Uracil-DNA Glycosylase

PURIFICATION AND PROPERTIES OF URACIL-DNA GLYCOSYLASE FROM MICROCOCCUS LUTUS*

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A uracil-DNA-glycosylase from Micrococcus luteus has been purified more than 3,000-fold. The enzyme preparation appears homogeneous, according to the results of sodium dodecyl sulfate-polyacrylamide gel electrophoresis. It is devoid of nonspecific endonucleases, specific endonucleases for apurinic and apyrimidinic sites, 3-methyladenine or 7-methylguanine-DNA-glycosylases. It behaves as a monomer protein of 19,400 daltons. It has an isoelectric point of 7.0 ± 0.1. It has an optimal activity between pH 5.0 and 7.0. It has no cofactor requirement and is not inhibited by EDTA. Uracil-DNA-glycosylase is highly specific for DNA containing dUMP residues, releasing uracil as product of the reaction. It is 2-fold more active on single-stranded DNA than on double-stranded DNA. If it releases uracil dimers from ultraviolet-irradiated PBS1 DNA, it is at the threshold of the detection. The apparent $K_m$ is $7 \times 10^{-4}$ M, and uracil acts as a noncompetitive inhibitor with a $K_i$ of $3.2 \times 10^{-4}$ M. Cis-syn cyclobutadiuracil also is a potent inhibitor, while some analogs, produced by x-irradiation of uracil and thymine, are weak inhibitors. Spermine, between 10 and 400 µM, increases the enzymatic activity by 50% and is not inhibitory at other concentrations. Spermidine activates the enzyme at concentrations of 40 to 120 µM, but becomes inhibitory at 200 and 400 µM.

A new finding is that drugs which intercalate in DNA, such as ethidium bromide and ellipticine, cause a 2- to 2.5-fold activation of this enzyme activity. The concentrations giving maximal activation depend on the drug. The enzyme does not behave as a processive enzyme during uracil excision.

Uracil residues can arise in DNA by two different mechanisms: incorporation of dUTP instead of dTTP during DNA synthesis (1, 2), or in situ deamination of cytosine residues. The latter process occurs spontaneously (3), during bisulfite or nitrous acid treatment (4, 5), and at a low rate during UV and x-ray irradiation (6, 7).

The uracil residues can be actively removed from DNA by a uracil-DNA glycosylase. This enzyme hydrolyzes the base-sugar bond, liberating free uracil and generating an apyrimidinic site. This enzyme was originally found in Escherichia coli (8) and purified to homogeneity (9). Such enzymatic activities have also been found in and partially purified from extracts of Bacillus subtilis (10), Micrococcus luteus (11, 12) human placenta (13), human fibroblasts (14), calf thymus (15), phytohemagglutinin-stimulated peripheral lymphocytes (16), and blast cells from acute myelocytic leukemia patients (17). However, only the latter, the E. coli and the B. subtilis enzymes, have been extensively purified (9, 10).

The enzymology of DNA repair has been best documented in E. coli and M. luteus. There exist two different pathways: base excision repair and nucleotide excision repair (for review see Ref. 18). In the case of M. luteus, the study of the properties of uracil-DNA glycosylase was hampered by the only partial purification of the preparation (11). We have purified to a considerable degree our M. luteus uracil-DNA glycosylase. Its physical and catalytical properties, as well as its mechanism of action, are described in this paper.

