418–422 Nucleic Acids Research, 1996, Vol. 24, No. 3

Novel activities of human uracil DNA *N*-glycosylase for cytosine-derived products of oxidative DNA damage

Miral Dizdaroglu[1,*], Asuman Karakaya[1,2], Pawel Jaruga[1,3], Geir Slupphaug[4] and Hans E. Krokan[4]

[1]Chemical Science and Technology Laboratory, National Institute of Standards and Technology, Gaithersburg, MD 20899, USA, [2]Faculty of Pharmacy, University of Ankara, Ankara, Turkey, [3]Department of Clinical Biochemistry, Medical Academy, 85-094 Bydgoszcz, Poland and [4]UNIGEN Center for Molecular Biology, University of Trondheim, N-7005 Trondheim, Norway

Received November 3, 1995; Accepted December 13, 1995

ABSTRACT

Uracil DNA *N*-glycosylase is a repair enzyme that releases uracil from DNA. A major function of this enzyme is presumably to protect the genome from pre-mutagenic uracil resulting from deamination of cytosine in DNA. Here, we report that human uracil DNA *N*-glycosylase also recognizes three uracil derivatives that are generated as major products of cytosine in DNA by hydroxyl radical attack or other oxidative processes. DNA substrates were prepared by γ-irradiation of DNA in aerated aqueous solution and incubated with human uracil DNA *N*-glycosylase, heat-inactivated enzyme or buffer. Ethanol-precipitated DNA and supernatant fractions were then separated. Supernatant fractions after derivatization, and pellets after hydrolysis and derivatization were analyzed by gas chromatography/isotope-dilution mass spectrometry. The results demonstrated that human uracil DNA *N*-glycosylase excised isodialuric acid, 5-hydroxyuracil and alloxan from DNA with apparent K_m values of ~530, 450 and 660 nM, respectively. The excision of these uracil analogues is consistent with the recently described mechanism for recognition of uracil by human uracil DNA *N*-glycosylase [Mol,C.D., Arval,A.S., Slupphaug,G., Kavil,B., Alseth,I., Krokan,H.E. and Tainer,J.A. (1995) *Cell*, 80, 869–878]. Nine other pyrimidine- and purine-derived products that were identified in DNA samples were not substrates for the enzyme. The results indicate that human uracil DNA *N*-glycosylase may have a function in the repair of oxidative DNA damage.

INTRODUCTION

Oxidative DNA damage generated by free radicals is the most frequent type encountered by living aerobic cells (reviewed in 1). Of free radicals generated *in vivo*, the hydroxyl radical (·OH) is the most reactive toward biomolecules and produces a myriad of modifications in DNA (reviewed in 2,3). DNA modifications are subject to cellular repair and may be removed from DNA by specific repair enzymes (reviewed in 4,5). DNA base lesions are repaired by both base excision and nucleotide excision mechanisms (4). In both bacteria and mammalian cells, repair enzymes with multiple activities toward products of oxidative DNA damage have been discovered (5). The efficiency of repair of DNA lesions is a determining factor for survival of a damaged cell and/or maintaining its genetic integrity. Of the DNA repair enzymes, *E.coli* uracil DNA *N*-glycosylase (UDG) catalyzes the excision of uracil from DNA by cleaving the glycosidic bond between uracil and the sugar moiety (6). This enzyme has no accompanying lyase activity (7). *E.coli* UDG also recognizes 5-fluorouracil (8) and two DNA products 5-hydroxyuracil and isodialuric acid, which are generated by ·OH attack on cytosine in DNA (9,10).

