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# A DNA glycosylase from Escherichia coli that releases free urea from a polydeoxyribonucleotide containing fragments of base residues

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ABSTRACT

A poly (dA, [2-$^{14}$C]dT) copolymer has been synthesized using terminal deoxynucleotidyltransferase. Treatment of the polydeoxyribonucleotide with potassium permanganate converts the thymine residues to urea and N-substituted urea derivatives, while the adenine residues are resistant to oxidation. This damaged polymer has been annealed with an equimolar amount of poly (dT) to generate a double-stranded polydeoxyribonucleotide containing scattered fragmented base residues, which are radioactively labeled selectively. On incubation of the latter with crude cell extracts from E. coli, free urea is released by a DNA glycosylase activity. The enzyme has been partly purified, and appears to be different from previously studied DNA glycosylases. It shows a strong preference for a double-stranded substrate, exhibits no cofactor requirement, and has a molecular weight of 20 000 - 25 000. Since fragmentation of pyrimidine residues is a major type of base lesion introduced in DNA by exposure to ionizing radiation, it seems likely this DNA glycosylase is active in repair of X-ray-induced lesions.

## INTRODUCTION

Most studies on DNA lesions induced by ionizing radiation have been concerned with the formation and fate of strand breaks. However, radiation-induced base damage in DNA is known to make an important contribution to the mutagenic and lethal effects observed in vivo (1). The characterization of X-ray-induced base alterations has been technically difficult, and consequently the different forms of base damage are not known to the same extent as those obtained by exposure of DNA to ultraviolet light or to simple alkylating agents. It has been shown that pyrimidine residues are more sensitive to ionizing radiation than purine residues, and a major pathway of degradation appears to involve attack of hydroxyl radicals at the 5,6 double bond of thymine and cytosine, with the generation of unstable hydroperoxides. In the case of thymine, these peroxides are converted to thymine glycol, followed by rupture of the 5,6 bond to generate formylpyruvylurea. The latter unstable derivative is further degraded to yield urea and N-substi-

tuted urea derivatives, which largely remain attached to deoxyribose residues in DNA (2). Although it seems likely that such base residue fragments can be removed by a DNA repair process, no enzymes acting on lesions of this type have been described.

A problem in preparing DNA substrates for repair enzymes, which may act at base lesions caused by ionizing radiation, has been that extensive chain breakage occurs simultaneously with base damage. Therefore, we have used group specific reagents to produce selectively one (or a few) of several forms of base lesions representative of those caused by ionizing radiation, without the accompanying chain scission. Here, we have employed potassium permanganate to cleave thymine residues into fragments. This reagent oxidizes thymine, cytosine, and guanine in DNA to urea and N-substituted urea residues which remain bound to deoxyribose; adenine residues appear resistant to degradation (3,4). In order to obtain a high-molecular weight polydeoxyribonucleotide radioactively labeled in fragmented base residues, a poly (dA) polymer containing a few scattered [2-$^{14}$C]dT residues was synthesized and then treated with potassium permanganate. This polymer, after addition of a complementary strand to generate a double helical structure, served as substrate for a previously unrecognized DNA glycosylase, which catalyzed the release of free urea residues from the polymer by cleavage of the urea-deoxyribose bonds.

Current knowledge of the pathway of degradation of DNA thymine residues by potassium permanganate is summarized in Fig. 1.

## MATERIALS AND METHODS

Synthesis of polydeoxyribonucleotides. Polymers were synthesized using calf thymus terminal deoxynucleotidyltransferase (6). The reaction mixture (1 ml) for synthesis of a poly (dA,[2-$^{14}$C]dT) copolymer of random sequence contained 40 mM potassium cacodylate, pH 6.8/40 mM KCl/1 mM $CoCl_2$/1 mM dATP/20 µM [2-$^{14}$C]dTTP (49 µCi/µmole)/5 µM oligo-$(dN)_4$/60 units terminal transferase. After 16 hr at 35°, the reaction was stopped by addition of 10 µl 0.2 M $Na_2$-EDTA, 100 µl 5 M NaCl, and 2.2 ml cold ethanol. Nonradioactive poly (dT) was synthesized in the same way in a reaction mixture containing 1 mM dTTP.