EXPERIMENTAL PROCEDURES

Nucleic Acids, Nucleotides, and Related Compounds—Purine and pyrimidine bases, nucleosides and nucleotides were purchased from Sigma; 3-methyladenine from Fluka, Buchs, Switzerland. The $[^{3}H]$ dimethyl-sulfate and $[^{3}H]$Juridine (49 Ci/mmol) were from Radiochemical Center, Amersham. $[^{3}H]$thymidine (16 Ci/mmol), $[^{3}H]$thy- midine (20 Ci/mmol), and $[^{3}H]$Thymidine (46 Ci/mmol) were from CEA, Saclay, France; the $[^{3}H]$-bromodeoxyuridine and $[^{14}C]$ were from New England Nuclear. The $[^{3}H]$- and $[^{14}C]$Juracil-labeled phage PBS1 DNAs were prepared as described by Lindahl and Nyberg (19). Bacteriophage PBS1 and its host B. subtilis QUA-18 (asa-), as well as wild type B. subtilis, were obtained from Dr T. Lindahl, Department of Medical Chemistry, University of Gothenburg, Sweden. The specific radioactivities of $[^{3}H]$PBS1 DNA were 1.9 x 10^{6} and 8.9 x 10^{6} cpm/µg and that of $[^{14}C]$DNA was 1.75 x 10^{6} cpm/µg. The purity of the PBS1-DNA preparations was checked by hot acid degradation to bases, followed by analysis by HPLC using an ODS-3 column (see below). The results were that 75% of the total radioactivity was eluted with uracil, 25% with cytosine, and less than 0.2% with thymine, thus excluding contamination by host DNA. In order to test a possible contamination by cellular RNA, PBS1 DNA was incubated with preheated RNase (20) and the mixture was acid precipitated. Less than 0.2% of the total radioactivity was acid soluble. To prepare single-stranded DNA, $[^{3}H]$PBS1 DNA was denatured just before use by heating for 10 min in a boiling water bath (10 µg/ml DNA in 0.007 M Hespe, 0.002 M NaEDTA, pH 7.0) and chilling in an ice bath. PM$_{2}$ $[^{3}H]$DNA was obtained by growing the phage in the presence of $[^{3}H]$thymidine and 250 µg/ml of guanosine, as previously described for unlabelled phage (21). H$_{4}$ DNA containing 5-14H bromouracil residues was a gift from Dr F. Laval, Institut G. Roussy, Villejuif, France. It was prepared from H$_{4}$ cells (epithelial cells from a rat hepatoma), as previously described

1 The abbreviations used are: HPLC, high performance liquid chromatography; Hespe, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; UraO'Ura dimer, cis-syn cyclobutadiuracil; SDS, sodium dodecyl sulfate.
(22). DNA containing 14C-labeled purine residues were prepared from B. subtilis as described by Lindahl and Nyberg (23). DNA containing ["H"]-methyladenine and ["H"]-methylguanine was prepared, as previously described (24), by alkylation of calf thymus DNA (Chooay, Paris) with ["H"]-dimethyl sulfite. Its specific activity was 1,200 cpm/mmol.

Irradiation of DNA—In order to introduce uracil dimers, DNA samples were irradiated (8 x 10^12 J/m^2) at 0 °C at a concentration of 64 μM in 0.1 M NaCl, using a 1-mm path length quartz cell and a UVS-11 lamp. A black ray ultraviolet monitor (Ultra-Violet Products, Inc., San Gabriel, CA) was used to measure the UV dose. The photoreversal of potential dimers, with one glycosidic bond broken, leaving a second uracil linked to sugar, was performed using ultraviolet irradiation at 3.5 x 10^13 J/m^2.

Cells and Cell Culture—M. luteus (ATCC 4698) was obtained from the American Type Culture Collection, Rockville, MD. The cells were grown at 32 °C in a medium containing 5 g of tryptone, 5 g of yeast extract, 1 g of casaminoacids, (Difco products), and 5 g of NaCl (pH 7.3). The cells were harvested in late log phase and washed in buffer A (0.01 M sodium phosphate, 0.002 M EDTA, pH 7.7).

Enzyme Assays—Uracil DNA-glycosylase was assayed using a modification of the procedure described by Lindahl et al. (9). The standard reaction mixture (50 μl) contained 0.125 nmol of native PBSI ["H"]DNA, 7.5 μM Hepes-NaOH, 37.5 mM NaCl, 2 mM Na2 EDTA, and 5 mM diethiothreitol (pH 6.8). The reaction was initiated by adding the enzyme, diluted if necessary, in the standard mixture where DNA was omitted, but supplemented with bovine serum albumin (200 μg/ml). After incubation at 37 °C for different lengths of time, the reaction was stopped by adding 10 μl of a solution containing 10 mM NaCl, 20 μg of uracil, and 10 μg of calf thymus DNA, followed by 100 μl of 0.8% perchloric acid. After 10 min at 0 °C, the samples were centrifuged at 4000 x g for 12 min at 2 °C. The radioactivity in the supernatant was determined in a liquid scintillation counter.

When the enzyme was omitted, less than 0.2% of the total radioactivity was found to be acid soluble after a 30-min incubation of the reaction mixture. The enzyme reactions were performed with time and at a concentration within the range of 0.02-0.05 nmol of hydroxylated substrate. The enzyme activity, over the linear portion of time, was found to be acid soluble after a 30-min incubation of the extract, as measured by linear regression analysis. Under the above conditions and using four different incubation times, one unit of uracil DNA-glycosylase activity was found to be acid soluble after a 30-min incubation of the extract.

