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An *N*-Glycosidase from *Escherichia coli* That Releases Free Uracil from DNA Containing Deaminated Cytosine Residues

(deoxyuridine/DNA repair/heteroduplex DNA)

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ABSTRACT **An enzyme that liberates uracil from single-stranded and double-stranded DNA containing deaminated cytosine residues and from deoxycytidylate-deoxyuridylate copolymers in the absence of Mg^{++} has been purified 30-fold from cell extracts of *E. coli*. The enzyme does not release uracil from deoxyuridine, dUMP, uridine, or RNA, nor does it liberate the normally occurring pyrimidine bases, cytosine and thymine, from DNA. The enzymatic cleavage of *N*-glycosidic bonds in DNA occurs without concomitant cleavage of phosphodiester bonds, resulting in the formation of free uracil and DNA strands of unaltered chain length that contain apyrimidinic sites as reaction products. The enzyme may be active in DNA repair, converting deaminated dCMP residues to an easily repairable form.**

Slow hydrolytic degradation of the primary structure of DNA occurs in neutral aqueous solution, and it seems likely that several types of spontaneous lesions are introduced into DNA at biologically significant rates under *in vivo* conditions (1–4). One type of hydrolytic event that may be of biological relevance is the deamination of cytosine residues to uracil in DNA (4, 5). The lability of cytosine in comparison with the other DNA bases raises the possibility that cells possess repair mechanisms to convert guanine·uracil base-pairs in DNA back to guanine·cytosine pairs. It is also interesting in this regard that bisulfite, which may be a common environmental mutagen, catalyzes the deamination of cytosine to uracil in nucleic acids (6). If this type of DNA lesion can be repaired, cells might be expected to contain enzymes specifically acting on DNA with deaminated cytosine residues. A search for such activities was, therefore, undertaken. The present report describes an enzyme from *Escherichia coli* that cleaves uracil–deoxyribose bonds in DNA, thereby converting deaminated dCMP residues to apyrimidinic sites.

MATERIALS AND METHODS

Nonradioactive pyrimidine derivatives, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (Hepes), snake venom phosphodiesterase, *E. coli* alkaline phosphatase, and *Aspergillus oryzae* crude α-amylase were obtained from Sigma. The phosphodiesterase was further purified according to Sulkowski and Laskowski (7). S_1 nuclease was purified from the crude α-amylase by heat treatment followed by gradient chromatography on DEAE-cellulose, and was used under conditions optimal for this enzyme (8). On incubation of DNA (20 μg/ml) with the S_1 nuclease (40 μg/ml of protein) for 1 hr at 37°, >95% of heat-denatured DNA but <1% of native DNA was acid-solubilized. Terminal deoxynucleotidyl transferase was purified from calf thymus (9).

[^{3}H]dUTP was obtained from the Radiochemical Centre, Amersham, and [2-^{14}C]dCTP, [8-^{14}C]dATP, [2-^{14}C]uracil, and [^{3}H]uridine were from New England Nuclear Chemicals. The [^{3}H]dUTP was freed from traces of dCTP prior to use by chromatography on DEAE-cellulose, with a linear gradient of 0–0.4 M KCl in 0.01 M citrate, pH 3.3. [^{3}H]dUMP and [^{3}H]deoxyuridine were made from [^{3}H]dUTP by treatment with either phosphodiesterase alone, or with phosphodiesterase and phosphatase together, followed by isolation by preparative paper chromatography.

The endonuclease I-deficient strain *E. coli* 1100 was a gift from Dr. H. Hoffmann-Berling. The bacteria were grown in a glucose-mineral salts medium, supplemented with 0.2% casamino acids and 0.1% yeast extract, and were harvested in the logarithmic growth phase.

