Properties of Purified Uracil-DNA Glycosylase from Calf Thymus

AN IN VITRO STUDY USING SYNTHETIC DNA-LIKE SUBSTRATES*

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The bovine uracil-DNA glycosylase previously isolated from thymocyte nuclei was further purified by 1 order of magnitude with the aid of affinity chromatography. The final preparation was totally devoid of DNase and apurinic or apyrimidinic (AP) endonuclease activities, and it corresponded to purifications of 457-fold over the nuclear extract and of about 2000-fold over the crude tissue homogenate.

Most of the general enzyme properties already described were confirmed. Furthermore, this mammalian uracil-DNA glycosylase was shown to bind specifically with polymerized and not with mononucleotide compounds, while having a preference for double-stranded forms. It cleaved N-glycosyl linkages only at the deoxyuridyl units located in internal positions of polynucleotide chains. The enzyme also used RNA-DNA hybrids as functional substrates and was practically ineffective on deoxyuridyl residues at the 3'-ends of nucleic acids.

The activity of the glycosylase was greatly impaired in assays with DNA substrates that contained amounts of AP sites exceeding 5 μM. The inhibitory concentrations of AP residues were about 100 times lower than those found equally effective for the other reaction product, i.e. free uracil, and were almost comparable to the K₅ values for deoxyuridyl nucleotides in the DNA substrates. This all appears as a modulation of the glycosylase catalysis by the relative amounts of its substrate and product structures in DNA. The data lead us to surmise that the removal of uracil from cellular DNA is functionally coupled to the expected elimination of the formed AP sites by specific endonucleases.

Base-exchange and base-insertion experiments with the purified enzyme yielded negative results under various conditions. The glycosylase behaved essentially as a hydrolase which has no associated base-insertase properties and irreversibly excises uracil from DNA by a mechanism for channeling the process to the next steps of the repair pathway.

Recently, a new class of enzymes implicated in the repair of altered or incorrect DNA bases was found in prokaryotic and in eukaryotic cells. These enzymes, called DNA glycosylases,
digestion according to Fasman and Loeb (14). The resulting gapped DNA was used as a template-initiator system in the reaction catalyzed by the Klenow fragment of E. coli DNA polymerase I with a standard incubation mixture containing [H]dUTP in place of dTTP. The final product consisted of short heterogeneous DNA duplexes with about 12.5% of the thymine residues substituted by [H]uracil (375 cpm/pmol of uracil) and had an average molecular weight of about 35,000.

Uracil-Polydeoxynucleotides (dT)\textsubscript{m}-(dU)\textsubscript{n}—Double-stranded DNA-like homopolymers or random copolymers containing H-labeled or unlabeled uracil residues in various proportions were synthesized according to the general procedures previously outlined (10). Preliminary experiments showed that the T/U ratios of the nucleotides incorporated in the polymeric products largely corresponded with the proportions of dTTP and dUTP substrates in the reaction mixtures. The synthesized polydeoxynucleotides were: (dT)\textsubscript{m}-(dU)\textsubscript{n} (23 cpm/pmol of uracil), (dA)\textsubscript{m}-(dF)\textsubscript{n}(dU)\textsubscript{350} (280 cpm/pmol of uracil), (dA)\textsubscript{m}-(dT)\textsubscript{n}U\textsubscript{350} with T/U = 1 (350 cpm/pmol of uracil), (dA)\textsubscript{m}-(dF)\textsubscript{n}(dU)\textsubscript{350} with T/U = 2 (290 cpm/pmol of uracil), and (dT)\textsubscript{m}-(dF)\textsubscript{n}(dU)\textsubscript{200} with T/U = 9 (350 cpm/pmol of uracil).