The poly (dA,[2-$^{14}$C]dT) polymer was dissolved in 0.2 ml 0.1 M $NaHCO_3$/NaOH, pH 9.0, and dialysed against this buffer. Under the conditions used, 60 % incorporation of dT and 35 % of dA into polymeric material was obtained as estimated from radioactivity and $A_{260}$ measurements. Thus, the copolymer apparently contained 96-97 % dA and 3-4 % dT residues.

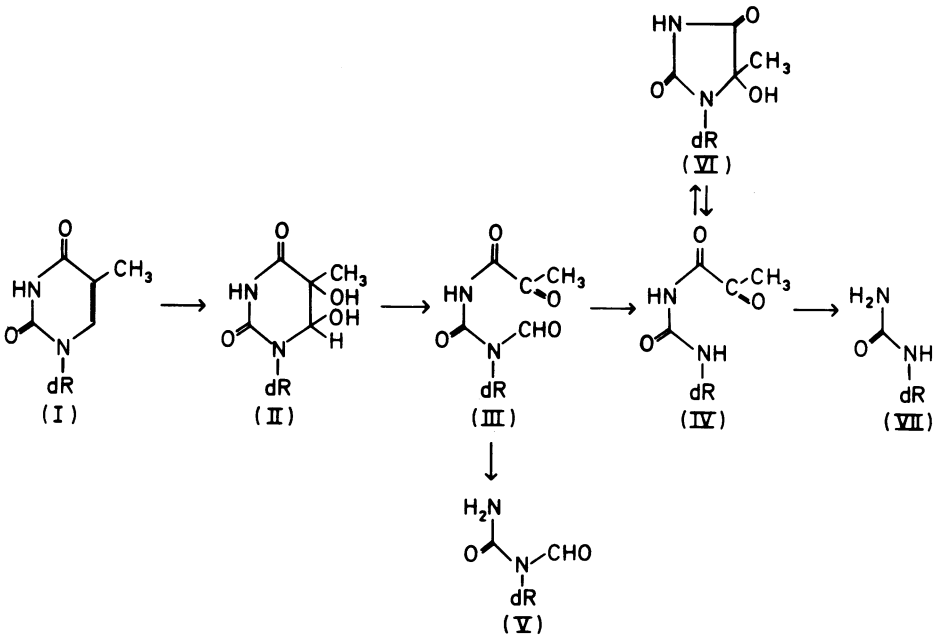


Figure 1. Degradation of thymine residues in DNA as a result of the action of $KMnO_4$ (2-5). Minor details in the scheme may require modification. The thymine residue in (I) is converted to thymine glycol (II). Oxidation of the 5,6 bond of this compound yields formylpyruvylurea (III), which is rapidly degraded to pyruvylurea (IV). In a side reaction, formylpyruvylurea is also degraded to N-formylurea (V). Pyruvylurea (IV) undergoes ring closure with the formation of 5-hydroxy-5-methylhydantoin (VI) and is slowly cleaved to generate urea (VII). The above reactions can occur when the derivatives remain bound to deoxyribose. More extensive treatment with $KMnO_4$ leads to the release of free 5-hydroxy-5-methylhydantoin, urea, and N-formylurea.

Oxidation of thymine residues with $KMnO_4$. A solution of the poly (dA,[2-$^{14}$C]dT) polymer (200 µg, $1.2 \cdot 10^6$ cpm) in 0.2 ml 0.1 M $NaHCO_3$, pH 9.0, was supplemented with 5 µl 0.1 M $KMnO_4$, freshly prepared in the same buffer. This solution, which had a final $KMnO_4$ concentration of 2.5 mM, was incubated in an Eppendorf microtube at 37° for 19 hours (3). At the end of the reaction period, a slight precipitate of $MnO_2$ was removed by centrifugation, and the solution was chilled and rapidly chromatographed at 4° on a column of Sephadex G-25 medium (0.9 x 7 cm) equilibrated with 0.1 M $NaHCO_3$, pH 9.0. The non-retarded fraction contained the polymer (95% of the total eluted radioactive material, 70% yield) largely free of $KMnO_4$. Since the column matrix is attacked by $KMnO_4$, each column was only used once. The polymer was

dialysed at 4° for 24 hr against 1 M NaCl/10 mM Tris·HCl, pH 7.5/0.2 mM EDTA and then for 16 hr against the same buffer without NaCl. The polydeoxyribonucleotide solution was stored at -20°.