Nucleic Acids. *E. coli* B DNA, ^{14}C-labeled in the cytosine residues (30,000 cpm/μg) was obtained from the uracil- and thymine-dependent mutant OK308 grown in the presence of [2-^{14}C]uracil and nonradioactive thymine (3). [^{14}C]Thymine-labeled DNA (21,000 cpm/μg) was prepared from the same strain after growth with nonradioactive uracil and radioactive thymine. Phage T7 [^{3}H] DNA (50,000 cpm/μg) was made according to Richardson (10), and [^{14}C]adenine-labeled poly(dA-dT), 44,000 cpm/μg, according to Schachman *et al.* (11). Salmon sperm DNA was purchased from Sigma. It was dissolved in 0.1 M NaCl, 0.01 M Hepes·KOH, 1 mM ethylenediaminetetraacetate (EDTA), pH 8.0, extracted with phenol, extensively dialyzed against the same buffer, and heat-denatured (100°, 5 min) before use. [^{3}H]Uridine-labeled ribosomal RNA (54,000 cpm/μg) was prepared from purified ribosomes (12) after growth of *E. coli* with [^{3}H]uridine. Yeast tRNA was made as described (13).

The copolymer poly(dC,[^{3}H]dU) was prepared with terminal deoxynucleotidyl transferase, with a tetranucleotide fraction from a DNase I digest of thymus DNA as primer. The synthesis of copolymers of random sequence from mixtures of several deoxynucleoside triphosphates and the synthesis of poly(dU) from dUTP with this enzyme have been reported (14). Pilot experiments showed that under the conditions used here the enzyme polymerized dCMP preferentially over dUMP. The reaction mixture finally employed (1.2 ml) contained 0.2 M K cacodylate, pH 7.0, 2 mM $CoCl_2$, 1 mM dCTP, 1 mM [^{3}H]dUTP (24 × 10^6 cpm), 50 μM $(dNMP)_4$, and 600 units of terminal transferase. After 20 hr at 35°, the

Abbreviations: Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid.

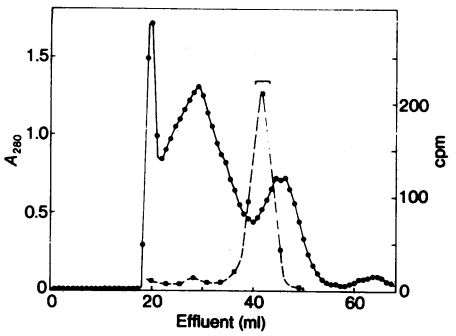

FIG. 1. Purification of the *N*-glycosidase activity by gel filtration on Sephadex G-100. (●), A_{280}. The ability to release [^{3}H]uracil (■) from poly(dC,[^{3}H]dU) by 0.25-μl aliquots of indicated fractions was assayed under standard reaction conditions.

reaction was stopped by addition of NaCl (to 0.5 M), EDTA (to 10 mM), and ethanol (2 volumes). The precipitate was recovered by centrifugation, dissolved in 0.1 M NaCl, 10 mM Hepes·KOH, 1 mM EDTA, pH 8.0, and dialyzed against the same buffer for 40 hr at 2°. At the end of the incubation period, 60% of the dCMP and 2.7% of the dUMP residues were in acid-insoluble form. One-half of this material was subsequently lost during the dialysis, indicating that a large part of the product was of oligonucleotide size. The nondialyzable material had a sedimentation coefficient of about 1.3 S, as determined by alkaline sucrose gradient centrifugation, and a specific activity of 2×10^7 cpm/μmole of dUMP residue. [^{14}C]-Labeled poly(dC), 2×10^5 cpm/μmole, was prepared in the same way, with the substitution of [2-^{14}C]dCTP for the [^{3}H]dUTP. The polymer chains were much longer in this case, and had a sedimentation coefficient of 9 S in alkaline solution.