The presence of a nonspecific deoxyribonuclease activity was checked by monitoring the nicking of supercoiled PM2 DNA by agarose gel electrophoresis. When the enzyme was omitted, less than 0.2% of the total radioactivity was found to be acid soluble after a 30-min incubation of the reaction mixture. The enzyme reactions were performed with time and at a concentration within the range of 0.02-0.05 nmol of hydroxylated substrate. The enzyme activity, over the linear portion of time, was found to be acid soluble after a 30-min incubation of the extract, as measured by linear regression analysis. Under the above conditions and using four different incubation times, one unit of uracil DNA-glycosylase activity was found to be acid soluble after a 30-min incubation of the extract.

Nonspecific Deoxyribonuclease and DNA-glycosylase Activities—The presence of a nonspecific deoxyribonuclease activity was checked by monitoring the nicking of supercoiled PM2 DNA by agarose gel electrophoresis, as previously described (25). A similar assay was used to check the presence of endonuclease activity specific for apurinic or apyrimidinic sites. The 5'-methylated DNA-agarose glycosylase, and unspecific phosphatases were assayed as previously described (23, 24, 26).

Determination of Uracil, Cytosine, and Cis-syn Cyclobutaduracil—It was performed by high performance liquid chromatography, using a Cecil model CE 212 variable wavelength detector (Cecil Instruments, Cambridge, England) equipped with a 0.46 cm, inner diameter). The column was packed with sterically exchange Bondapak AX-Corasil stationary phase (Waters Associates, Milford, MA) which has a particule size of 37-50 μm, by the “tap fill” technique. The mobile phase was an aqueous solution of 0.01 M KH2PO4, buffered at pH 5.5 with potassium hydroxide. Aqueous solutions were filtered using Millipore HA 0.45 μm cellulos filters prior to use.

The relative elution order of the following: Ura > U (K' = 0.46) > cyt (K' = 1.00) > uracil (K' = 1.42). The capacity factor Vᵣ/V₀, where Vᵣ is the elution volume of the peak and V₀ the dead volume of the column. The absence of any detectable amount of radioactive contaminant in the elution profile of the DNA control experiment at the position of Ura or U allows accurate determination of low level of Ura or Ura in the various irradiated samples.

Analysis of the Enzymic Reaction Products—It was performed by Biogel P-2 chromatography as described by Khym (27), using a column (0.5 cm2 x 50 cm). The acid-soluble products of the reaction were adjusted to pH 8.9 and chromatographed together with dAMP, dGMP, dTMP, and dUMP. The UV-transmission of the effluent was continuously monitored at 254 nm, with an LKB recorder. Fractions were collected and their radioactivities measured.

Electrophoresis—Polyacrylamide gel electrophoresis was performed according to the method of Ornstein (28), using 7.5% acryl- amide. Prior to each experiment, the gels were equilibrated to a voltage of 65 V, at 4 °C, for 12 h. Thirty units of enzyme (50 μl) were loaded on the top of each gel, together with bromophenol blue as marker. The run was performed at 300 V and at 4 °C. After the end of the run, one of the gels was stained with Coomassie blue, while the other was sliced in 5-mm fractions. Each fraction was crushed with a glass rod and extracted at 0 °C with 100 μl of a solution containing 14 mM Hepes-NaOH, 2 mM EDTA, 5 mM dithiothreitol, 20% glycerol, 500 μg/ml of bovine serum albumin (pH 6.5). The enzymatic activity was measured in each extract. The radioactive labeling of proteins by chemical iodination and the method for electrophoresis under denaturating conditions has previously been described (25). Kodirex ROPMat film was used for autoradiography. The film was scanned with a Joyce Loebel densitometer.

Sedimentation Analysis of Protein—Sucrose gradient centrifugation was carried out as previously described (25), but modified by using a linear 5-20% sucrose gradient in 5 mM potassium phosphate, 300 mM KCl, 5 mM dithiothreitol, at pH 6.5.

Cell Filtration Analysis—Exclusion chromatography on Sephadex G-75 (fine) was used to measure the Stokes radius of uracil-DNA glycosylase, according to the method of Siegel and Monty (29), as previously detailed (25), but modified as follows: the equilibration buffer was the same used for sucrose centrifugation.

Materials—The origin of protein markers, protease inhibitors, chromatographic supports, and various specific reagents has already been described (25, 30). Hydroxyapatite Ultragel was purchased from Bio-Rad, and Poly A Sepharose was Pharmacia products (Piscataway, N.J.).