The hybrid (dA)\textsubscript{m}-(dF)\textsubscript{n}(dU)\textsubscript{200} with T/U = 0.5 (180 cpm/pmol of uracil) was prepared enzymatically. The (dA)\textsubscript{m}-(dF)\textsubscript{n}(dU)\textsubscript{200} was used to prime the polymerization of dTTP-Mg and [H]dUTP-Mg substrates that was catalyzed by the avian myeloblastosis virus reverse transcriptase.

Polydeoxynucleotide substrates containing terminal [H]deoxyuridyl units were produced by the addition reactions catalyzed by terminal deoxynucleotidyltransferase with (dT)\textsubscript{m} and (dA)\textsubscript{m} initiators and [H]UTP substrates in the presence of a 1 mol% mixture of Mg\textsuperscript{2+} and Co\textsuperscript{2+} as metal cofactors. The products, namely (dT)\textsubscript{m}-(dF)\textsubscript{n}(dU)\textsubscript{350} (300 cpm/pmol of uracil) and (dA)\textsubscript{m}-(dF)\textsubscript{n}(dU)\textsubscript{350} (430 cpm/pmol of uracil), were used either directly or after annealing with equimolar concentrations of their complementary deoxypolymers.

Uracil-Polyribonucleotides, (rU)\textsubscript{m}—A (dT)(rU)\textsubscript{n} random oligonucleotide with T/U = 4 (180 cpm/pmol of uracil) was enzymatically synthesized from a mixture of dTTP-Mg and [H]dUTP-Mg substrates that was catalyzed by the avian myeloblastosis virus reverse transcriptase.

Purification of Uracil-DNA Glycosylase on Agarose—The radioactive uracil released from the substrate was first separated from the nucleotide material by TLC on silica gel and then counted by a liquid scintillation method as already reported (10). One unit of uracil-DNA glycosylase is defined as the amount that releases 1 nmol of free uracil per min from the DNA-like substrate under the conditions of the standard assay. This definition of enzyme unit has been originally proposed by Caradonna and Cheng (9) for a human uracil-DNA glycosylase. It is 10 times larger than the unit we defined in the preceding paper (10) and differs by a factor of 10\textsuperscript{4} from that adopted by Lindahl et al. (11) for the very active enzyme from E. coli.

Assay for Base Incorporation in DNA-like Polymers Containing Uracil—The possible enzymatic incorporation of pyrimidinic bases into uracil-polydeoxynucleotides by both a base-exchange and a base-insertion mechanism was tested. The experiments were carried out for 15 min at 37 °C under the conditions of the standard assay using (dA)\textsubscript{100}-(dT)(rU)\textsubscript{100} with or without apyrimidinic sites as substrates and various 14C-labeled pyrimidine compounds as donors. The release of [H]uracil from the substrates was measured as indicated above. The incorporation of 14C-labeled pyrimidinic base into the DNA-like polymers was determined from the amount of acid-insoluble 14C-radioactive materials immobilized on a glass fiber paper disc (18). The radioactivity was measured in a liquid scintillation spectrometer adjusted for the differential counting of 14C and H.

Assay for Nuclease Activities—The possible presence of DNases contaminating our final uracil-DNA glycosylase preparation was investigated by monitoring the acid-soluble nucleotide release from uracil-containing DNA substrates and various 14C-labeled pyrimidine compounds as donors. The reactions were carried out in a 1:ml incubation mixture under the conditions described by Thibodeau and Verly (19) using 8 units of uracil-DNA glycosylase Fraction 5 and specific DNA substrates, namely 5 μg of [14C]DNA for endo- and exonuclease assays and 10 μg of alkylated-depurinated [3H]DNA for the AP-endonuclease assay. For comparative purposes with uracil-DNA glycosylase, a unit of either AP-endonuclease or DNases is defined as the catalytic activity releasing 1 nmol of acid-soluble nucleotides per min at 37 °C from the specific substrates in the respective enzyme assays.