Deamination of cytosine residues in DNA and poly(dC) by NaOH treatment was performed according to Ullman and McCarthy (15). In strong alkali, deamination of cytosine is the prevalent mode of degradation of DNA, but alkali-catalyzed depurination and chain breakage also occur. To [^{14}C]-cytosine-labeled DNA (600 μg/ml), an equal volume of freshly made 2 M NaOH was added, followed by incubation at 70° for 30 min. The alkali-treated DNA was neutralized and dialyzed against 10 mM Hepes·KOH, 1 mM EDTA. An aliquot of this DNA was analyzed for dUMP content as described (4), and another aliquot was characterized by alkaline sucrose gradient centrifugation. Before the NaOH treatment, <0.05% of the DNA deoxycytidine residues were deaminated, but after treatment 3.2% were present as deoxyuridine. The sedimentation coefficient of the DNA in alkaline solution simultaneously decreased from 36 S to 9 S. The [^{14}C]poly(dC) was deaminated in the same fashion, except that the NaOH treatment was only for 15 min. The polymer contained <0.1% dUMP residues before treatment and 1.5% after treatment. One-half of the partly deaminated DNA was reassociated by addition of NaCl (to 1 M) to the DNA solution (150 μg/ml), followed by incubation at 65° for 20 hr. This treatment converted the DNA from a form >95% acid-solubilized by S_1 nuclease treatment to a form in which only 18% of the DNA was sensitive, indicating that most of the sequences had reassociated to a double-stranded form.

TABLE 1. *Purification of the N-glycosidase activity*

Fraction	Volume (ml)	Protein (mg)	Specific activity (units/mg)	Total activity (units)
I. Crude extract	17	159	2.7	430
II. Ammonium sulfate	1.0	32	10	320
III. Sephadex G-100	2.7	1.4	80	110

Paper Chromatography. Details of the chromatographic procedures have been described previously (3, 4). The following solvent systems were used: I. Isobutyric acid–water–0.1 M EDTA–concentrated ammonia–toluene (160:22:3:2:20). II. Isobutyric acid–water–0.1 M EDTA–concentrated ammonia (66:33:1:1). III. Isopropyl alcohol–concentrated HCl–water (170:41:39). IV. Upper phase from ethyl acetate–*n*-propyl alcohol–water (4:1:2). V. 1-butyl alcohol–concentrated ammonia–water (86:5:14).

Enzyme Assay. The assay measures the conversion of radioactive uracil in deoxynucleotide form to free uracil. The standard reaction mixture (50 μl) contained 0.7 μg (2000 cpm) poly(dC,[^{3}H]dU) and 0.5 μg of denatured salmon sperm DNA in 70 mM Hepes·KOH, pH 7.8, 1 mM EDTA, 1 mM dithiothreitol, and a limiting amount of enzyme (<0.02 units). After incubation at 37° for 20 min, the reaction was stopped by heating at 100° for 2 min. Five microliters each of 0.5% uracil and 0.5% deoxyuridine were then added, and the mixture was applied to a notched Whatman 3 MM paper and chromatographed in System I for 22 hr (16). The paper was subsequently cut in 1-cm pieces, and the radioactivity of each fraction was determined (3, 4). One enzyme unit was defined as the amount that would liberate 1 nmole of uracil under the standard assay conditions.

Enzyme Purification. A summary of the purification procedure is given in Table 1. All operations were performed at 0–4°, and centrifugations were carried out for 15 min at 12,000 × *g* when not otherwise stated.