The concentration of the poly (dA,[2-$^{14}$C]dT) polymer, which contained 96-97% dA residues, was estimated from the known absorption coefficient of poly (dA) (7). For the preparation of a double-stranded polydeoxyribonucleotide, this co-polymer was mixed with an equimolar amount of poly (dT) in 10 mM Tris·HCl, pH 7.5/0.2 mM EDTA at 20°.

Reference compounds. Thymine and urea were purchased from Merck, and N-formylurea from Koch-Light Laboratories, Ltd. Radioactively labeled thymine glycol was prepared by treatment of [2-$^{14}$C] thymine with $OsO_4$ (8) or with $Br_2$ (9). Formylpyruvylurea was made from thymine glycol by treatment with sodium metaperiodate (10). 5-Hydroxy-5-methylhydantoin, the ring-closed form of pyruvylurea, was obtained by heating an aqueous solution of formylpyruvylurea for 3 hr at 100° (11), and also by condensation of [$^{14}$C] urea with pyruvic acid (12). In the latter synthesis, 0.3 M urea (2 000 cpm/μmole) was incubated with 1 M pyruvic acid for 16 hr at 56°. At the end of this incubation period, 80% of the radioactive material had the chromatographic properties expected of 5-hydroxy-5-methylhydantoin (2,13).

Chromatographic analysis of hydrolysates. Aliquots of $KMnO_4$-treated polymers were hydrolysed in 1 M HCl at 37° for 24 hrs, and then neutralized with NaOH. This treatment releases free urea residues from damaged DNA containing such fragmented bases and also converts N-substituted urea derivatives to urea (3,4). In an alternative procedure (2), polymers were hydrolysed with 98% formic acid at 90° for 16 hrs. Acid hydrolysates were analysed by descending chromatography on Whatman 3MM paper. Degradation products of bases, enzymatically released from polymers as ethanol soluble material, were also analysed by paper chromatography. The solvent systems employed were: System I. Ethyl acetate/n-propyl alcohol/$H_2O$, upper phase (4:1:2). System II. n-Butyl alcohol/ethanol/$H_2O$ (4:1:1). System III. Isopropyl alcohol/conc. $NH_3$/$H_2O$ (7:1:2). System IV. n-Butyl alcohol/$H_2O$/pyridine (1:1:1). System V. Isobutyric acid/$H_2O$/0.1 M EDTA/conc. $NH_3$/toluene (160:22:3:2:20). The solvent fronts were usually allowed to run off the papers in order to increase separation. Reference compounds were run separately on the same papers. After drying, individual lanes containing radioactive samples were cut transversely in 1-cm pieces, and each piece shredded and put into a scintillation counting vial. After elution with 2 ml $H_2O$ at room temperature for 2-16 hrs, 15 ml of Triton X-based scintillation liquid

were added and the radioactivity of the fraction determined. Radioactive reference compounds were localized in the same way. Thymine was detected by its ultraviolet absorption, while urea and N-formylurea were visualized by their yellow colour after spraying with Ehrlich reagent. The latter reagent was prepared by mixing 1 volume of 10% p-dimethylaminobenzaldehyde in conc. HCl with 4 volumes of acetone immediately before use.

Enzyme preparations. Partly purified enzyme fractions (about 10-fold purified) were obtained from cell-free extracts of E. coli B by streptomycin treatment, ammonium sulfate fractionation, and gel chromatography on Sephadex G-75, following the same protocol used for partial purification of the DNA glycosylase catalyzing the release of substituted formamidopyrimidine residues (14). E. coli uracil-DNA glycosylase and 3-methyladenine-DNA glycosylase were prepared as described (15,16). Calf thymus terminal deoxynucleotidyltransferase was made according to Yoneda and Bollum (17).