Electrophoresis—Sodium dodecyl sulfate-slab gel electrophoresis was performed by the method of Laemmli and Favre (20) using a model 220 vertical slab gel apparatus from Bio-Rad. The separating gel was polymerized with 9% acrylamide, 0.24% bisacrylamide, 0.375 M Tris-HCl, pH 8.8, 0.1% SDS, 0.05% ammonium persulfate, and 0.002% N,N,N',N''-tetramethylethylenediamine. The stacking gel was prepared with 3% acrylamide, 0.24% bisacrylamide, 0.125 M Tris-HCl, pH 6.8, 0.1% SDS, 0.1% ammonium persulfate, and 0.004% N,N,N',N''-tetramethylethylenediamine. The running buffer contained 50 mM Tris, 385 mM glycine, and 0.1% SDS. The electrophoresis was carried out on gels of 1.5 mm thickness at 22 °C and at an initial voltage of 150 V for I h, the voltage was increased to 250 V and the run was continued for another 4 h. The gel was stained overnight in an aqueous solution containing 0.05% (W/V) Coomassie blue, 45% (V/V) ethanol, and 10% (V/V) acetic acid. The destainer consisted of the ethanol-acetic acid solution without added dye.

RESULTS

Purification of Uracil-DNA Glycosylase on Agarose Polyribonucleotides—The bovine uracil-DNA glycosylase enzyme from thymocyte nuclei and isolated and described in a preceding paper (10) was further purified by affinity chromatography on agarose-poly(rU). The preparation was from 280 g of ground thymus tissue and all procedures were carried out at 2 °C.

The active proteins of the enzyme Fraction 4, recovered after the final gel filtration step on Sephadex G-100, were precipitated with (NH\textsubscript{4})\textsubscript{2}SO\textsubscript{4} at 75% saturation, collected by centrifugation, redissolved in a small volume of fluid, and then extensively dialyzed against 10 mM potassium phosphate, pH 7.2. The dialysate (2 ml) was pipetted onto a 5-ml column of agarose-poly(rU) equilibrated with the dialysis buffer. The column was washed with the 50 mM potassium phosphate solution and the enzyme was displaced from the gel by raising the phosphate concentration to 200 mM in the medium. The data from a typical affinity chromatography of the glycosylase

Methods

Preparation of Uracil-DNA Glycosylase—The enzyme was purified from the nuclear extract of calf thymus cells according to the procedure previously outlined (10). The active fraction recovered from the final gel filtration step on Sephadex G-100 (Fraction 4) was salted out with 75% saturated (NH\textsubscript{4})\textsubscript{2}SO\textsubscript{4} and used for subsequent purification.

Determination of Protein—The protein concentration was determined by the method of Bradford (16, 17) with bovine γ-globulin as a standard.

Assay for Uracil-DNA Glycosylase—The enzyme activity was measured under conditions similar to those described in the preceding paper (10). The reaction mixture for the standard assay contained 10 mM Tris-HCl, pH 7.8, 5 mM EDTA, 1 mM dithiothreitol, (dA)\textsubscript{100}-(dT)(rU)\textsubscript{100} with a T/U ratio of 2:1 as a substrate at the concentration of 75 μM total nucleotides, and enzyme. The incubation was at 37 °C for 3 to 10 min. Any variation from the standard test will be noted in the text.

The radioactive uracil released from the substrate was first separated from the nucleotide material by TLC on silica gel and then counted by a liquid scintillation method as already reported (10).
are reported in Table I. The new step introduced in the isolation procedure is fairly reproducible and has been repeated with very high recoveries of glycosylase units and an average purification factor of about 10. It should be anticipated that elution of the agarose-poly(rU) by a linear concentration gradient of potassium phosphate might yield an enzyme peak emerging from the column well after 200 mM salt.