E. coli 1100 cells (3.5 g) were suspended in 17.5 ml of an extraction buffer containing 50 mM Hepes·KOH, pH 7.7, 1 mM EDTA, 0.1 mM dithiothreitol, and were disrupted by sonication. After 30 min, the debris was removed by centrifugation at 20,000 × *g* for 30 min. The crude extract (Fraction I) was recovered, and an equal volume of 1.6% streptomycin sulfate in the extraction buffer was slowly added. After 30 min, the precipitate was removed by centrifugation, and 0.01 volume of 0.1 M dithiothreitol was added. To this solution (33 ml), solid ammonium sulfate (6.6 g) and concentrated ammonia to keep the pH at 7.0–7.5 were also added, and after 30 min the precipitate was removed by centrifugation. An additional 6.6 g of ammonium sulfate were then added, and after 30 min the resulting precipitate was recovered by centrifugation and suspended in 0.5 ml of the extraction buffer. After dialysis for 5 hr against 1 M NaCl, 10 mM Hepes·KOH, pH 7.4, 1 mM EDTA, 1 mM dithiothreitol, a small remaining precipitate was removed by centrifugation. The supernatant solution (Fraction II) was applied to a Sephadex G-100 (Pharmacia Fine Chemicals) column, 1.3 × 86 cm, equilibrated with the dialysis buffer, and 0.9-ml fractions were collected. A single peak of enzyme activity was obtained (Fig. 1), and the three most active fractions were pooled and stored at −70° in several small tubes (Fraction III).

RESULTS

Properties of Enzyme. Cell-free extracts of *E. coli* were found to release free uracil from poly(dC,dU) and from partly deaminated DNA, but not from dUMP. This *N*-glycosidase activity was purified 30-fold by ammonium sulfate fractionation and gel filtration. The partly purified enzyme had a pH optimum at 7.8, and showed 50% of maximal activity at pH 7.0 and 8.5. Some requirements of the activity are shown in Table 2. The enzyme showed no dependence on Mg^{++} or phosphate, and was not inhibited by tRNA. In order to suppress nuclease activity, most experiments were, therefore, performed in the absence of Mg^{++}; the apparent partial inhibition by Mg^{++} (Table 2) was also observed with partly deaminated DNA as substrate, but could have been due to activation of contaminating nucleases. Addition of NaCl to 0.1 M caused 80–90% inhibition, and heating of the enzyme at 70° for 5 min caused >95% inactivation. As estimated from the gel filtration data (Fig. 1) and a separate calibration experiment of the same column with several proteins of known size, the Stokes radius of the enzyme activity was 23 Å, corresponding to a molecular weight of 20,000–25,000 for a globular protein (17).

Substrate Specificity. With short chains of poly(dC,[^{3}H]dU) as substrate, the release of free uracil by Fraction III was proportional to enzyme concentration up to 0.4 units/ml, when 21% of the total uracil had been liberated. However, with 2- to 10-fold higher amounts of enzyme, only 23–24% of the uracil in the polymer could be released (Table 2). The reason for the refractoriness of the remaining uracil in the polymer is unknown, but possibly the *N*-glycosidase activity was inactive on polymeric dUMP residues at 3′-termini. A larger proportion of the uracil residues could be removed from a poly(dC,dU) preparation of higher molecular weight, made by partial deamination of [^{14}C]poly(dC) (see below). When alkali-denatured [^{14}C]DNA containing 3.2% deaminated cytosine residues was instead employed as a substrate for Fraction III, most or all of the DNA uracil residues (90 ± 10%) were released by enzyme concentrations above 0.1 unit/ml. Such DNA thus was a better substrate for the *N*-glycosidase activity than the approximately 40 times shorter poly(dC, [^{3}H]dU) chains. Under the standard assay conditions (50 μl), 0.003 units of Fraction III released 50 pmoles of uracil from the DNA (1 μg containing 100 pmoles of uracil residues) and 0.01 unit released 90 pmoles of uracil. This DNA had a denatured secondary structure, as treatment with S_1 nuclease caused >95% acid-solubilization. After reassociation of the DNA to 82% resistance to S_1 nuclease digestion, >80% of the uracil residues were still released in free form by ≥0.1 units/ml of Fraction III. It is concluded that the *N*-glycosidase activity liberates uracil from both single-stranded and double-stranded DNA.