Enzyme assay. The standard reaction mixture (50 µl) was composed of 0.1 M KCl/0.07 M Hepes·KOH, pH 7.8/1 mM EDTA/1 mM dithiothreitol/0.4 µg double-stranded polydeoxyribonucleotide that contained $^{14}$C-labeled urea and N-substituted urea residues (1000 cpm)/a limiting amount of E. coli enzyme. After 20 min at 37°, the reaction mixtures were chilled to 0°, and 10 µl of a 0.2% solution of heat-denatured calf thymus DNA, 5 µl of 5 M NaCl, and 150 µl of cold ethanol were added. After 20 min at -70°, the samples were centrifuged for 15 min in an Eppendorf 5412 centrifuge, and 150 µl of each supernatant were recovered. The volume of the solutions was reduced to about 30 µl under vacuum, and the samples were analysed by paper chromatography in System I for 7 hours. The radioactive material co-migrating with urea was determined.

Enzymatic release of 2,6-diamino-4-hydroxy-5-N-methyl-formamidopyrimidine from DNA containing such altered base residues was performed as described previously (14).

## RESULTS

Poly (dA) containing a small number of $^{14}$C-labeled (dT) fragments. A poly (dA,[2-$^{14}$C]dT) co-polymer was synthesized using terminal deoxynucleotidyltransferase. The polymer contained 3-4% dT residues (see Materials and Methods). On hydrolysis of an aliquot of this polydeoxyribonucleotide with 1 M HCl for 24 hrs at 37°, the radioactive material remained in a form that did not move detectably from the origin in the paper chromatographic systems employed. Under these hydrolysis conditions, a macromolecular

apurinic acid containing a few (dT) residues was presumably generated. The hydrolysate contained less than 0.5% of its radioactivity in the form of free urea (data not shown).

After oxidation of the intact polymer with 2.5 mM $KMnO_4$ for 19 hours at 37° (ref. 3), 85-90% of the radioactive material was found to be present as urea after hydrolysis with 1 M HCl (Fig. 2a). Since N-substituted urea derivatives are degraded to urea under such conditions (3,4), the relative proportions of unsubstituted versus N-substituted urea in the polymer were not obtained, but little or no intact thymine remained after the $KMnO_4$ treatment. However, chromatography of formic acid hydrolysates of the oxidized polymer in systems I and II indicated that only about 25% of the urea residues were present in unsubstituted form. Following chromatography of the single-stranded, $KMnO_4$-treated polymer on Sephadex G-100, more than 95% of the radioactivity was recovered in the non-retarded fraction. Thus, the radioactive material remained in polynucleotide form after oxidation with $KMnO_4$, as expected from the resistance of a poly (dA) chain to this agent. The procedure is summarized in Fig. 3.

Enzymatic release of free urea. When a double-stranded poly (dA)·poly (dT) polymer, containing a minority of fragmented, $^{14}C$-labeled bases in the (dA) chain, was incubated with a crude cell extract of E. coli K-12 (final protein conc. 0.4 mg/ml) under the standard reaction conditions (see Enzyme assay, Materials and Methods), 10-15% of the radioactive material was released in ethanol-soluble form in different experiments. On paper chromatographic analysis of this material, 8-11% was found to have the chromatographic properties of urea, while about 4% remained at the origin. The activity that apparently catalyzed the release of free urea was purified about 10-fold by ammonium sulfate fractionation and chromatography on Sephadex G-75. The major, or only, product released from the polymer by this enzyme fraction seemed to be urea (Fig. 2b).