In this instance, the peak of glycosylase activity contained proteins with up to 580 units per mg, as found in separate enzyme purifications which were carried out subsequently. In the overall fractionation recorded in Table I and referring to the present study, the enzyme was purified as many as 2000-fold over the crude homogenate of calf thymus and 457-fold over the original nuclear extract which contained proteins with enriched glycosylase activity in terms of units per mg. At the final stage of purification, the uracil-DNA glycosylase was found practically devoid of contaminating DNases and of an associated AP-endonuclease activity. In fact, 1 mg of protein with 320 units of glycosylase displayed AP-endonuclease and total nucleases activities corresponding to less than 0.8 and 0.2 unit, respectively.

Properties of the Newly Purified Enzyme—The availability of a more purified mammalian uracil-DNA glycosylase without interfering nucleases offered the opportunity to re-evaluate both the biochemical properties and the catalytic requirements of the enzyme.

It was confirmed that the glycosylase has a small molecular size. Repeated measurements of its sedimentation coefficient in sucrose density gradients and of the Stokes’ radius in gel filtration experiments according to the procedures previously described (10) yielded values ranging from 2.50 to 2.85 S and from 24.5 to 25.2 Å, respectively. This corresponds to calculated molecular weights from 26,000 to 28,700 by the methods outlined in the preceding paper (10). These differences are not significant and are within the experimental errors of such measurements.

The protein composition of the isolated enzyme fractions was investigated by SDS-slab gel electrophoresis. Fig. 1 displays the results of an analysis carried out on 20-µg protein aliquots of enzyme Fractions 3, 4, and 5 as compared to the pattern obtained with a mixture of markers. It may be observed that a band with a Rf of 0.6 corresponding to a polypeptide of Mr = 28,300 was enriched throughout the purification and amounted in the final Fraction 5 to more than two-thirds of the detectable protein material.

The identification of this polypeptide with the purified uracil-DNA glycosylase is suggested by the similarity of the molecular weight of the 0.6 Rf electrophoretic band with that observed for the enzymatic activity in sucrose density gradients and gel filtration analyses. In aqueous solutions at low ionic strength and neutral pH, the enzyme proteins tended to aggregate if their concentration exceeded 1 to 2 mg/mL. The formed supramolecular complexes were eluted with the void volume in gel filtration experiments on Sephadex G-100 columns and rapidly lost activity in an irreversible manner.

The pattern of the glycosylase catalysis under various assay conditions related to changes in pH or ionic strength and to the presence of divalent cations was similar to that previously outlined. Notable exceptions were the effects on the enzymatic reaction by millimolar concentrations of uracil, thymine, and its derivatives. This will be further considered below.

Enzyme Activity with Different Types of Substrates—Table II lists the rates of the reaction catalyzed by the glycosylase with DNA and various polynucleotides containing uracil. The enzyme efficiently released the pyrimidine base from deoxyuridyl units located in internal positions of nucleic acids and seemed to prefer the substrates with higher proportions of uracil. In fact, the reaction rate with (dA)280-([3H]dU)280 was the highest and far exceeded that measured with the standard substrate (dA)100-([3H]dU)100 which contained only 33% of uracil in the pyrimidine strand. On the other hand, a possible dependence of the Kcat for uracil on the T:U ratio in the DNA substrates could not be inferred from the data obtained with our small series of polydeoxynucleotides. With the various DNA-like substrates, the Kcat of the glycosylase for uracil fell around the value of 10^{-6} M, ranging from 0.5 to 1.4 × 10^{-6} M for the standard polymer (dA)100-([3H]dU)100 with T:U = 2 and the (dA)70-([3H]dU)70 with T:U = 9, respectively. These data are well in agreement with the previous measurements (10). The Kcat for uracil in DNA was 5 × 10^{-6} M.

Table II shows also that the glycosylase was scarcely active on deoxyuridyl residues at the 3'-end of polynucleotide chains. It should be added that the hydrolytic reaction on the uracil-terminated substrates remained negligible even when the enzyme was used in excess (see legend of Table II).