Recently, the major human UDG was overexpressed in *E.coli*, purified to homogeneity and characterized (11). The crystal structure and mutational analysis of this enzyme identified the active-site groove, which at its base has a rigid uracil-binding pocket that confers selectivity for uracil over the pyrimidine bases normally present in DNA (12). Human UDG, like its bacterial analog, could possibly recognize a number of DNA base modifications resulting from oxidative damage, since many of these modifications are small and in positions that would not exclude binding in the uracil-binding pocket. To test this hypothesis, we investigated the ability of this enzyme to excise such products from DNA. For this purpose, we utilized the technique of gas chromatography-mass spectrometry (GC/MS). This technique facilitates the measurement of numerous purine- and pyrimidine-derived products, thus permitting the determination of the substrate specificity of a DNA repair enzyme toward a multitude of DNA base modifications under the same conditions (10,13–15).

MATERIALS AND METHODS

Materials and preparation of DNA substrates

Modified DNA bases, their stable isotope-labeled analogues and other materials for GC/MS were obtained as described (16). DNA

* To whom correspondence should be addressed

substrates were prepared by γ-irradiation of aerated aqueous solutions of calf thymus DNA at a dose of 50 Gy according to the published procedures (10).

Isolation of human uracil DNA *N*-glycosylase

Construction of the expression system, conditions for fermentation of *E.coli* strain NR8052 harboring the expression construct pTUNGΔ84 and purification of the enzyme has been described previously (11). Briefly, the fermented biomass was disintegrated using s Dyno-Mill type KDL homogenizer. Homogeneous enzyme was then isolated by protamine sulfate precipitation, DEAE–cellulose and CM-Sephadex C-50 chromatography followed by gel filtration on Superdex 75 and finally MonoS HR chromatography.

Enzymatic assays

The standard reaction mixture contained 50 mM phosphate buffer (pH 8), 1 mM EDTA, 1 mM dithiothreitol, bovine serum albumin (0.1 mg/ml) and 50 µg of irradiated DNA. Aliquots of 1–5 µg of active or inactivated human UDG were added to each mixture. The total volume of the mixture was 120 µl. The inactivation of the enzyme was done by heating at 140°C for 30 min. Some samples contained no human UDG, but they were added the equivalent amount of the enzyme buffer. Three replicates of each mixture were incubated at 37°C for 5, 10, 15, 30 or 60 min. Following incubation, 270 µl cold ethanol (–20°C) was added to each sample. Samples were kept at –20°C for 2 h and then centrifuged at 4°C for 30 min at 10 000 r.p.m. The DNA pellets and supernatant fractions were separated. The pellets were washed with 100 µl of a cold mixture (–20°C) of ethanol and water (80:20; v/v). The pellets and supernatant fractions were dried in a SpeedVac under vacuum. The determination of the excised amounts of the substrates as a function of the substrate concentration was done as described previously (10). The amount of human UDG added to each sample was 5 µg and the incubation time was 30 min. Following incubation, the samples were treated as described above.

Hydrolysis, derivatization and GC/MS

The amount of DNA in the pellets was determined by the absorbance at 260 nm (absorbance of 1 = 50 µg of DNA/ml). The recovery of DNA by precipitation with ethanol was close to 100%. Aliquots of stable isotope-labeled analogues of modified DNA bases were added as internal standards to DNA pellets and to supernatant fractions (16). Samples were then lyophilized. Dried pellets were hydrolyzed with 0.5 ml of 60% formic acid in evacuated and sealed tubes at 140°C for 30 min. The hydrolyzates were lyophilized. Supernatant fractions were not hydrolyzed. Dried supernatant fractions and hydrolyzates of DNA pellets were derivatized with 100 µl of a mixture of bis(trimethylsilyl)trifluoroacetamide (with 1% trimethylchlorosilane) and acetonitrile (80:20; v/v) at 120°C for 30 min in vials sealed under nitrogen with teflon-coated septa. The derivatized samples were analyzed by GC/MS with selected-ion monitoring (SIM) as described (17). The quantification of modified DNA bases was performed by isotope-dilution mass spectrometry using their stable isotope-labeled analogues as internal standards (16).