In the partly deaminated DNA preparations, most of the radioactivity (97%) was still present as cytosine. Nevertheless, no free cytosine (<0.1%) was released by Fraction III (0.002–0.1 unit) under the standard assay conditions. Further, no thymine (<0.1%) was enzymatically released in similar experiments with thymine-labeled DNA, and no adenine (<0.1%) was liberated from adenine-labeled poly(dA-dT). Fraction III (0.01–0.1 unit) also did not cause any detectable cleavage of dUMP (2000 cpm, 100 pmoles), deoxyuridine (2000 cpm, 100 pmoles), or uridine (15,000 cpm, 100 pmoles) under standard conditions, as analyzed by chromatography in System I. Further, when the poly(dC;dU) was degraded to mononucleotides by treatment with snake venom phosphodiesterase (2 μg of enzyme, 60 min, 30°) in the standard reaction mixture supplemented with 5 mM $MgCl_2$ prior to incubation with Fraction III, no release of free uracil was observed (Table 2). These results show that the *N*-glycosidase activity was not merely due to the combined effect of *E. coli* nucleases, nucleotidases, and nucleosidases, as it specifically cleaved only deoxyuridine residues in macromolecular form. Further, Fraction III (0.02–0.1 unit) did not release free uracil when RNA was substituted for DNA in the standard reaction mixture; with 100 pmoles of uracil in [^{3}H]uridine-labeled ribosomal RNA (0.12 μg), no detectable radioactivity (<0.5%) was released. In this experiment, the radioactive RNA remained >90% acid-insoluble at the end of the incubation period, so the lack of activity on RNA was not due to rapid degradation of this substrate by contaminating RNases. The *N*-glycosidase activity studied here is not unique to *E. coli*, because a similar enzyme that releases free uracil from poly(dC,dU) but not from dUMP in the absence of Mg^{++} was also found in *Bacillus stearothermophilus* cell extracts and ammonium sulfate fractions.

Products of Reaction. On incubation of 0.1 unit of Fraction III with poly(dC,[^{3}H]dU) in the standard reaction mixture (50 μl) supplemented with 500 pmoles of [^{14}C]uracil (45,000 cpm), less than 1 pmole of [^{14}C]uracil was incorporated into an acid-insoluble form when 23 pmoles of [^{3}H]uracil were enzymatically released. The cleavage of the *N*-glycosidic bond of deoxyuridine residues in the polymer thus was essentially irreversible under the conditions used here, and no indications of an exchange of uracil at the sites of reaction were observed.

The radioactive material released by Fraction III from poly(dC,dU) and from partly deaminated DNA cochromatographed with authentic uracil in five different solvent systems. It is therefore very likely that the enzymatically liberated compound is free uracil, and not some derivative form of uracil. The following substances had clearly different R_f values in several of the solvent systems employed, and were not reaction products: deoxyuridine, dUMP, cytosine, deoxycytidine, dCMP, thymine, thymidine, dTMP, uridine,

TABLE 2. *Requirements of the N-glycosidase activity*

Components added	Uracil released from poly(dC,[^{3}H]dU)(pmoles)
Standard reaction mixture with 0.014 enzyme units	14
−Enzyme	<0.5
Substrate pretreated with venom phosphodiesterase	<1
Tris·HCl instead of Hepes·KOH	13
+10 mM K_2HPO_4	13
+5 mM $MgCl_2$	9
+ 20 μg/ml tRNA	16
Enzyme heated at 70° for 5 min	<1
+ 0.1 M NaCl	2
Standard reaction mixture with 0.03 enzyme units	23
Standard reaction mixture with 0.1 enzyme units	24