It is worthwhile to note that the glycosylase worked proficiently also on a hybrid RNA-DNA duplex with uracil inserted in the deoxyribonucleotide strand. As a matter of fact, the reaction rates observed for the substrate (rA)300-([3H]dT)300 were

**Table I**

| Purification of uracil-DNA glycosylase from thymocyte nuclei | Enzyme fraction | Total protein | Specific activity | Total activity |
|-------------------------------------------------------------|-----------------|--------------|------------------|---------------|
|                                                             | mg              | units/mg     | units             | units         |
| 1. Nuclear extract                                          | 2500            | 0.7          | 1750             |               |
| 2. DEAE-cellulose, 75% saturated (NH4)2SO4                  | 1375            | 1.2          | 1650             |               |
| 3. P-cellulose, 75% saturated (NH4)2SO4                     | 95             | 17.0         | 1615             |               |
| 4. Sephadex G-100, 75% saturated (NH4)2SO4                  | 35             | 31.0         | 1085             |               |
| 5. Agarose-poly(rU)                                         | 2.9            | 320.0        | 928              |               |
In addition to the nucleic acid substrate, the incubation mixtures contained: Tris-HCl, EDTA, and dithiothreitol as in the standard assay, glycosylase units from Fraction 5 as indicated. In terms of hydrolyzable [3H]uracil residues, the final concentrations of the polynucleotide substrates in the assays were 12.5 μM for those internal uracil and 4 μM for those with 3'-terminal uracil. The incubation was at 37 °C for time intervals ranging from 3 to 10 min. The measurements of the initial reaction rates were corrected for the differences of enzyme concentration. The results are expressed as percentages of the maximal value which was obtained in the assay with the (dA)200 - (dT)[H]U200 substrate and corresponded to 0.84 nmol/ml of [3H]uracil released per min.

**Table II**

| Substrate          | T:U ratio | Enzyme units/ml | % rate of control |
|--------------------|-----------|-----------------|------------------|
| Internal Ura residues |           |                 |                  |
| Uracl-DNA          | 7:1       | 0.96            | 10               |
| Uracl-polydeoxyribonucleotides |       |                 |                  |
| (dA)200 - (dT)[H]U200 | 0.32     | 100             |                  |
| (dT)500 -(dU)[H]U500 | 0.17:1   | 0.32            | 12               |
| (dA)200 -(dT)[H]U200 | 1:1       | 0.32            | 42               |
| (dA)200 -(dT)[H]U200 | 2:1       | 0.32            | 38               |
| (dA)200 -(dT)[H]U200 | 9:1       | 0.32            | 30               |
| (rA)200 -(dT)[H]U200 | 0.5:1     | 0.32            | 77               |
| Uracl-polyribonucleotides |       |                 |                  |
| (dA)200 - 2(dT)[H]U200 | 1.6:16   | 10              | 0.05             |
| (dA)200 - 8(dT)[H]U200 | 4:1       | 1.6:16          | 0.25             |
| 3'-Terminal Ura residues |        |                 |                  |
| (dT)100 -(dU)[H]U100 | 16       | <0.1            |                  |
| (dA)200 - 2(dT)100 -(dT)[H]U200 | 16  | <0.1            |                  |
| (dA)200 -(dT)[H]U200 | 16       | <0.5            |                  |
| (dT)100 -(dA)200 -(dU)U100 | 16 | <0.1            |                  |

and of their nucleosides and 5'-nucleotides from both the ribose and the deoxyribo- seres. With the exception of free uracil, all the compounds of this set could be added to the incubation mixtures up to 1 mM concentrations without affecting appreciably the rates of the catalyzed reaction. As reported in previous investigations, partial experiments with the deoxy derivatives of the pyrimidine bases had yielded similar results for the compounds of uracil and cytosine but not for those of thymine (10). On the other hand, the antagonistic effects on the glycosylase by the old samples of thymine, deoxythymidine, and dTMP were confirmed also with the enzyme preparation isolated by affinity chromatography, in contrast with the observations made using fresh solutions of such substances. Obviously, a potent enzyme inhibitor was present in the early assays. However, the attempts to identify this reactive chemical species, which was either introduced as a contaminant in the aqueous samples of thymine and its derivatives or was formed during their storage in the frozen state, did not succeed.