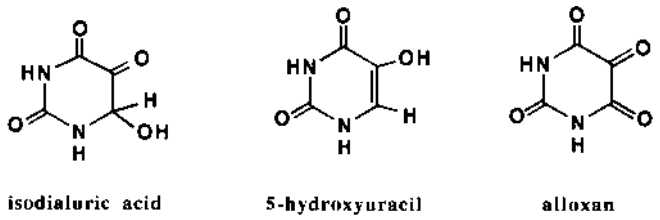

Figure 1. Structures of isodialuric acid, 5-hydroxyuracil and alloxan.

RESULTS

The analysis of DNA pellets by GC/MS-SIM revealed the formation of seven pyrimidine-derived and five purine-derived products in DNA upon exposure to ionizing radiation in aerated aqueous solution. These were 5-hydroxy-5-methylhydantoin (5-OH-5-MeHyd), 5-hydroxyhydantoin (5-OH-Hyd), 5-(hydroxymethyl)uracil (5-OHMeUra), 5-hydroxyuracil (5-OH-Ura), 5-hydroxycytosine (5-OH-Cyt), 5,6-dihydroxyuracil (5,6-diOH-Ura), thymine glycol (Thy glycol), 4,6-diamino-5-formamidopyrimidine (FapyAde), 8-hydroxyadenine (8-OH-Ade), 2-hydroxyadenine (2-OH-Ade), 2,6-diamino-4-hydroxy-5-formamidopyrimidine (FapyGua) and 8-hydroxyguanine (8-OH-Gua). Of these products, the uracil derivatives with the exception of 5-OHMeUra are products resulting from OH attack on cytosine in DNA (2,14). 5,6-Dihydroxyuracil is the enol form of isodialuric acid, which is the prevalent form in aqueous solution (or in DNA) (10,18). Isodialuric acid is converted into its enol form during derivatization and is detected as 5,6-diOH-Ura by GC/MS (19). 5-Hydroxyhydantoin is formed during hydrolysis by decarboxylation of alloxan, which is the prevalent form in DNA (14,16).

Table 1. Amounts of modified bases in DNA pellets after various incubations of DNA samples with human UNG[a]

Modified base	Incubation with		
(nmol/mg of DNA)[b]	No enzyme	Inactive enzyme	Active enzyme
5-OH-5-MeHyd	0.641 ± 0.069	0.709 ± 0.136	0.706 ± 0.064
5-OHMeUra	0.042 ± 0.020	0.052 ± 0.007	0.040 ± 0.003
5-OH-Cyt	0.706 ± 0.017	0.730 ± 0.004	0.714 ± 0.010
Thy glycol	1.701 ± 0.074	1.559 ± 0.095	1.673 ± 0.023
FapyAde	1.155 ± 0.073	1.264 ± 0.020	1.178 ± 0.056
8-OH-Ade	0.952 ± 0.050	0.917 ± 0.007	0.926 ± 0.044
2-OH-Ade	0.039 ± 0.001	0.038 ± 0.006	0.039 ± 0.007
FapyGua	1.027 ± 0.057	1.034 ± 0.069	0.963 ± 0.028
8-OH-Gua	2.211 ± 0.203	2.017 ± 0.130	2.057 ± 0.074

[a]Incubation conditions were as in Figure 2.
[b]Values were obtained from the analysis of three independently prepared samples (mean ± standard deviation).

The results obtained with quantification of modified DNA bases showed that three of the aforementioned 12 products were excised from DNA by human UDG. These were isodialuric acid, 5-OH-Ura and alloxan. The structures of these compounds are illustrated in Figure 1. First, the excisions of isodialuric acid,

