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The Role of Deoxyuridine Triphosphate Nucleotidohydrolase, Uracil-DNA Glycosylase, and DNA Polymerase α in the Metabolism of FUdR in Human Tumor Cells

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SUMMARY

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The lack of evidence demonstrating FdUMP residues in DNA or FdUTP in cells after exposure to FUdR could be due either to the breakdown of FdUTP by dUTP nucleotidohydrolase (dUTPase) precluding incorporation of FdUTP into DNA via DNA polymerase or to the action of uracil-DNA glycosylase which would remove fluorouracil from DNA. Evidence presented indicates that the human cell has the capacity to form FdUTP and that FdUTP, once formed, will act as a substrate for dUTPase as well as α DNA polymerase. In addition, if FdUTP escapes hydrolysis and is incorporated into DNA, uracil-DNA glycosylase is capable of removing the FU moiety from DNA, creating an apyrimidinic site which conceivably would undergo repair synthesis. Various cultured cell lines and cells obtained from patients with different forms of leukemia were analyzed for dUTPase and glycosylase activity. The specific activity for dUTPase ranged from 0.5 to 14 nmol dUMP formed/min/mg protein. For the glycosylase, the activity ranged from 0.3 to 42 nmol uracil released/min/mg protein. Cytosols prepared from cultured HeLa S3 and KB cells were used to show that FdUTP can be formed, however, only in the presence of high concentrations of dUTP (1 mM), which presumably slows down the hydrolysis of FdUTP by dUTPase. Enzymatic studies utilizing partially purified nucleoside monophosphate kinase and highly purified thymidylate kinase illustrate that both of these enzyme species have the capability of phosphorylating FdUMP to FdUDP. NDP kinase is in turn responsible for the conversion of FdUDP to FdUTP. The K_m value of FdUMP for the partially purified HeLa S3 CMP kinase (species having a p*I* value of 4.8) is 4 mM. The K_m value of FdUMP for the highly purified TMP kinase is 225 μM. FdUTP is a substrate for the purified human dUTPase (K_m of 1.2 μM) which catalyzes the conversion of FdUTP to FdUMP. Purified HeLa α DNA polymerase will catalyze the incorporation of FdUTP into DNA. The K_m of FdUTP for α DNA polymerase is 4.8 μM. In turn the incorporated fluorouracil will act as a substrate for uracil-DNA glycosylase, having a K_m value of 1.4 μM.

INTRODUCTION

5-Fluorouracil was developed by Heidelberger and associates in 1957 as a structural analogue of uracil in an attempt to develop a selective anticancer agent (1). The rationale which led to development of this analogue was based on the observations that transplanted rat and mouse tumors utilize uracil for nucleic acid biosynthesis to a much greater extent than the normal counterpart tissues (2). It was envisioned that an antimetabolite, resembling uracil closely in structure but differing in one important respect, might be utilized preferentially by tumors and therefore have a specific tumor inhibitory effect. Moreover, since in the formation of thymine a methyl group is attached to the 5-carbon of the uracil ring, it was predicted that 5-fluorouracil should block formation of thymine (3). The prediction for the inhibition of thymine formation was later confirmed by a number of investigators (4–6). These workers determined

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that the deoxynucleoside of 5-FU[2] upon phosphorylation to the monophosphate acts as an inhibitor of thymidylate synthetase. One of the possible modes of cytotoxicity of 5-FU appears to be its inhibitory effect on thymidylate synthetase. Subsequent to this are a decrease in cellular thymidylate and an increase in cellular deoxyuridylate (7–11). The phrase "thymineless death" has been formulated to express the idea of depleted thymidylate, leading to interference of DNA synthesis and ultimately cell death. Alternate target sites have been postulated for 5-FU action. One involves the formation of 5-fluorouridine and its subsequent interference with RNA processing and function (12). More recently, evidence has appeared suggesting that 5-fluorouracil can induce membrane changes (13). The data suggest that the effects of 5-FU are associated with drug-induced interference with the biosynthesis of a cell surface glycoprotein.

