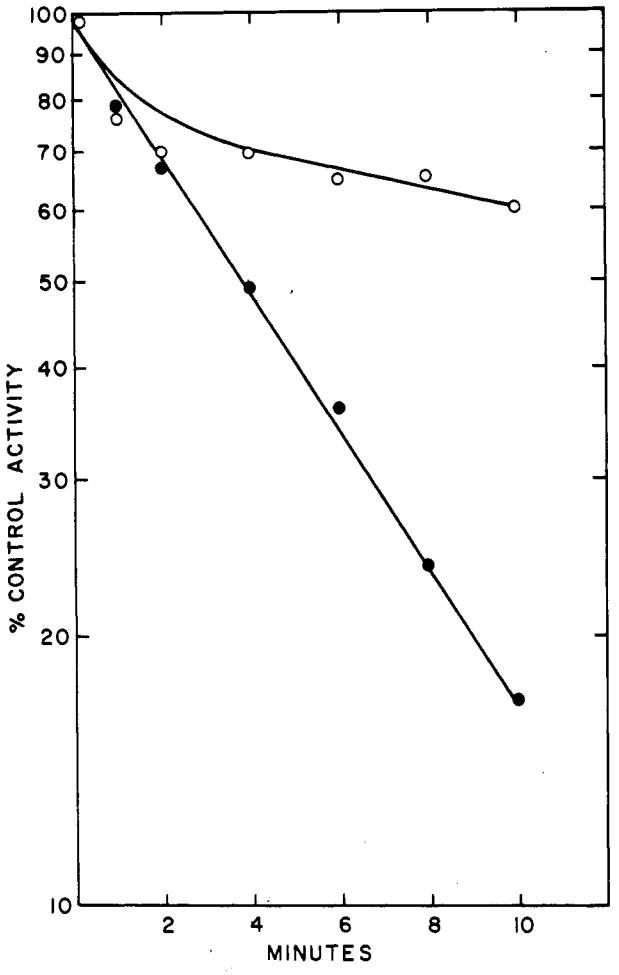

FIGURE 5: Heat inactivation of nuclear and mitochondrial uracil-DNA glycosylase. Two sets of tubes containing fraction VI nuclear uracil-DNA glycosylase (●) or fraction VI mitochondrial uracil-DNA glycosylase (○) were incubated at 45 °C. At the times indicated, standard reaction components were added to enzyme samples and incubated for 30 min at 37 °C as described under Experimental Procedures. Samples which were not incubated at 45 °C contained 0.03 and 0.02 units of nuclear and mitochondrial uracil-DNA glycosylase, respectively.

blast cells (Caradonna & Cheng, 1980) and from several bacterial systems (Lindahl et al., 1977; Leblanc et al., 1982). This property is somewhat unique to the uracil class of DNA glycosylase since DNA glycosylases which recognize other DNA adducts exhibit little or no activity against damaged single-stranded DNA (Lindahl, 1982). The biological significance of this substrate specificity is not clear; however, it does suggest that uracil excision repair could be initiated on either single- or double-stranded DNA.

Our results indicate that 3′-terminal dUMP residues are not efficiently removed by the nuclear enzyme. Hence, a dUMP residue incorporated at the 3′ terminus during DNA synthesis would not represent an effective substrate until it was internalized by chain elongation. Thus, uracil-DNA glycosylase would not be expected to frequently generate 3′-apyrimidinic sites at the ends of replicating forks. If 3′-baseless sites were generated during DNA synthesis, they would impede further chain elongation since baseless sites are refractory for priming in vitro DNA synthesis by DNA polymerase α or β (Mosbaugh & Linn, 1983). The exact number of nucleotides that a uracil residue must be internalized before becoming an efficient substrate was not specifically determined. However, our data suggest that uracils located less than 21 bases from the 3′ end of poly(dA-dT) represent suboptimal substrates.