Compounds Capable of Influencing the Uracil-DNA-Glycosylase Activity—Three classes of compounds were studied. Uracil analogs were obtained from Sigma, and 5-fluorouracil was a gift from Roche Laboratories, Paris. Degradation products of cytosine and thymine, which can be induced by x-rays were synthesized according to published procedures: 5,6-dihydrothymine (31), 5,6-dihydroxy-5,6-dihydrothymine (32), cis-syn cyclobutadiuracil (33), other compounds (34). All solutions were freshly prepared from powder and used within an hour to obviate spontaneous decomposition. A second class of compounds covalently bound to DNA was purchased from Sigma. Among intercalating drugs, ethidium bromide was obtained from Boots Pure Drug Co., Nottingham, England; 9-amino-acridine, ellipticine acetate (35) and ethidium homodimer (36) were gifts from Drs. C. V. Paoletti and J. B. Le Pecq, Institut G. Roussy, Villejuif, France. The third class of compounds, compounds that covalently bound to DNA, were purchased from Drs. C. V. Paoletti, D. R. Saucier, Institut G. Roussy, Villejuif, France. In order to compare various drugs with different affinities for DNA, the results were expressed as the ratio r of the intercalated drug per nucleotide, as described by Le Pecq and Paoletti (38), using the published apparent dissociation constants and numbers of binding sites per nucleotide (39).

RESULTS

Purification

Crude Cell Extract—The cells (25 g), grown as described under "Experimental Procedures" and suspended in buffer A, were lysed with lysozyme in the presence of the protease inhibitor, sonicated, and centrifuged, as described for the preparation of AP-endonucleases (25). This method yielded 100 ml of fraction I.

Poly(ethylene glycol) Dextran Phase Partition—The procedure for the preparation of AP-endonucleases (25) was followed to yield fraction II, which was concentrated to 120 ml with poly(ethylene glycol) and dialyzed for 1 h against 2 liters of 20 mM sodium phosphate, 2 mM Na2 EDTA, and 5 mM 2-mercaptoethanol, pH 7.7 (buffer B) (fraction II).

DEAE-cellulose Chromatography I—A column of DEAE-
cellulose (12.5 cm² × 12 cm) was equilibrated with buffer B. Fraction II was loaded on the column and washed with 200 ml of buffer B. Uracil DNA-glycosylase eluted with the unadsorbed proteins. Under these conditions AP-endonucleases A and B remained adsorbed on the resin. Elution of the column with up to 0.5 M potassium phosphate released no uracil DNA-glycosylase activity. The active fractions, which did not adsorb to the resin, were pooled, concentrated 5 times with poly(ethylene glycol) and dialyzed against 2 liters of 2 mM sodium phosphate, 5 mM 2-mercaptoethanol, pH 7.7 (buffer C), yielding fraction III.

**DEAE-cellulose Chromatography II**—A column of DEAE-cellulose (2 cm² × 12 cm) was equilibrated with buffer C. Fraction III was loaded on the column and washed with 50 ml of buffer C. The uracil-DNA glycosylase activity was recovered in the effluent. The active fractions were pooled and slowly titrated with 0.2 M H₃PO₄ to pH 6.8 (fraction IV).

**Hydroxyapatite-Ultrogel Chromatography**—A column of hydroxyapatite-Ultrogel (0.3 cm² × 12.7 cm) was equilibrated with buffer D: 5 mM potassium phosphate, 5 mM 2-mercaptoethanol, 200 µg/ml of bovine serum albumin, pH 6.8 (buffer D). Fraction IV was loaded on the column. The column was washed with 20 ml of buffer and the enzyme was eluted with a linear gradient of 0 to 0.3 M potassium phosphate in buffer D (total volume 100 ml). The active fractions were pooled and dialyzed for a total of 90 min against 1 liter of buffer and the enzyme was eluted with 0.2 M H₃PO₄ to pH 6.7 (fraction V).

**Poly U-Sepharose Chromatography**—Fraction V was applied to a column of Poly U-Sepharose (0.38 cm² × 3.1 cm) previously equilibrated with buffer E. The column was washed with 10 ml of buffer E, then another 10 ml of buffer E containing dextran sulfate (200 µg/ml). The latter wash was used to eliminate contaminating proteins which did not bind strongly to the column. This treatment had already been found useful during the purification of AP-endonucleases (25). The column was again washed with 10 ml of buffer E and the enzyme was eluted with a linear gradient of 0 to 0.5 M KCl in buffer E (total volume, 20 ml). The pooled active fractions were concentrated with glycerol. The resultant fraction was stable for several months in 50% glycerol at −20 °C. Unless otherwise stated, it was used in all subsequent steps of enzyme characterization.