Recent attention in our laboratory has focused on the metabolism of 5-FU and the question of why cellular FdUTP or FdUMP residues in DNA have not been detected in cells exposed to 5-FU. Previous attempts have been made to locate either intracellular levels of FdUDP and FdUTP (14) or FdUMP residues in DNA (15). These attempts have failed to uncover measurable levels of either component. Conclusions drawn from these studies speculate on the inability of cells to phosphorylate FdUMP to the di- and triphosphate levels. Evidence has been established for the phosphorylation and incorporation into DNA of the thymidine analogues, 5-bromo-2′-deoxyuridine and 5-iodo-2′-deoxyuridine (16). Despite this, the reason why 5-fluoro-2′-deoxyuridine does not follow a similar metabolic course has never been elucidated. Two possibilities exist: (i) FUdR, once phosphorylated to the monophosphate level, will not proceed further; and (ii) FUdR can be phosphorylated to the triphosphate level, but once formed, FdUTP is rapidly metabolized. In regard to the second possibility, it has been determined that an enzymatic activity (dUTP nucleotidohydrolase) exists in mammalian cells which catalyzes the hydrolysis of dUTP to dUMP (17, 18). Since FdUMP is a structural analogue of dUMP, the possibility exists that this enzyme will also hydrolyze FdUTP to FdUMP.

Recently, Ingraham and co-workers (19) have made the initial observation that the enzyme dUTPase will hydrolyze FdUTP to FdUMP. In addition, these investigators show that the enzyme uracil-DNA glycosylase will remove 5-FU from DNA containing FdUMP residues. The study presented here is an attempt to further elucidate the metabolic pathway by which FdUMP proceeds to the triphosphate level and becomes incorporated into DNA. Data will also be presented on the kinetic parameters of FdUTP hydrolysis by dUTPase and 5-FU removal from FdUMP-containing DNA by uracil-DNA glycosylase.

[2] Abbreviations used: Tris, tris(hydroxymethyl)aminomethane; DTT, dithiothreitol; BSA, bovine serum albumin; PEI, polyethyleneimine; EDTA, ethylenediaminetetraacetic acid; 5-FU, 5-fluorouracil; FUdR, 5-fluorodeoxyuridine; FUR, 5-fluorouridine; FdUMP, 5-fluorodeoxyuridine monophosphate; NDP kinase, nucleoside diphosphate kinase; TMP kinase, thymidylate kinase; MEM, minimal essential medium; ALL, acute lymphoblastic leukemia; MTX, methotrexate.

MATERIALS AND METHODS

Chemicals. Unlabeled bases, nucleosides, and nucleotides were purchased from Sigma Chemical Co. [2-^{14}C]FUdR (50 mCi/mmol) and [2-^{14}C]FdUMP (50 mCi/mmol) were purchased from Moravek Biochemicals. [5-^{3}H]-dUTP (26 Ci/mmol) was purchased from New England Nuclear. Ag 1-X2 (200–400 mesh) was purchased from Bio-Rad Laboratories. PEI/uv$_{254}$-cellulose was purchased from Brinkman Instruments Inc. Ampholytes (pH range, 3.5–10) was supplied by LKB. *Escherichia coli* polymerase I was purchased from Worthington Biochemicals.

Cell culture. Cells in culture were grown as previously reported (20) in Eagle's minimum essential medium supplemented with 10% calf serum.

Clinic samples. Blast cells from untreated patients having the indicated leukemic conditions were provided by the Department of Medicine A of Roswell Park Memorial Institute. Cells obtained by leukophoresis were washed six times to remove contaminating erythrocytes by hypotonic shock as described (21). Washed cells were stored at −20°C until use.