In order to confirm the identity of the released material with unsubstituted urea, it was chromatographed in five different solvent systems together with unsubstituted and N-substituted ureas as reference compounds. Several N-substituted urea derivatives that are generated from thymine by $KMnO_4$ treatment, i.e. N-formylurea, pyruvylurea, and formylpyruvylurea, were well separated from unsubstituted urea in System I (Fig. 2a) and in at least one additional solvent system. Thymine glycol was not resolved clearly from urea in System I, but these two compounds were well separated in systems IV and V. The radioactive material released by the enzyme chromatographed as urea in

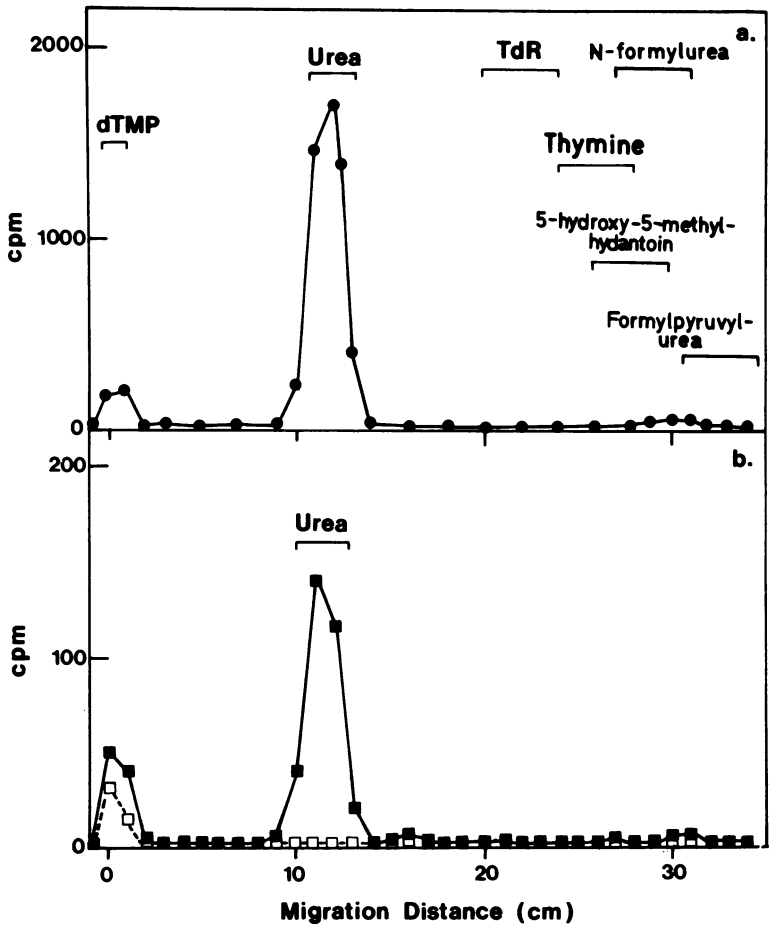


Figure 2. Release of free urea from poly (dA) containing a small number of $^{14}C$-labeled, fragmented base residues. (a) Acidic hydrolysis. The polymer was incubated with 1 M HCl for 24 hrs at 37° prior to analysis by chromatography in System I. Under these conditions, N-substituted urea derivatives were further degraded to urea (3,4). (b) Enzymatic hydrolysis. The radioactive polymer was mixed with an equimolar amount of nonradioactive poly (dT) before use. The ethanol-soluble material released by a 10-fold purified E. coli enzyme fraction (final protein concentration 40 µg/ml) was analysed by chromatography in System I (filled squares). The open symbols show the results from a parallel incubation without enzyme. Reference compounds, as indicated, were run in separate lanes. The fastest migrating compound, formylpyruvylurea, had an $R_f$ value of 0.83.