All together these findings and those summarized in Tables II and III indicate that the glycosylase does not combine with mononucleotides but it binds with high affinity to polymerized nucleotides, the proficiency of catalysis being dependent upon the presence of internal deoxyuridylic residues in the nucleic acids.

**Inhibition of Uracl-DNA Glycosylase by Reaction Products—**Fig. 2 shows that concentrations of uracil above 0.1 mM in the assay inhibited the glycosylase and 1.4 mM uracil caused a 50% reduction in its rate of catalysis. This pyrimidine base is a product of the enzymatic reaction and it is to be expected that high levels of such a product in the incubation mixtures will impair the measured hydrolysis of uracil from the DNA substrates. The observed enzyme inhibition by uracil is at variance with our preceding report where glycosylase assays were found to be unaffected by concentrations of added uracil up to 1 mM (10). The new data were obtained by using the same number of enzyme units as previously and a 10-fold more purified preparation. In these experiments, the inhibitory concentration was increased thus permitting longer and more easily detectable effects which amounted to a 70% reduction of the reaction rates at 2.5 mM uracil.

Also, the AP sites that were left in the DNA substrates, after removal of uracil, appeared to induce inhibition of the
glycosylase catalysis. The enzyme activity in the assays started to be affected when the accumulation of AP sites in the DNA substrates reached values as low as 4 μM and was massively blocked when the concentration of AP structures approached 10 μM (Fig. 3).

A simple comparison of Figs. 2 and 3 reveals a difference by more than 2 orders of magnitude between the inhibitory concentrations of the two products of the glycosylase reaction, namely free uracil and AP residues in DNA. This is also related to the type of enzyme catalysis which occurs on macromolecular DNA substrates where the uncommon deoxyuridyl nucleotides are changed into AP sites. The produced uracil diffuses away whereas the AP residues formed in the DNA substrate nearby the specific uracil sites are more susceptible to interact nonproductively with the enzyme. Therefore, the amounts of the two reaction products that would interfere with the glycosylase catalysis might well be different. This consideration holds quite regardless of the enzyme acting by a processive or a distributive mechanism.

Moreover, the AP sites of DNA hindered the enzyme catalysis at concentrations that fell approximately within the same range of values as the measured Kₚ values for deoxyuridyl units in DNA (see above). Thus, the activity of the uracil-DNA glycosylase from calf thymocyte nuclei appears to be modulated in vitro by the proportion between its subunits. The activity of the enzyme was found to be inversely related to the type of enzyme catalysis which occurs on macromolecular DNA substrates where the uncommon deoxynucleotides are changed into AP sites. The produced uracil diffuses away whereas the AP residues formed in the DNA substrate nearby the specific uracil sites are more susceptible to interact nonproductively with the enzyme. Therefore, the amounts of the two reaction products that would interfere with the glycosylase catalysis might well be different. This consideration holds quite regardless of the enzyme acting by a processive or a distributive mechanism.

As recorded in the upper part of Table IV, the tests failed to show appreciable base-exchanges between uracil of the DNA substrates and the radioactively labeled pyrimidines of the donors. Equally negative results were obtained for the thymine and uracil bases as for their deoxynucleosides and deoxynucleoside triphosphates. It is noteworthy that the conversion of uracil into thymine was not considered definitive proof for these claims. A search for uracil exchanges in polydeoxynucleotide substrates, such as the one that yielded negative results with the glycosylase from E. coli (5), should be properly complemented by testing whether the enzyme promotes the direct replacement of the missed base in the AP sites of DNA. Therefore, this matter was specifically studied as reported below.