**Comments on the Purification**—A highly purified enzyme was also obtained by substituting P-cellulose and DNA-agarose chromatography by hydroxyapatite-Ultrogel and Poly U-Sepharose. A comparison of the different enzyme preparations showed that their purities were similar. However, with the latter method the recovery was very poor. We have also compared the efficiency of other potential affinity columns, namely DNA-agarose (25), Poly A-Sepharose, and Poly U-Sepharose for the purification of uracil DNA-glycosylase. The enzyme bound to all the columns, the highest affinity being for Poly U-Sepharose, followed by DNA agarose, since the ionic strengths used to elute the enzyme were 0.2 and 0.1 M KCl, respectively. Attempts to scale up the purification procedure, in order to obtain stable enzyme without bovine serum albumin, were unsuccessful. In preparations purified without protective protein (see below), the amount of protein after hydroxyapatite chromatography was less than 10 µg/ml. This indicates that, after this step, the enzyme has been purified more than 3000 times (Table I).

**Physical Properties of the Uracil-DNA Glycosylase**

**Degree of Purity of the Preparation**—When the purified preparation of uracil-DNA glycosylase (fraction VI prepared without added bovine serum albumin) was analyzed, after iodination, by SDS-polyacrylamide gel electrophoresis, a single sharp symmetrical peak was observed (Fig. 1). To correlate this peak with enzymatic activity, an aliquot of fraction VI (stabilized by the addition of bovine serum albumin) was analyzed by continuous polyacrylamide gel electrophoresis under non-denaturing conditions. As shown in Fig. 2, the enzyme activity was found associated with one stained band, the other band representing bovine albumin.

**Absence of Contaminating Enzymatic Activities in Purified Preparations**—Fraction VI showed no nicking activity on either native supertwisted PM2DNA (Fig. 3), or under similar conditions on supertwisted PM2DNA carrying AP-sites (data not shown). When DNA containing [³H]-3-methyladenine and [³H]-7-methylguanine, substrates for the corresponding DNA-glycosylases, was incubated with 10 units of fraction VI, less than 1% of the radioactivity became ethanol-soluble. This amount can be accounted for by chemical de-purination of the alkylated bases during incubation.

**Molecular Weight**—The molecular weight was determined by electrophoresis in SDS-polyacrylamide slab gels, exclusion chromatography on Sephadex G-75, and sucrose zone centrifugation. From the electrophoretic experiments in SDS-polyacrylamide slab gels where the mobility of the uracil-DNA glycosylase was compared with the mobilities of marker proteins of known molecular weight, it was estimated that the polypeptide chain had a Mᵣ = 19,000 ± 1,900.

The Stokes radius was estimated by chromatography on a Sephadex G-75 column. The enzyme eluted as a single symmetrical peak. From the relation between the elution volumes of the proteins and their sizes, a Stokes radius of 20 ± 1 Å was evaluated, corresponding to a Mᵣ = 19,400 ± 1,000 (29).

The sedimentation coefficient of the enzyme, measured in Table I.

<table>
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<tr>
<th>Fraction</th>
<th>Volume</th>
<th>Protein</th>
<th>Total Activity</th>
<th>Specific Activity</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>ml</td>
<td>mg/ml</td>
<td>units x 10⁻⁶</td>
<td>units/mg of protein</td>
</tr>
<tr>
<td>I. Crude extract</td>
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<td>40.0</td>
<td>28.7</td>
<td>71</td>
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<tr>
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<td>10.3</td>
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<td>0.08</td>
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<td>BSA⁹</td>
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<td>VI. Poly U-Sepharose</td>
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<td>BSA²</td>
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* Bovine serum albumin (BSA) was added to the column effluent to protect the enzyme from denaturation.

**Fig. 1.** SDS-polyacrylamide slab gel electrophoresis of purified uracil-DNA glycosylase (fraction VI). The enzyme was prepared without the addition of bovine serum albumin. The overall enzyme recovery after purification was less than 1% and the enzyme was extremely unstable. Fifty microliters of the preparation was iodinated and analyzed by electrophoresis. A film was exposed to the gel, developed, and scanned. The arrow shows the origin of migration. For details see "Materials and Methods."
sucrose gradients containing marker proteins of known coefficients, was $d_{20w} = 2.1 \pm 0.1$.

Isoelectric Point—Its value was $7.0 \pm 0.1$ as measured by electrophoresis in a pH gradient. Another measurement was performed on the poly(ethylene glycol) fraction in order to ascertain that the extract did not contain another uracil-DNA glycosylase activity which could have been lost during the purification procedure. A single symmetric peak of activity was found at pH 6.8 $\pm$ 0.2 (data not shown).