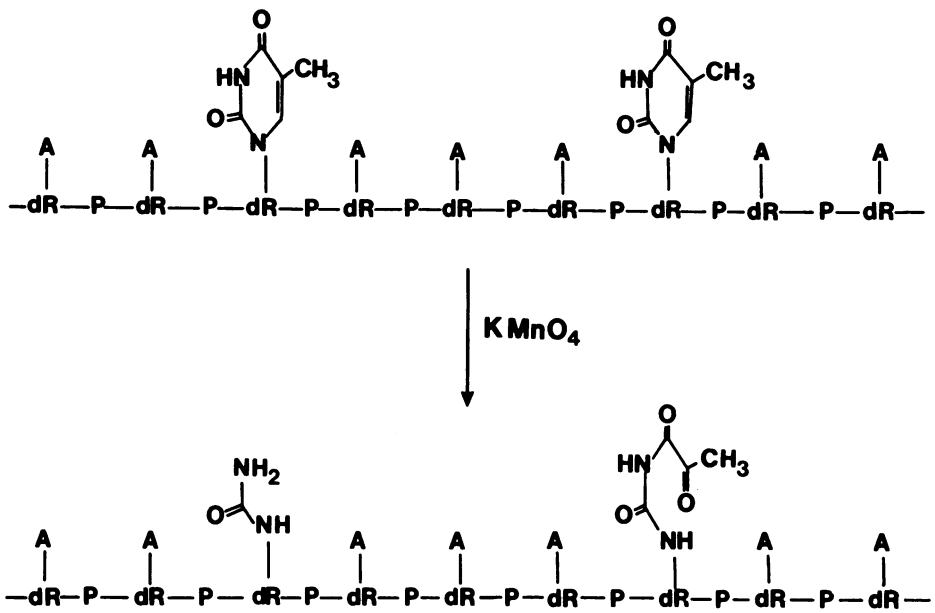


Figure 3. Schematic diagram of the preparation of a polynucleotide substrate for a DNA glycosylase that catalyzes the release of free urea from DNA containing fragmented base residues. A poly (dA,⌈2-$^{14}$C⌉dT) copolymer was oxidized with $KMnO_4$ to degrade the thymine residues to a mixture of urea and N-substituted urea under defined conditions (see Materials and Methods). More drastic treatment with $KMnO_4$, which might convert N-substituted urea derivatives to urea, could also lead to glycosyl bond cleavage and loss of urea from the polymer. The poly (dA) chain, which contained scattered fragmented base residues, was mixed with an equimolar proportion of poly (dT) before use.

these systems, while no thymine glycol was detected (data not shown).

The enzyme activity exhibited a strong preference for a double-stranded substrate, and urea was released only very slowly from a single-stranded polydeoxyribonucleotide that lacked a complementary (dT) chain (Fig. 4). A similar preference for double-stranded DNA has been previously observed for several other DNA glycosylases that act on different lesions (14,16,18). Only 8% of the radioactive material in the polymer was released rapidly by the enzyme in a form that was ethanol-soluble (Fig. 4). The reason for this limited release is unclear, but addition of more enzyme to reaction mixtures resulted in very little additional release of urea (Fig. 4). Similar results were obtained with several different polydeoxyribonucleotide and enzyme preparations. Since the polymer substrate employed contained a mix-

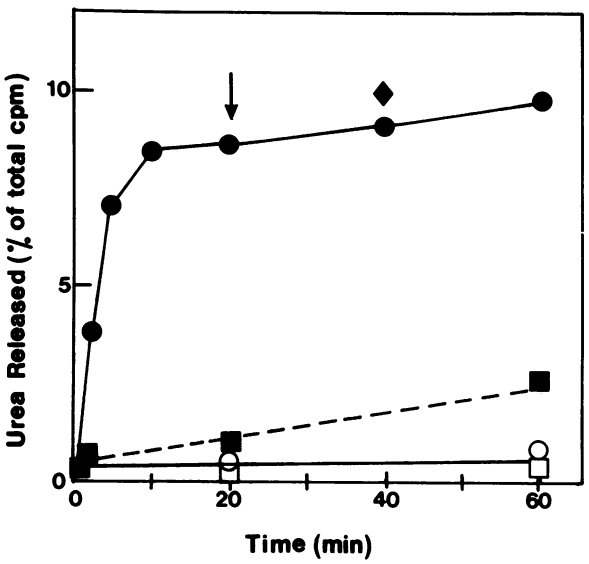


Figure 4. Kinetics of enzymatic release of [$^{14}$C] urea from a poly (dA)·poly (dT) polymer containing $^{14}$C-labeled fragmented base residues in the (dA) chain (filled circles). The reaction was allowed to proceed for different times under the standard reaction conditions and terminated by ethanol precipitation. A 10-fold purified E. coli enzyme fraction (final concentration 40 µg/ml protein) was employed. After 20 min (arrow), an equivalent amount of enzyme was added, and the reaction was in this case continued for an additional 20 min (♦). A reaction mixture containing a single-stranded polynucleotide substrate without a complementary (dT) chain was also employed (filled squares). The open symbols show the same reaction mixture incubated without enzyme.