Tests for Base-Exchange and Base-Insertion Reactions—Although our preceding observations favored a totally hydrolytic action of the mammalian uracil-DNA glycosylase (10), we investigated the enzyme ability to catalyze base-exchange and base-insertion reactions between DNA and various thymine or uracil donors. Synthetic polydeoxynucleotides containing uracil served as DNA substrates for the base-exchange assays, whereas a similar tailored polymer carrying apyrimidinic residues was used in the base-insertion experiments.

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**TABLE IV**

*Base-exchange and base-insertion reactions with various compounds as donors*

| Base donor | DNA substrate | Release of [3H]Ura pmol | Incorporation of donor base pmol |
|------------|---------------|-------------------------|-------------------------------|
| None       | (dA)₃₅₀.(dT,dU)₃₅₀ | <2                      | <2                            |
| [14C]Thymine | (dA)₃₅₀.(dT[^3H]dU)₃₅₀ | 2250                    | 2275                          |
| [14C]Deoxythymidine | (dA)₃₅₀.(dT[^3H]dU)₃₅₀ | 2240                    | <3                            |
| [14C]dTTP | (dA)₃₅₀.(dT[^3H]dU)₃₅₀ | 2210                    | <3                            |
| [3H]Uracil | (dA)₃₅₀.(dT[^3H]dU)₃₅₀ | 2000                    | <3                            |
| [3H]Deoxyuridine | (dA)₃₅₀.(dT[^3H]dU)₃₅₀ | <1                      | <1                            |
| [3H]dUTP | (dA)₃₅₀.(dT[^3H]dU)₃₅₀ | <2                      | <2                            |

**Base insertion**

| Base donor | DNA substrate | Incorporation of donor base pmol |
|------------|---------------|---------------------------------|
| None       | (dA)₃₅₀.(dT,dU)₃₅₀ with AP sites | <1                            |
| [14C]Thymine | (dA)₃₅₀.(dT,dU)₃₅₀ with AP sites | <2                            |
| [14C]Deoxythymidine | (dA)₃₅₀.(dT,dU)₃₅₀ with AP sites | <2                            |
| [14C]dTTP | (dA)₃₅₀.(dT,dU)₃₅₀ with AP sites | <2                            |
| [3H]Uracil | (dA)₃₅₀.(dT,dU)₃₅₀ with AP sites | <5                            |
| [3H]Deoxyuridine | (dA)₃₅₀.(dT,dU)₃₅₀ with AP sites | <2                            |

![Fig. 2 (top). Inhibition of the uracil-DNA glycosylase reaction by added uracil. The incubation system (0.1 ml) contained the reactants of the standard assay, free uracil as indicated, and 0.02 unit/ml of enzyme. The incubation was at 37 °C. V₀ represents the amount of uracil released in 6 min in the absence of added uracil and corresponded to a value of 1.93 nmol/ml. Vₐ is the reaction rate under similar assays containing free uracil.](image)
centration of free uracil used in the base-exchange experiments was well below the value found to induce significant inhibition of the glycosylase reaction. As a matter of fact, the enzymatic release of label from uracil by the enzyme was practically the same as in the glycosylase assay run in parallel for control. The absence of any detectable incorporation of label in the polydeoxynucleotide substrate under the stated conditions suggests that the equilibrium of the enzyme catalysis was almost completely shifted toward the hydrolysis of the uracil-deoxyribose bond in DNA.

The data listed in the lower part of Table IV about the partially apyrimidinic polynucleotide substrates indicate that the glycosylase did not catalyze base-grafting processes in the AP sites of DNA. The inability of the enzyme to incorporate uracil in DNA without pyrimidine residues, opposite to deoxyadenyl nucleotides, represents compelling evidence that the glycosylase reaction is practically irreversible. On the other hand, our failure to restore the AP sites of these DNA substrates by using in the assays either thymine or base donors demonstrates that the enzyme does not behave in vitro as a correcting insertase.