**Enzymatic Properties of the Uracil-DNA Glycosylase**

**pH**—The optimal pH for enzymatic activity was determined in different buffers, using native and heat denatured DNA. The results are shown in Fig. 4. An optimal activity occurred between pH 6.0 and 7.0 at $37^\circ$C, using a saturating substrate concentration. Under all conditions, the activity was proportional to time, thus excluding a possible denaturation at the measured pH values.

Effects of Different Ions and Compounds—The monovalent ions K$^+$ and Na$^+$ behaved in the same manner. They were activators at low concentrations (between 20 and 50 mM) and inhibitors at higher concentrations: 83%, 24%, and 10% of the maximal activity were measured at concentration of 100, 200, and 300 mM, respectively. Mg$^{2+}$, Mn$^{2+}$, and Ca$^{2+}$ were slightly inhibitory at 5 mM (10 to 30% inhibition). They did not activate at lower or higher concentrations. Co$^{2+}$ and Zn$^{2+}$ at 1 mM were potent inhibitors. Mercaptoethanol and EDTA, at concentrations ranging from 1 to 50 mM, had no effect.

$K_m$—The initial velocity of the enzyme was measured using various concentrations of native DNA as substrate. The results were plotted according to Lineweaver and Burk. The $K_m$ found was $7 \times 10^{-8}$ M.

**Substrate Specificity**—The initial cleavage rate of native PBS1 DNA was 50% that of heat denatured DNA (Fig. 5). It should be noted that in the case of denatured DNA, only the initial velocity was increased and that at the end of the reaction the plateau values were the same for the two DNAs.

Uracil-DNA-glycosylase was highly specific for DNA containing dUMP residues: the amount of enzyme which, under saturating concentration of substrate, liberated 50% of the uracil from PBS1 DNA in the standard assay, released less than 1.0% of acid or ethanol-soluble products when it acted on the following substrates: $[^{3}H]$adenine-$[^{3}H]$guanine B. subtilis DNA, $[^{3}H]$cytosine E. coli DNA, $[^{3}H]$thymine E. coli DNA, $[^{3}H]$T5 depurinated DNA, 5$'$-$[^{3}H]$bromouridine H4 PBS1 DNA and either no enzyme (A), or 4.0 units of uracil-DNA glycosylase. The incubation mixtures contained 1.6 nmol of native PM$_2$ DNA containing 12% form II DNA (C). For details, see "Materials and Methods." The arrows indicate the position of covalently closed circular duplexes (I) and nicked molecules (II).

**Fig. 3.** Densitometric tracing of an agarose gel electrophoresis of native PM$_2$ DNA after treatment with uracil-DNA glycosylase. The incubation mixtures contained 1.6 nmol of native PM$_2$ DNA and either no enzyme (A), or 4.0 units of uracil-DNA glycosylase (B), or aged PM$_2$ DNA containing 12% form II DNA (C).

**Fig. 4.** Activity of uracil-DNA glycosylase as a function of pH, buffer, and tertiary structure of DNA. The determination of the enzymatic activity was performed as described under "Materials and Methods." $\triangle - \triangle$, 0.007 M Hapes, 0.0375 M NaCl, native DNA; $\square - \square$, same solvent and heat-denatured DNA; $\odot - \odot$, 0.007 M Hapes, 0.15 M NaCl, native DNA; $\square - \square$, potassium phosphate buffer, native DNA.
inhibitor of uracil-DNA glycosylase. Using a constant substrate concentration, the activity of the enzyme was measured at 15°C, 25°C, and 45°C, using the Arrhenius equation.

The inhibition at a given concentration of uracil (0.0036 M) was modulated by the pH and the ionic strength of the solvent. At low ionic strength (0.0375 M NaCl) and a uracil concentration of 3.5 × 10^{-3} M, the inhibition was independent of the pH: 18% and 15% residual activity at pH 5.5 and 7.5, respectively. At high ionic strength (0.15 M NaCl), the residual activity was 2-fold higher at pH 5.5 than at pH 7.5.

Several uracil analogs were also tested as inhibitors, focusing on products which may arise from nucleotides or DNA by UV- or x-irradiations. Table II shows that in addition to uracil, several compounds were found to be potent inhibitors. The other compounds were weak inhibitors even at high concentrations. In addition, we also tested at 2 mM final concentration: thymine, adenine, 3 methyladenine, cytosine, hypoxanthine, ATP, dUMP, cis-syn cyclobutadiuridine. No inhibition of enzymatic activity was observed (less than 5%) in the presence of these compounds.