ture of radioactive unsubstituted and N-substituted ureas (3,4), little or no enzymatic release of the N-substituted urea derivatives seemed to occur in these experiments. Possibly, some of the urea residues in the polymer may have been localized at termini or in single-stranded regions, and therefore have been relatively resistant to the enzyme. A search for conditions that would generate a larger proportion of susceptible urea residues in the polymer was unsuccessful. Thus, different times of $KMnO_4$ treatment, a 10 times higher $KMnO_4$ concentration during treatment, or addition of different amounts of complementary poly (dT) chains to the oxidized polymer did not markedly improve the quality of this enzyme substrate.

General properties of the enzyme. The DNA glycosylase activity that catalyzed the liberation of free urea appeared as a single distinct peak on Sephadex G-75 gel chromatography (Fig. 5). In comparison with several ref-

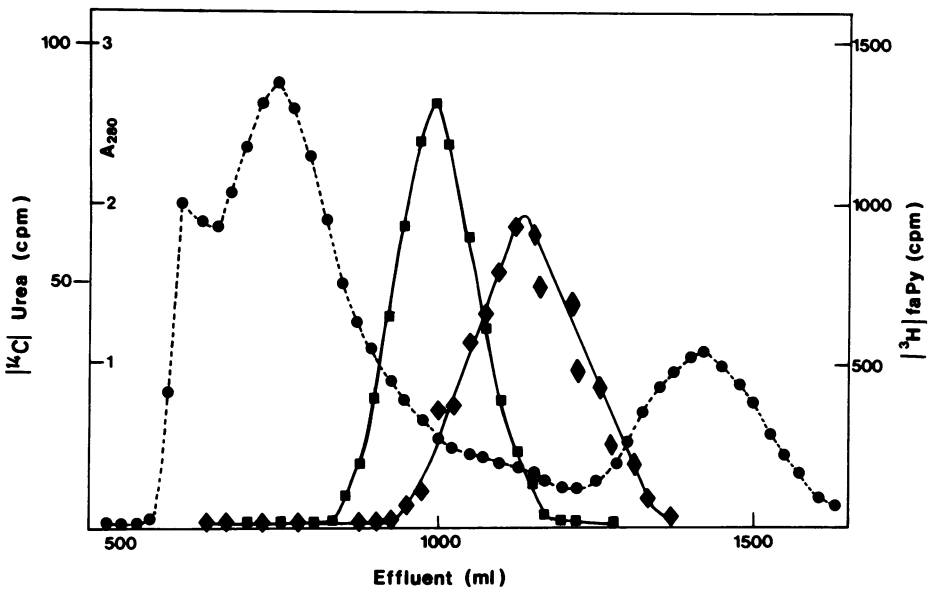


Figure 5. Gel chromatography on Sephadex G-75 of an ammonium sulfate-fractionated cell extract (45-70 % saturation) from E. coli K-12. The column (3 × 100 cm) was equilibrated with 0.5 M KCl/0.05 M Tris·HCl, pH 7.5/1 mM EDTA/1 mM 2-mercaptoethanol/5 % glycerol. Fractions, as indicated, were examined for: $A_{280}$ (●), release of formamidopyrimidine (faPy) residues from DNA damaged by alkylation and subsequent alkali treatment (■), release of free urea from a polydeoxyribonucleotide that contained fragmented base residues (◆). Two µl of each fraction were used in the enzyme assays. The most active fractions containing the urea releasing activity were pooled, concentrated 10-fold in an Amicon ultrafiltration cell equipped with a Diaflo PM10 membrane, and used as the enzyme source.