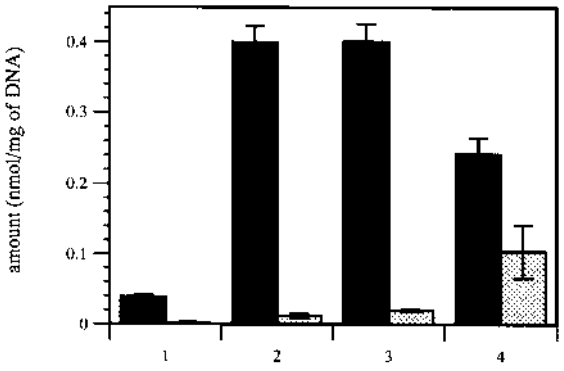

Figure 2. Amounts of isodialuric acid in pellets and supernatant fractions of DNA samples. Dark columns, pellets; light columns, supernatant fractions. 1, Unirradiated DNA; 2, γ-irradiated DNA after incubation without human UDG; 3, γ-irradiated DNA after incubation with the inactivated human UDG (5 µg); 4, γ-irradiated DNA after incubation with the active human UDG (5 µg). Incubation time was 1 h. Each column represents the mean (± standard deviation) of the values obtained from the analysis of three independently prepared samples. One nmol of a lesion/mg of DNA corresponds to ~32 lesions/10^5 DNA bases.

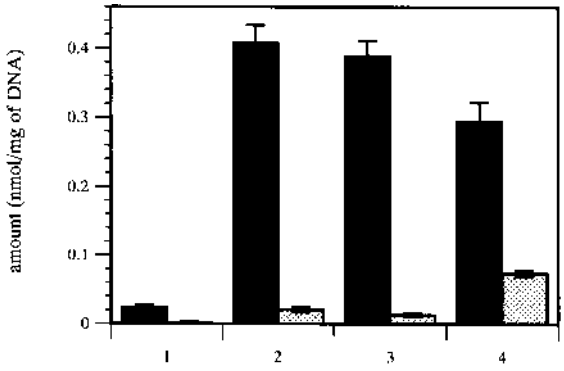

Figure 3. Amounts of 5-OH-Ura in pellets and supernatant fractions of DNA samples (other details as in Fig. 1).

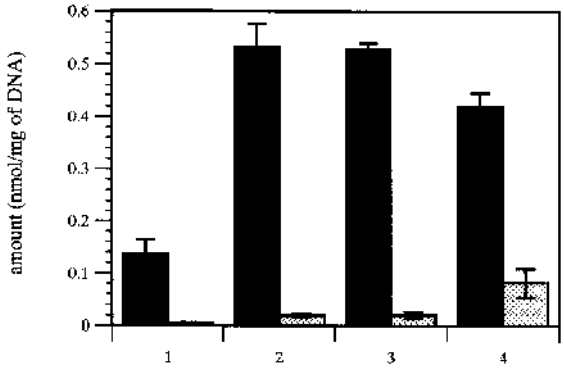

Figure 4. Amounts of alloxan in pellets and supernatant fractions of DNA samples (other details as in Fig. 1).

5-OH-Ura and alloxan were determined as a function of the amount of human UDG (1–10 µg). The excisions progressed up to 5 µg of the enzyme and reached a plateau thereafter (data not shown). In the following experiments, 5 µg of human UDG were used. Figures 2, 3 and 4 illustrate the amounts of isodialuric acid, 5-OH-Ura and alloxan (detected as 5-OH-Hyd in pellets) in pellets and supernatant fractions of irradiated DNA and in control DNA. Irradiation of DNA samples significantly increased the amounts of these compounds over their background levels. Their amounts in pellets of DNA samples incubated without the enzyme or with the inactivated enzyme were similar (Figs 2–4). By contrast, significant decreases totaling to 40, 28 and 21%, respectively, in these amounts were observed following incubation of DNA samples with the active enzyme. The excisions were confirmed by the presence of isodialuric acid, 5-OH-Ura and alloxan in supernatant fractions of DNA samples incubated with the active enzyme. Small amounts of these products were also detected in supernatant fractions of DNA samples incubated without UDG or with inactivated UDG, but these were significantly smaller than those found after incubation with active UDG (Figs 2–4). The amounts in the supernatant fractions of DNA samples incubated with active UDG corresponded to the excised amounts. The other nine modified bases were not excised from DNA by active human UDG as evident from Table 1. The amounts of each compound in all three cases of incubation were similar.