Cell synchronization. Synchronized HeLa cells were generously provided by Paterson and Cass of the University of Alberta, Canada. Synchronous cells were obtained by selective detachment of mitotic cells from monolayers grown in roller bottles. Procedures, as outlined here, were obtained from Paterson and Cass. Prior to seeding (48 h), eight roller bottles were conditioned at 37°C with 50 ml/bottle of MEM supplemented with 10% calf serum and antibiotics. Each roller bottle was then inoculated with 600 ml of HeLa cell suspension (mycoplasma free) containing 2×10^8 cells. Bottles were then supplemented with $CaCl_2$ to a final concentration of 0.9 mM. The bottles were equilibrated with 10% CO_2 in air, allowed to incubate for 24 h at 37°C, and rotated at a speed of 1 mm/s. Beginning 24 h after seeding, each roller bottle was subjected at 15-min intervals to two strippings. Following the second stripping, the entire contents of the eight roller bottles were discarded. Each bottle was rinsed three times with 100 ml of 5% calf serum and MEM containing antibiotics. Fifty milliliters of fresh 5% calf serum and antibiotic containing MEM was added to each roller bottle and the bottles were rolled for 30 min at a speed of 2 mm/s. Following the 30-min incubation at 37°C the contents of three roller bottles were pooled to obtain 150 ml and this medium was passed successively through the eight roller bottles for collection of mitotic cells (the remaining 5×50 ml of culture medium was discarded). The yield was approximately 150 ml containing $3–4 \times 10^7$ cells. The cells were aerated with 5% CO_2 in air and incubated at 37°C to the appropriate time point in spinner flasks before harvesting for enzyme extraction (cells were stored at −20°C and transported to Roswell Park Memorial Institute before enzyme extraction was performed). The generation time for these mitotically selected cells was 18 h. [^{3}H]Thymidine incorporation into the acid-insoluble portion of the cell was performed by taking 1 ml of the cell suspension and incubating for 15 min with 1 μCi of [^{3}H]thymidine. The cells were transferred to filter paper, washed three times with 5% trichloroacetic acid, and analyzed for ra-

dioactivity by liquid scintillation. The authors wish to express their deep appreciation to Dr. Paterson and associates for supplying this information as well as the synchronized cells.

Preparation of cytosol fractions. HeLa S3 or KB cells were harvested by centrifugation and washed with phosphate-buffered saline. The cell pellet (5×10^7 cells) was then washed twice in buffer A. This buffer contained 10 mM Tris–Cl, pH 7.5; 6 mM β-mercaptoethanol; 1 mM EDTA; 4 mM $MgCl_2$; and 1 mM ATP-Mg. The cells were resuspended in buffer A and allowed to stand on ice for 15 min. The cells were then dounced 30 times in a 15-ml Dounce homogenizer (type A pestle). The homogenate was centrifuged and the supernatant was saved. This supernatant contained the enzymes necessary for the phosphorylation of FdUMP.

Column isoelectricfocusing. Isoelectrofocusing was performed on an LKB ampholine electrofocusing column. The pH gradient was formed using carrier ampholytes with a pH range of 3.5 to 10. A sucrose gradient (0–20%, w/v) was used to stabilize the pH gradient. Cytosols of either HeLa S3 or KB prepared as described were mixed with the gradient solutions. The cathode solution consisted of 1 M NaOH and the anode solution contained 0.1 M phosphoric acid. Focusing was conducted at a constant voltage of 800 V for 24 h at 4°C. Upon completion, 2-ml fractions were collected. The pH of each fraction was determined. The fractions were then assayed for enzyme activity.

Synthesis of [2-^{14}C]FdUTP. Quantitative phosphorylation of FdUMP to FdUTP was accomplished enzymatically using the partially purified enzyme preparation of HeLa S3, obtained from isoelectric focusing of the cytosol preparation (species having a p*I* of 4.8). The reaction mixture consisted of 75 mM Tris–Cl, pH 7.5, 50 mM DTT, 5 mM ATP, 5 mM MgCl, 100 μg/ml BSA, 560 μM [2-^{14}C]FdUMP (50 mCi/nmol), and a 50-μl aliquot of enzyme in a total volume of 0.5 ml. The reaction was allowed to proceed until about 60% of the FdUMP was converted to the triphosphate. The reaction was heated to 70°C for 5 min and then applied to a 1.5 × 13-cm column of Ag 1-X2 (200- to 400-mesh) chloride form, previously equilibrated with deionized water. Contaminating FdUMP and FdUDP were eluted with 0.6 M ammonium bicarbonate. This step also appeared to eliminate ATP as determined spectrophotometrically. FdUTP was eluted with 1 M ammonium bicarbonate. The fractions containing FdUTP were pooled and lyophilized. Radiolabeled FdUTP was resuspended in 10 mM Tris–Cl and used for subsequent studies. Verification of the purity of FdUTP was accomplished by thin-layer chromatography as described below. The partially purified enzyme preparation used in this synthesis contained both the nucleoside monophosphate and the diphosphate kinase.