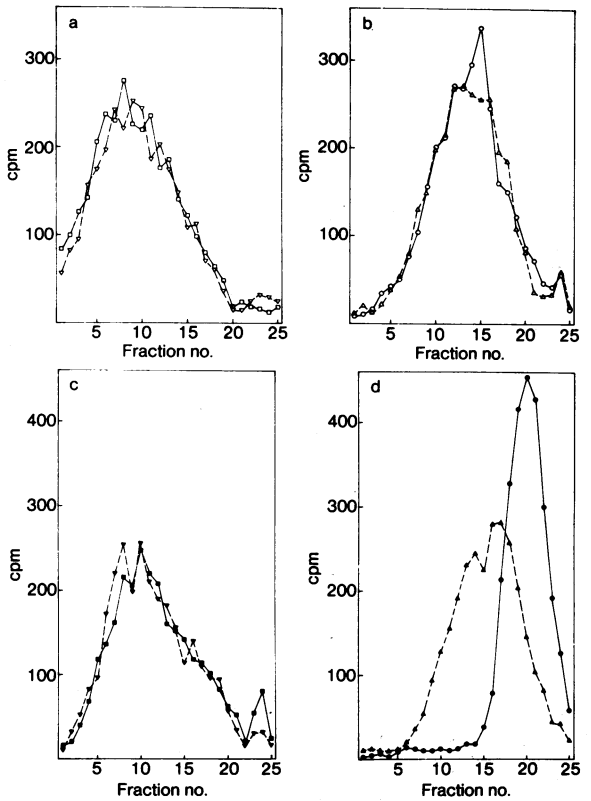

FIG. 2. Enzymatic conversion of dUMP residues to apyrimidinic sites in partly deaminated [^{14}C]poly(dC). Solid lines show polymer incubated with enzyme, broken lines show controls without enzyme. "Neutral" sucrose gradients (5–20%) contained 0.15 M NaCl, 50 mM Hepes·KOH, 1 mM EDTA, pH 8.5, and were centrifuged for 14 hr at 40,000 rpm and 5° in a Spinco SW 50.1 rotor. Alkaline sucrose gradients (5–20%) contained 0.9 M NaCl, 0.2 M NaOH, 1 mM EDTA, and were centrifuged for 16 hr under the same conditions. At the end of the runs, 0.2-ml fractions were collected from the bottom of the tubes, and their radioactivities were determined. Reference experiments with phage T7 DNA (4 hr of centrifugation) and with tRNA ("neutral" gradients only) were done under the same conditions. (*a*) Nondeaminated poly(dC), "neutral" gradient; (*b*) partly deaminated poly(dC), "neutral" gradient; (*c*) nondeaminated poly(dC), alkaline gradient; (*d*) partly deaminated poly(dC), alkaline gradient.

dihydrouracil, 5-hydroxyuracil, 6-hydroxyuracil, 2-thiouracil, and orotic acid. When paper chromatograms were cut in transverse strips and analyzed (3, 4), no radioactive material other than free uracil and polymeric, nonmigrating material was found in the reaction mixtures.

After enzymatic release of uracil from DNA or polydeoxynucleotides, the chains might be expected to contain alkali-labile apyrimidinic sites. Endonucleases that specifically cleave DNA at apurinic and apyrimidinic sites only act on double-stranded DNA (18) and do not release free uracil from DNA containing deaminated dCMP residues (unpublished). The *E. coli* endonuclease II (19, 20), therefore, should not interfere with attempts to characterize the products from a reaction between single-stranded poly(dC,dU) and the *N*-glycosidase. The results of such an experiment are shown in Fig. 2. [*2*-^{14}C]poly(dC) was partly deaminated by incubation in 1 M NaOH at 70° for 15 min. After neutralization, the polymer contained 1.5% dUMP residues, as determined by chromatography of a hydrolysate (4). The polymer was dialyzed against 10 mM Hepes·KOH, pH 8.0, 1 mM EDTA, and then incubated with 2 units/ml of Fraction III and 20 μg/ml of tRNA under the standard assay conditions, except that dithiothreitol was excluded from the reaction mixture, and the reaction was stopped by addition of 0.1 volume 2% Sarkosyl instead of by heating. This treatment released 60% of the uracil residues in the polymer in free form, as determined by chromatography of an aliquot of the reaction mixture. One-half of the enzyme-treated polymer was immediately centrifuged in a "neutral" sucrose gradient (actual pH 8.5) to measure the chain length with apyrimidinic sites remaining in the polymer. The other half of the material was incubated with an equal volume of 2 M glycine–NaOH, pH 13.1, for 3 hr at 25° to cleave the chains at apyrimidinic sites (21), and then analyzed by alkaline sucrose gradient centrifugation. Controls without enzyme or containing nondeaminated poly(dC) were processed in the same fashion. A slight decrease in chain length occurred as a consequence of the NaOH treatment at 70° (Fig. 2*c* and *d;* broken lines). However, no endonuclease activity was observed, as the nondeaminated poly(dC) retained its chain length after incubation with Fraction III (Fig. 2*a* and *c*). In the partly deaminated poly(dC), Fraction III introduced lesions revealed as chain breaks in alkaline solution (Fig. 2*d*) but not in neutral solution (Fig. 2*b*). The sedimentation coefficient of the enzyme- and alkali-treated poly(dC,dU) was 3 S, corresponding to a chain length of about 100 nucleotides. These results show that Fraction III introduced approximately one apyrimidinic site in the polymer for each uracil residue released in free form, and that there was no simultaneous cleavage of phosphodiester bonds.