The overall observations summarized in Table IV strongly suggest that the bovine uracil-DNA glycosylase does not occur through an exchange-transfer mechanism, but it is basically a simple hydrolytic process. The same conclusions had been forwarded for the bacterial enzyme (11).

**DISCUSSION**

The present investigation complements our preceding studies on the uracil-DNA glycosylase from calf thymocyte nuclei and largely confirms the pattern of enzyme properties that was already outlined (10). The slightly higher molecular weight and the greater affinity for double-stranded DNA as still remain as differential traits of this mammalian glycosylase with respect to the analogous bacterial one.

Enzyme assays with different sets of substrates revealed new features. The glycosylase acted proficiently also on RNA-DNA hybrids carrying deoxyuridyl units and was hindered in its function if an excess of nucleic acids was included in the assay mixtures. It then appears that the enzyme is strongly polymerized nucleotides and was able to promote a hydrolytic type of reaction only with the deoxyuridyl residues linked to uracil.

Further remarks are appropriate for the glycosylase assays performed with polydeoxynucleotide substrates containing AP sites. Quite surprisingly, the enzyme activity started to decline when the overall AP residues of DNA in the reaction medium attained the 10⁻⁶ M level. Such a concentration is about 100-fold lower than the one found inhibitory for added uracil and is almost comparable to that of K₅₀ values for deoxyuridyl units in nucleic acid substrates. As already mentioned under “Results,” the glycosylase works on DNA and the reaction product which accumulates in the nucleic acid is likely to affect the enzyme catalysis to a greater extent than one which is released free in solution, such as uracil.

The finding that deoxyuridyl and AP residues of DNA affected the glycosylase assays at about the same concentrations, but with opposite responses, implies the possibility that the enzyme activity is modulated by the balance of substrate and product forms present in DNA at a given time. Certainly, such DNA structures are not likely to be encountered in mammalian cells at the concentrations used for the in vitro studies. However, a discrete inhibition of uracil-DNA glycosylase in vivo by the number of AP sites accumulated in DNA should not be disregarded. The observed effects concern the activity of the enzyme and have no bearing on the reaction equilibrium, which lies totally on the side of hydrolysis as shown by the base-exchange and base-insertion experiments. Therefore, the inhibition concerns the velocity at which the enzymatic removal of uracil from DNA can take place rather than the final outcome of the process. This does not impair the function of uracil-DNA glycosylase in DNA repair. In fact, the modulation of the enzyme activity according to the ratio of deoxyuridyl to AP residues in DNA, might have the consequence of coupling tightly the glycosylase reaction in the cell nucleus with the next inciding step of DNA repair by AP-endonucleases. The slowing down of the catalysis by uracil-DNA glycosylase in DNA substrates will allow the removal of the unproper base from the genetic material without piling up the unstable AP sites. This may represent a way of channeling directly the uracil-DNA glycosylase reaction to a biochemical pathway of DNA repair.

The base-exchange assays with our purified DNA glycosylase are consistent with the fact that the enzyme acts through a simple hydrolytic mechanism by releasing uracil from DNA and does not require an acceptor molecule for a transfer reaction. The repair of an altered base in DNA by a transfer process has been recently suggested for the enzymatic removal of O²-methyl guanine from alkylated DNA (21). In this instance, however, only the abnormal methyl groups, not the entire base residue, are excised and AP sites are not produced.

As shown by the experiments with apyrimidinic DNA substrates, the uracil-DNA glycosylase from bovine thymocytes nuclei is completely devoid of base-insertase activity of the type described by Deutsch and Linn in cultured human fibroblasts (3) and by Livneh et al. in E. coli (4). The enzyme cleaves off the incorrect base from DNA and initiates a sequence of repair reactions, being functionally linked to the following biochemical operation presumably due to AP-endonucleases.

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