Figures 5, 6 and 7 illustrate the time course of excision of isodialuric acid, 5-OH-Ura and alloxan, respectively. In the case of isodialuric acid, the excision progressed up to 30 min and reached a plateau thereafter (Fig. 5). The rate of excision of 5-OH-Ura and alloxan did not increase after 15 min (Figs 6 and 7, respectively). Using the data in Figures 5–7, the logarithms of the ratios of the initial amounts to the remaining amounts of these products in DNA were plotted as a function of the incubation time (20). These plots yielded linear relationships up to 30 min of incubation for isodialuric acid and up to 15 min for 5-OH-Ura and alloxan (plots not shown), revealing that the excisions followed first-order kinetics. The rate constants calculated from the data up to 30 min for isodialuric acid and up to 15 min for 5-OH-Ura and alloxan in Figures 5–7 and the calculated half-lives are given in Table 2. Isodialuric acid and alloxan had similar rate constants, whereas 5-OH-Ura had a slightly higher one. The rate constant for excision of isodialuric acid by human UDG is comparable to the previously reported value of 0.011 min^{-1} for its excision by *E.coli* UDG (10). The excisions of isodialuric acid, 5-OH-Ura and alloxan by human UDG were also determined as a function of their concentrations in DNA. Apparent K_m and V_{max} values were obtained from the analysis of the results by Lineweaver–Burk plots of initial velocity versus product concentration. As an example, Figure 8 illustrates a Lineweaver–Burk plot of the data for the excision of 5-OH-Ura. The K_m and V_{max} values obtained from the Lineweaver–Burk plots are given in Table 2.

Table 2. Kinetic constants of excision of isodialuric acid, 5-OH-Ura and alloxan by human UDG from DNA

Modified base	Rate constant[a] (min^{-1})	Half-life[b] (min)	K_m (nM)	V_{max} (nM min^{-1})
Isodialuric acid	0.0157 ± 0.0025	44 ± 6	~530	~5
5-OH-Ura	0.0204 ± 0.0017	34 ± 3	~450	~3.3
Alloxan	0.0153 ± 0.0008	45 ± 2	~660	~7

[a]Rate constant = $1n(a_0/a)$/time (a_0 is the initial amount and a is the remaining amount of the product in DNA after incubation with active UNG) (20). The values were calculated from the data in Figures 5–7. The numbers represent the mean of four (isodialuric acid) or three (5-OH-Ura and alloxan) values ± standard deviation.
[b]Half-life = 0.693/rate constant (20).

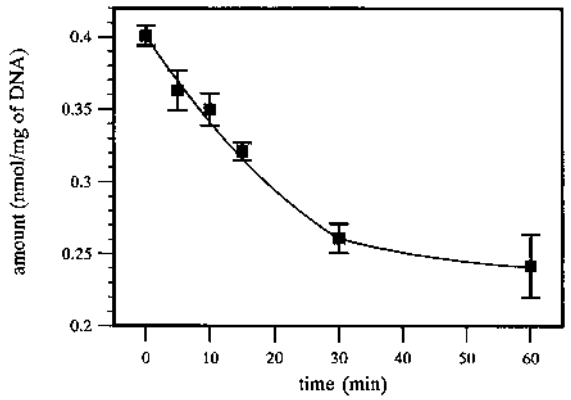

Figure 5. The excision of isodialuric acid from DNA by human UDG as a function of the incubation time. Enzyme amount was 5 µg. Each data point represents the mean ± standard deviation (n = 3).

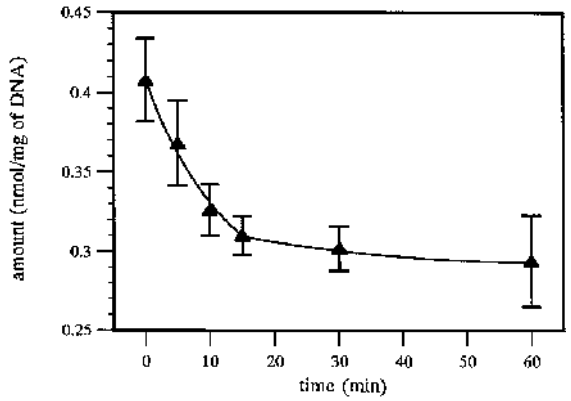

Figure 6. The excision of 5-OH-Ura from DNA by human UDG as a function of the incubation time (other details as in Fig. 5).