DISCUSSION

Deoxyuridine is of similar stability as other deoxynucleosides, and thymidine and deoxyuridine are slowly cleaved by the same reaction mechanism in neutral aqueous solution (22). In single-stranded, partly deaminated DNA, the rate of cleavage of uracil-deoxyribose bonds by spontaneous hydrolysis at pH 7.4 is also very slow ($k = 6 \times 10^{-8}$ sec^{-1} at 95°; ref. 4). It can therefore be ruled out that nonenzymatic cleavage of *N*-glycosidic bonds occurred to a relevant extent in the present experiments. The *E. coli* enzyme activity that catalyzes such cleavage is highly specific for dUMP residues in polymeric form, as uracil was not released from deoxyuridine, dUMP, or RNA, nor were other bases released from DNA. Both single-stranded and double-stranded DNA containing uracil residues were apparently substrates for the enzyme. In the latter case, the DNA substrate contained guanine·uracil base pairs. One possible function of this enzyme, acting in concert with endonuclease II, an exonuclease, a DNA polymerase, and DNA ligase, would be the reversion of guanine·uracil base-pairs in DNA to guanine·cytosine pairs by excision-repair. The incorporation of uracil instead of thymine in newly synthesized DNA is probably prevented by other mechanisms (23, 24).

Enzymatic degradation of nucleic acids by cleavage of *N*-glycosidic bonds instead of phosphodiester bonds has not been observed previously, but there are a number of apparently analogous reactions. In precursor molecules to tRNA, a minority of the uridine residues are enzymatically converted to pseudouridine in a Mg^{++}-dependent reaction, and a likely mechanism involves the initial enzymatic cleavage of the *N*-glycosidic bond, followed by rotation of the uracil residue and

formation of a *C*-glycosidic linkage by the same enzyme (25, 26). Further, enzymes that cleave free uridine to uracil and ribose by an irreversible hydrolytic reaction in the absence of phosphate and Mg^{++} have been found in yeast (27) and in *Lactobacillus* (28, 29), and it seems likely that the enzyme studied here acts in the same fashion on DNA containing deaminated dCMP residues. Two recent reports on enzyme activities that may be similar to the present *N*-glycosidase have appeared. Kirtikar and Goldthwait (30) discovered that a 1600-fold purified preparation of *E. coli* endonuclease II released free 3-methyladenine and O^6-methylguanine, but not 7-methylguanine, from alkylated DNA. It is not known if this *N*-glycosidase activity was due to the endonuclease itself or to a different enzyme with similar fractionation properties. Carrier and Setlow (31) found an enzyme activity in *Micrococcus luteus* extracts that introduced single-strand breaks at dUMP residues in DNA. However, their experiments do not distinguish between an endonuclease attacking directly at dUMP residues and the combined action of a *N*-glycosidase and a nuclease acting at apyrimidinic sites.

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