Energy of Activation—The apparent energy of activation was found to be 21.5 kcal mol^{-1} when native PBSI DNA was used as substrate and 14.5 kcal mol^{-1} with the same DNA but heat denatured. These values were computed from the initial velocity of the enzyme measured at 15°C, 25°C, 37°C, and 45°C, using the Arrhenius equation.

Mechanism of Action of Uracil-DNA Glycosylase

Identification of the Acid-soluble Product Released by the Enzyme as Ura—The assay for enzymatic activity consisted in following the release of radioactivity from DNA containing labeled dUMP and dCMP moieties. In order to ascertain that the enzyme was a DNA-glycosylase, it was necessary to show that the product of the reaction was uracil. This identification was done by chromatography on Biogel P2, as shown in Fig. 6. Under the conditions used, nucleotides, nucleosides, pyrimidic and purinic bases were separated. The radioactivity (more than 98%) co-chromatographed with the uracil marker. The same results were obtained using HPLC chromatography (data not shown). In the latter chromatographic system, which allows the separation of uracil and cytosine, all the radioactivity was associated with uracil. After the second UV irradiation, no radioactivity was found at the position of uracil. This result rules out, at the level of detection used, any appreciable

otides. All the radioactivity migrated with uracil when the uracil-DNA glycosylase was freed from the AP-endonucleases which remained adsorbed on DEAE-cellulose I (25).

Can Uracil-DNA Glycosylase Excise Uracil Dimers?—After ultraviolet irradiation, PBSI DNA was found to contain pyrimidine dimers (Ref. 40 and data not shown). When such a substrate, containing uracil or uracil dimers (2% of the total uracil) was used as substrate for the uracil-DNA glycosylase, the initial velocity of the enzyme was lower than that observed with unirradiated substrate. The product of the reaction was analyzed in order to determine if Ura or Ura dimers were released. Such a release was barely detected (about 2% of the radioactivity were found at the position of Ura or Ura and 98% at the position of uracil, data not shown). However, the possibility existed that one bond of the dimer might be broken, leaving the second uracil of the dimer attached to its respective sugar. In order to check this possibility, ultraviolet irradiated PBSI DNA was treated with uracil-DNA glycosylase, the products of the reaction were separated by ethanol precipitation, the DNA was recovered, dialyzed, and again heavily irradiated with UV. This treatment is known to break the cyclobutane ring and to release the pyrimidine if one glycosylic bond is broken (41). After the second UV irradiation, no radioactivity was found at the position of uracil. This result rules out, at the level of detection used, any appreciable
Effects of Polyamines on the Uracil-DNA-glycosylase Activity—It has been shown that the activity of human uracil-DNA-glycosylase was enhanced by the presence of spermine or spermidine in the incubation mixture (17). We have studied the effect of these two polyamines on the activity of our enzyme. At concentrations ranging from 10 μM to 400 μM, spermine increased the activity by 30 to 50% and was not inhibitory at other concentrations. At concentrations ranging from 40 to 120 μM, spermidine increased the enzymatic activity by 50%, while at 200 and 400 μM an inhibition of 50% was observed. Since the influence of polyamines on the enzyme seemed rather discrete, the mechanism of action of these compounds was not investigated further.

Activation of Uracil-DNA-Glycosylase by DNA Intercalating Drugs—Since drugs which intercalate in DNA modify its structure and depress DNA repair, this class of compounds was investigated for their possible influence on uracil-DNA-glycosylase. The results (Fig. 7) show that drugs which intercalate in DNA as monomers are activators of the enzyme roughly to the same extent (the curves have a bell shape), but with different characteristics. An optimal activation is obtained when one molecule of 9-aminoacridine, or of 2-methyl-9-hydroxellipticinium is intercalated/10 nucleotides. With ethidium bromide, the optimal activation is obtained near saturation, that is, when one molecule is intercalated/about 6 nucleotides. At saturation, all the drugs tested were inhibitors. Ethidium homodimer showed a very weak activity, either as an activator at low concentration or as an inhibitor at high concentration. Irehdiamine, which does not intercalate in DNA, had almost no influence on the uracil-DNA-glycosylase activity.

Is Uracil-DNA-Glycosylase a Processive Enzyme?—The hydrolysis of glycosidic bonds by uracil-DNA-glycosylase can occur by two different mechanisms. Either the enzyme dissociates from the DNA molecule after each catalytic event (distributive enzyme), or it remains bound to the DNA molecule until all the dUMP residues of the molecule are excised (processive enzyme).