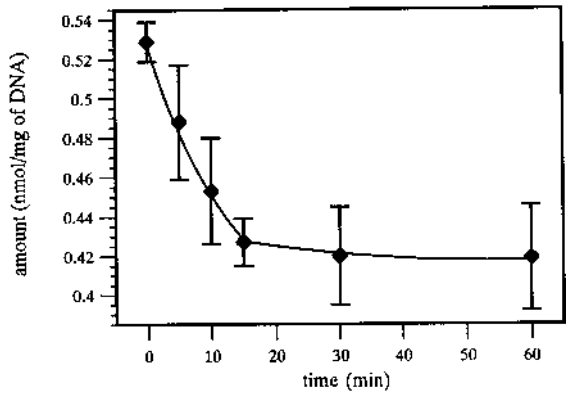

Figure 7. The excision of alloxan from DNA by human UDG as a function of the incubation time (other details as in Fig. 5).

DISCUSSION

The results provide evidence for novel activities of human UDG that excise isodialuric acid, 5-OH-Ura and alloxan from free radical-damaged DNA. Measurements of kinetics showed that

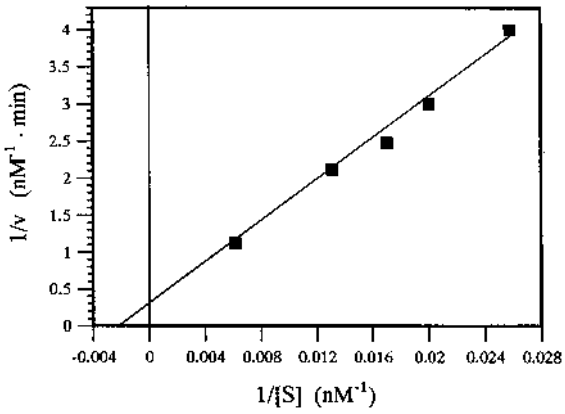

Figure 8. Lineweaver–Burk plot for the excision of 5-OH-Ura by human UDG. [S], concentration of 5-OH-Ura; v, initial velocity.

these products were excised by similar rates. Other base lesions that were also produced in DNA were not substrates for human UDG. These three products were identified as free bases in non-hydrolyzed supernatant fractions of DNA samples incubated with active human UDG. This fact proves that human UDG acted on them as an *N*-glycosylase. Isodialuric acid, 5-OH-Ura and alloxan are produced in DNA by reactions of ·OH with cytosine (2,14). These are among major products of oxidative DNA damage and their yields are comparable to those of other products as shown in this work and by previous studies done with cultured mammalian cells and experimental animals *in vivo* (3,21–23).

Our previous work showed that neither 5-OH-Ura nor alloxan were excised from DNA by *E.coli* UDG under the same experimental conditions as in this work (10). On the other hand, the excision of 5-OH-Ura by *E.coli* UDG from a 45 base pair-oligodeoxynucleotide containing 5-OH-Ura as the only modified base has been reported (9). In that study, endonuclease IV was used to cleave abasic sites created by *E.coli* UDG, which has no lyase activity, in order to prove the activity of *E.coli* UDG for 5-OH-Ura. Experimental differences may account for the discrepancy between the two works. Under similar experimental conditions, human UDG and *E.coli* UDG have a common substrate, which is isodialuric acid. On the other hand, the additional excision of 5-OH-Ura and alloxan by human UDG from DNA containing numerous base lesions indicates that these two enzymes may have differences in their mechanism of action on products of oxidative DNA damage. The reason why the human enzyme have additional substrates may also have to do with differences in the way in which these products fit into the substrate binding pocket of the enzyme. It should be pointed out that alloxan and 5-OH-Ura are substrates for *E.coli* endonuclease III (9,14). In addition, 5-OH-Ura is recognized by Fpg protein (9). By contrast, *E.coli* and human UDGs are the only enzymes shown to recognize isodialuric acid (10 and this work). The kinetic constants determined in the present study were in the same range, indicating that, under our experimental conditions, the three substrates were excised by human UDG from DNA by rates comparable to one another. The K_m value for excision of isodialuric acid is 4-fold higher than that determined for its excision by *E.coli* UDG (10). The reason for this may be that *E.coli* UDG has in general a lower K_m value than human UDG (6,11).