Anderson & Friedberg (1980) have reported the presence of nuclear and mitochondrial-associated uracil-DNA glycosylases in subcellular extracts of human KB cells. The results presented in this paper provide further evidence that rat liver nuclear and mitochondrial enzymes exist and that the purified enzymes are distinguished by different biochemical properties. The two rat liver species are characterized by a difference in chromatographic properties, molecular weight, sensitivity to NaCl, and stability to thermal inactivation. Two chromatographically distinct species of uracil-DNA glycosylase have also been partially purified from whole cell extracts of human lymphocytes (Sirover, 1979) and MPC-11 mouse cells (Hollstein et al., 1984). However, the subcellular localization of these forms has not been established. Similarly, two distinct species have been isolated from WI-38 human fibroblasts which have been described as nuclear and mitochondrial enzymes (Gupta & Sirover, 1981). These two fibroblast enzymes appear to be separately regulated during cellular proliferation. Taken together, these results suggest that the nuclear and mitochondrial enzymes represent distinctly different polypeptides and are perhaps expressed as different gene products.

We have also investigated the possibility that proteolysis could be involved in generating the subcellular forms of uracil-DNA glycosylase. This was of particular interest since the single-strand DNA binding endo-exonuclease of *Neurospora crassa*, another putative mitochondrial DNA repair enzyme, has been shown to be processed by a trypsin-like enzyme (Chow & Fraser, 1983). Although we cannot exclude the possibility that the nuclear and mitochondrial uracil-DNA glycosylases might be produced via proteolysis, the addition of PMSF upon purification did not alter the molecular weight of either enzyme. A more direct analysis of the primary polypeptide sequence or nucleotide sequence of the gene(s) is required to establish whether a structural relationship between the two rat liver enzymes exists.

Registry No. Uracil-DNA glycosylase, 59088-21-0.

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The Membrane Attack Complex of Complement: Lipid Insertion of Tubular and Nontubular Polymerized C9†

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ABSTRACT: The membrane-restricted photoactivatable carbene generator 3-(trifluoromethyl)-3-(*m*-[^{125}I]-iodophenyl)diazirine [Brunner, J., & Semenza, G. (1981) *Biochemistry 20*, 7174-7182] was used to label the subunits of the membrane attack complex of complement (C5b-9). C5b-9 complexes either were assembled from serum on erythrocyte membranes or were reconstituted from purified components on liposomes. After irradiation, most of the probe is bound to C9 independent of the membrane system used, indicating that the wall of the transmembrane channel is predominantly composed of C9. No difference was observed whether polymerized C9 was in the tubular or nontubular form [Podack, E. R., & Tschopp, J. (1983) *J. Biol. Chem. 257*, 15204-15212], showing that tubule closure is not essential for successful lipid insertion. The same label distribution between the two forms of polymerized C9 was obtained by analyzing zinc-polymerized C9 in the absence of C5b-8. Since the photoreactive probe reacted with at least two distinct polypeptide segments within C9, lipid interaction does not occur via a single segment of hydrophobic amino acids.

Association of the hydrophilic complement proteins C5b, C6, C7, C8, and C9 leads to a multimolecular structure termed the membrane attack complex (MAC)[1] (Podack & Tschopp, 1984). During the assembly process, the proteins expose lipid binding sites and insert into membranes (Bhakdi & Tranum-Jensen, 1983). Lipid insertion occurs during the formation of the intermediate complex C5b-7 (Mayer, 1982). Once associated with the membrane, C5b-7 binds C8. Several copies of C9 then interact with the membrane-bound C5b-8 forming the so-called membrane attack complex (MAC). Bound C9 then undergoes a conformational change allowing the formation of tubular polymers [poly(C9)] of different sizes

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[1] Abbreviations: EDTA, ethylenediaminetetraacetic acid disodium salt; ER, rabbit erythrocytes; MAC, complex of the complement proteins C5b, C6, C7, C8, and C9; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; TAN, 20 mM Tris, 90 mM NaCl, 0.2 mM EDTA, and 0.02% NaN_3, adjusted to pH 8.1 with acetic acid; TBS, 10 mM Tris and 150 mM NaCl, adjusted to pH 7.5 with HCl; [^{125}I]TID, 3-(trifluoromethyl)-3-(*m*-[^{125}I]iodophenyl)diazirine; Tris, (hydroxymethyl)aminomethane.

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