erence proteins of known size, which were run separately on the same column, the urea-DNA glycosylase had a molecular weight of 20 000 - 25 000. The activity showed a pH optimum at 7.4 - 7.8, and at pH 6.8 the reaction proceeded at 40% of the maximal rate. Exclusion of KCl from the reaction mixture, or increasing the KCl concentration from 0.1 M to 0.2 M, caused a 30% decrease in the rate of release of free urea. Addition of 5 mM $MgCl_2$ to the reaction mixture did not detectably stimulate the activity. This observation is in agreement with the previous finding that DNA glycosylases do not require $Mg^{2+}$ or other cofactors (14,19). No detectable product inhibition occurred. Thus, addition of 1-2 mM urea to standard reaction mixtures caused less than 20% inhibition.

Comparison with previously known DNA glycosylases. Highly purified preparations of E. coli uracil-DNA glycosylase (ung$^+$ gene product) and 3-methyladenine-DNA glycosylase (tag$^+$ gene product) did not catalyze the release of detectable amounts of free urea from the polydeoxyribonucleotide substrate employed here. Moreover, cell extracts from E. coli ung and tag mutant strains contained levels of the urea-liberating activity similar to that found in extracts from wild type strains. The partial purification scheme employed here serves to remove the hypoxanthine-DNA glycosylase (18); in the 10-fold purified enzyme preparation, that activity could not be detected. Furthermore, a DNA glycosylase that catalyzed the release of another type of base residue with a cleaved ring (7-methylguanine residues whose imidazole rings have been opened) was partly separated from the present activity during chromatography on Sephadex G-75 (Fig. 5). We conclude that the enzyme investigated here has an activity different from those of previously characterized DNA glycosylases. However, a DNA glycosylase activity associated with E. coli endonuclease III has recently been shown to catalyze the release of thymine glycol from irradiated DNA (20). In addition, a minor enzyme activity that can liberate 3-methyladenine from alkylated DNA, but which is different from the tag$^+$ gene product, has been detected in E. coli (21). A clarification of the relationship, if any, of the DNA glycosylase described here to these newly discovered activities will require further enzyme purification and characterization.

## DISCUSSION

Ring saturation, ring cleavage, and fragmentation of base residues are common types of lesions found in DNA exposed to ionizing radiation (1,2). Some forms, at least, of these damaged bases are actively removed by excision-repair during postirradiation incubation of cells (1,14,20). Here we have attempted to investigate DNA repair enzymes acting on lesions of this type. A polynucleotide substrate was constructed, which allowed the identification of DNA glycosylases that catalyze the release of fragments of pyrimidine residues from a damaged polymer. An enzyme that liberates free urea has been discovered by this approach. It may be predicted that after the action of this DNA glycosylase, an excision-repair process can take place utilising an endonuclease specific for apurinic and apyrimidinic sites, followed by the excision of a deoxyribose-phosphate residue, gap filling, and ligation (19).

It is unclear whether the activity described here is absolutely specific

for unsubstituted urea residues in DNA, or if it can also liberate any N-substituted urea derivatives. If the enzyme described here catalyzes only the release of unsubstituted urea residues, related enzyme activities may exist for the removal of other types of base fragments from DNA. One enzyme acting on a DNA base residue that had undergone ring cleavage was known previously, a DNA glycosylase that liberates a purine residue whose imidazole ring has been opened (14). It is clearly a different enzyme from the one investigated here (Fig. 5).

The molecular changes in DNA responsible for the mutagenic effect of ionizing radiation are presently unknown. Identification of the major DNA repair processes occurring in genetically well defined E. coli cells after exposure to X-rays should allow further progress in this area of radiobiology. The characterization of enzymes that act specifically on lesions such as those induced in DNA by radiation (14,20) represents a first step in this direction. In the present work, we give a preliminary description of an enzyme which catalyzes the release of a fragment of a pyrimidine residue from DNA. With the aid of such enzyme probes in purified form, and E. coli mutants deficient in one or several of these enzymes, the various mutagenic and lethal effects of ionizing radiation damage could be evaluated in more precise and quantitative terms than is now possible.

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