The present work extends the substrate specificity of human UDG. It shows that substituents at the C-5 atom of the uracil ring such as the hydroxyl group (5-OH-Ura) or such as carbonyl oxygen with a hydroxyl group (isodialuric acid) or carbonyl oxygen (alloxan) at the C-6 atom do not inhibit the action of human UDG on the uracil ring. These facts are consistent with the previous findings that substituents at the uracil C-5 atom, provided they are not large, do not affect the binding of the uracil ring to human or *E.coli* UDGs (12,24,25). The tight fit of the active site, particularly at the C-5 of the uracil ring discriminates against larger substituents (7,8). The C-5 carbonyl oxygen in both isodialuric acid and alloxan, as well as the C-5 hydroxyl group in 5-OH-Ura are apparently sufficiently small to be accommodated in the active site pocket. Modifications at the C-6 atom appear to be less critical due to the relatively large space around the C-6 atom (12). In agreement with this, 6-aminouracil was found to inhibit human UDG to the same extent as does uracil (24) and also docked into the uracil-binding pocket of crystals of human UDG (12). The substrate specificity of UDG from *E.coli*, which does not remove 5-OH-Ura and alloxan under the conditions used by us (10), is apparently more narrow than that of the human enzyme. The explanation for this discrepancy may have to await information on the structure of the bacterial enzyme. However, the apparent K_m for removal of uracil by UDG from *E.coli* (6) is several-fold lower than that of the human enzyme (11). This indicates that the active sites may be slightly different, in spite of the high degree of conservation of UDGs from different sources (26). Like uracil, isodialuric acid, alloxan and 5-OH-Ura may be required to become extrahelical, or 'flipped out' from the DNA helix, to be recognized by the active site of the enzyme (12,25). In addition, these compounds must meet requirements of extensive interactions of human UDG with the O-2, N-3 and O-4 atoms of the uracil ring (12). This part of the uracil ring is not modified in these molecules. 5-OH-Cyt is not a substrate for human UDG probably because the amino group at the C-4 position would clash with the amino group in Asn-204 of human UDG (12).

Of the three new substrates of human UDG, 5-OH-Ura has been found to mispair with adenine *in vitro* (27). Thus this product has the potential to be a pre-mutagenic lesion leading to C→T transitions. On the other hand, the likely cytotoxic and mutagenic effects of isodialuric acid and alloxan are not known. However, the fact that they are recognized and removed by human UDG may indicate that they are deleterious.

In conclusion, the present study indicates that human UDG may be involved in the repair of major modifications caused by oxidative damage to DNA. Whether this activity of human UDG is efficient enough to be biologically important compared to alternative repair systems remains to be elucidated by *in vivo* experiments and this should constitute an important area for further exploration.

ACKNOWLEDGEMENT

Certain commercial equipment or materials are identified in this paper in order to specify adequately the experimental procedure. Such identification does not imply recommendation or endorsement by the National Institute of Standards and Technology, nor does it imply that the materials or equipment identified are necessarily the best available for the purpose.