The mechanism of action was investigated by means of a DNA challenge experiment (data not shown). 3H or 14C native PBS1 DNA, used at the same concentration, were equally good substrates for the enzyme. When the enzymatic reaction was initiated with [3H]DNA and an equal amount of [14C] DNA was added later, the sum of the 14C and 3H-labeled uracil released was equal to the amount of uracil liberated when the enzyme acted on only one substrate. There was no preferential release of [3H]uracil. Therefore uracil-DNA-glycosylase acted as a distributive, and not as a processive enzyme.

**DISCUSSION**

From extracts of *M. luteus* we have purified a uracil-DNA-glycosylase more than 3000-fold and to apparent homogeneity as judged by polyacrylamide electrophoresis analysis. The enzyme preparation was shown to be free of contaminating nonspecific deoxyribonucleases, AP-endonucleases, 3-methyladenine-DNA-glycosylase, 7-methylguanine-DNA glycosylase.

In comparison with the two other DNA glycosylases obtained as homogeneous preparations from *E. coli* (9) and from blast cells (17), the *M. luteus* enzyme is a smaller molecule: 19,000 daltons, to be compared with 24,500 daltons for the *E. coli* enzyme and 30,000 daltons for the mammalian enzyme. None of these enzymes requires specific divalent cations for optimal activity and they all are fully active in the presence of low concentrations of EDTA. The $K_m$ of the bacterial enzymes are of the same order of magnitude: $4 \times 10^{-6}$ M and $7 \times 10^{-5}$ M for the *M. luteus* and the *E. coli* enzymes, respectively, which is 10 times lower than the $K_m$ of the mammalian enzyme. The three enzymes are 2-fold more active on denatured than on native DNA and they are highly specific for DNA containing dUMP residues. The three enzymes are inhibited by uracil which acts as a noncompetitive inhibitor, with a $K_i$ ranging from $1.2 \times 10^{-4}$ M for the *E. coli* enzyme to $5 \times 10^{-4}$ M for the *M. luteus* enzyme. The activation energy is higher for native than for denatured DNA. This difference may reflect the requirement for a local melting of the double-stranded DNA (to give rise to a better substrate for the enzyme). The difference between the activation energies for native and for denatured DNA is of the same order of magnitude as the energy required for a partial melting of double-stranded DNA (42).

It should be noticed that if uracil-DNA-glycosylase excise uracil dimers, it is barely detectable. Duker et al. (43) found no excision of uracil-dimers by the *B. subtilis* enzyme. However they were using paper chromatography and HPLC was shown to be a more efficient method of separation of cyclobutyl pyrimidine dimers (43). We have been unable to show the incision for one of the glycosidic bonds of the dimer, as is observed in *M. luteus* for thymine thymine dimer (41). However, uracil dimers are inhibitors of the enzyme. It should also be pointed out that products arising from bases by x-irradiation may be slight inhibitors at high concentrations. This raises the question of the possible role of the enzyme in the removal of some x-rays induced lesions.

The effects of polyamines are less pronounced on the bacterial enzymes than on the blast cells enzyme (17), although in both cases they behave as activators; this suggests a more general role of the polyamine in the modulation of uracil-DNA glycosylase activity.
The degree of stimulation of uracil-DNA-glycosylase by intercalating drugs depends on the proportion between intercalated drug and nucleotides. The activation is more pronounced with monomers than with dimers. It has been shown (44, 45) that the intercalation of ethidium bromide causes an alteration of the glycosidic torsional angle. If so, it might give rise to structures more easily recognized by the enzyme. The optimal values of \( r \) observed for maximal initial velocity of the enzyme are 0.1 for both 9-aminoacridine and 2-methyl-9-hydroxyellipticine, and 0.2 for ethidium bromide. This result may be related to the observations that intercalating drugs give different alteration of the glycosidic torsional angle (46). It should be noted that the observed effects of intercalating drugs on \( M. \) \( luteus \) uracil-DNA-glycosylase do not apply to the two enzymes extracted from H4 rat fibroblast.

The role of uracil-DNA-glycosylase is well established as that of a repair enzyme. We have now direct in vitro evidence that it prevents transition mutations by preventing the incorporation of DMP in the de novo synthesized strand in positions opposite the dUMP residues of the template. It has been suggested (47) that uracil-DNA-glycosylase may have a regulatory role during DNA synthesis and that the removal of uracil may induce events such as recombination. It has been shown that in eucaryotic cells, the DNA repair pathways depend on the stage of cell proliferation (48) and that a nuclear uracil-DNA-glycosylase is induced prior to the initiation of DNA replication in WI-38 cells (49).

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