REFERENCES

1 Halliwell, B. and Gutteridge, J. M. C. (1989) *Free Radicals in Biology and Medicine*, 2nd ed., Clarendon, Oxford, UK.
2 Téoule, R. (1987) *Int. J. Radiat. Biol.*, **51**, 573–589.
3 Dizdaroglu, M. (1992) *Mutat. Res.*, **275**, 331–342.
4 Demple, B. and Harrison, L. (1993) *Annu. Rev. Biochem.*, **63**, 915–948.
5 Wallace, S. S. (1994) *Int. J. Radiat. Biol.*, **66**, 579–589.
6 Lindahl, T., Ljungquist, S., Siegert, W., Nyberg, B. and Sperens, B. (1977) *J. Biol. Chem.*, **252**, 3286–3294.
7 Sakumi, K. and Sekiguchi, M. (1990) *Mutat. Res.*, **236**, 161–172.
8 Mauro, D. J., De Riel, J. K., Tallarida, R. J. and Sirover, M. A. (1993) *Mol. Pharmacol.*, **43**, 854–857.
9 Hatahet, Z., Kow, Y. W., Purmal, A. A., Cunningham, R. P. and Wallace, S. S. (1994) *J. Biol Chem.*, **269**, 18814–18820.
10 Zastawny, T. H., Doetsch, P. W. and Dizdaroglu, M. (1995) *FEBS Lett.*, **364**, 255–258.
11 Slupphaug, G., Eftedal, J., Kavli, B., Bharati, S., Helle, N. M., Haug, T., Levine, D. W. and Krokan, H. E. (1995) *Biochemistry*, **34**, 128–138.
12 Mol, C. D., Arval, A. S., Slupphaug, G., Kavil, B., Alseth, I., Krokan, H. E. and Tainer, J. A. (1995) *Cell*, **80**, 869-878.
13 Boiteux, S., Gajewski, E., Laval, J. and Dizdaroglu, M. (1992) *Biochemistry*, **31**, 106–110.
14 Dizdaroglu, M., Laval, J. and Boiteux, S. (1993) *Biochemistry*, **32**, 12105–12111.
15 Dizdaroglu, M., Zastawny, T. H., Carmical, J. R. and Lloyd, R. S. (1995) *Mutat. Res.*, In press.
16 Dizdaroglu, M. (1994) *Methods Enzymol.*, **234**, 3–16.
17 Doetsch, P. W., Zastawny, T. H., Martin, A. M. and Dizdaroglu, M. (1995) *Biochemistry*, **34**, 737–742.
18 Behrend, R. and Roosen, O. (1889) *Liebig's Ann. Chem.*, **251**,235–256.
19 Dizdaroglu, M. (1985) *Anal. Chem.*, **144**, 593–603.
20 Laidler, K. J. (1963) *Reaction Kinetics*. Pergamon Press, Oxford, Vol. 1, pp 1–38.
21 Mori, T., Hori, Y. and Dizdaroglu, M. (1993) *Int. J. Radiat. Biol.*, **64**, 645–650.
22 Kasprzak, K., Zastawny, T. H., North, S. L., Riggs, C. W., Divan, B. A., Rice, J. M. and Dizdaroglu, M. (1994) *Chem. Res. Toxicol.*, **7**, 329–335.
23 Zastawny, T. H., Altman, S. A., Randers-Eichhorn, L., Madurawe, R., Lumpkin, J. A., Dizdaroglu, M. and Rao, G. (1995) *Free Radic. Biol. Med.*, **18**, 1013–1022.
24 Krokan, H. and Wittwer, C. U. (1981) *Nucleic Acids Res.*, **9**, 2599–2613.
25 Savva, R., McAuley-Hecht, K., Brown, T. and Pearl, L. (1995) *Nature*, **373**, 487–493.
26 Olsen, L. C., Aasland, R., Wittwer, C. U., Krokan, H. E. and Helland, D. E. (1989) *EMBO J.*, **8**, 3121–3125.
27 Purmal, A. A., Kow, Y. W. and Wallace, S. S. (1994) *Nucleic Acids Res.*, **22**, 72–78.