BASE, nucleoside, and nucleotide chromatography. Descending paper chromatography on 3-mm paper was used to analyze 5-flubrouracil, uracil, and the corresponding deoxynucleosides. The solvent system consisted of ethylacetate, formic acid, and water at a ratio of 60:5:35. Thin-layer chromatography was utilized to monitor triphosphate formation and purity. PEI-cellulose was used in a solvent system consisting of 0.5 M LiCl and 2 N acetic acid.

Enzyme assays. (a) Analysis of the phosphorylation of FdUMP was accomplished using a modification of the TMP kinase assay previously described (22). The reaction mixture contained, in a 0.1-ml volume, 50 mM Tris–Cl, pH 7.5, 2 mM DTT, 4 mM ATP, 4 mM $MgCl_2$, 100 μg/ml BSA, and 0.4 mM [2-^{14}C]FdUMP (1 mCi/nmol). The following assays have been described previously: (b) TMP kinase assay (22), (c) CMP kinase assay (23), (d) dUTPase assay (18), and (e) uracil-DNA glycosylase assay (24). The DNA polymerase α assay was a modification of the method of Weissbach *et al.* (25). The reaction mixture contained 50 mM Tris–Cl, pH 7.5, 8 mM $MgCl_2$, 0.5 mM DTT, 0.5 mg/ml BSA, 100 μM each dATP, dCTP, and dGTP, and 120 μg/ml activated calf thymus DNA. The concentration of the limiting substrate [^{3}H]dUTP or [^{14}C]FdUTP was varied at a constant specific activity in order to obtain the kinetic parameters. The reaction was initiated by the addition of enzyme. Reactions were terminated by spotting 50-μl aliquots of reacted assay mix on 3-mm paper disks (2.3-cm diameter) and washing three times for 5 min each in 5% trichloroacetic acid and 10 mM sodium pyrophosphate. Disks were subsequently washed once in 95% ethanol, dried, and placed in vials for scintillation counting. All enzyme reactions were performed at 37°C.

Enzyme purification. The source and purification of dUTPase and uracil-DNA glycosylase used in this study have been described in previous publications (18, 24). TMP kinase was obtained from HeLa S3 cells and purified by affinity chromatography according to Lee and Cheng (26). The specific activities of an average purification for each of the enzymes studied are as follows: (a) dUTPase, 8800 nmol dUMP formed/min/mg protein; (b) uracil-DNA glycosylase, 688 nmol uracil released/min/mg protein; and (c) TMP kinase, 809 nmol TDP formed/min/mg protein. α DNA polymerase was kindly provided by Mr. D. Derse from this laboratory. The purification procedure for α DNA polymerase followed that of Fisher and Korn (27) for the obtainment of a nearly homogeneous enzyme preparation.

Synthesis of DNA containing dUMP and FdUMP residues. Activated calf thymus DNA was used as substrate to obtain uracil- and fluorouracil-containing DNA which was utilized for the characterization of uracil-DNA glycosylase. The reaction mixture contained (in a 0.5-ml volume) 120 μg/ml activated calf thymus DNA, 50 mM Tris–Cl, pH 7.5, 2 mM DTT, 8 mM $MgCl_2$, 100 μg/ml BSA, 100 μM each dATP, dCTP, and dGTP, and either 0.02 mM [5-^{3}H]dUTP or 0.03 mM [2-^{14}C]FdUTP. Reactions were catalyzed by the addition of *E. coli* polymerase I (fraction VIII) (28). The reaction was incubated at 37°C. Reactions were terminated by the addition of 20 mM EDTA and then heated to 70°C for 5 min. The samples were dialyzed against buffer containing 10 mM Tris–Cl, pH 7.5, 1 mM EDTA, and 0.5 M NaCl to eliminate unreacted nucleotides. The samples were then dialyzed against the same buffer minus NaCl. Specific activities for FdUMP-containing DNA and dUMP-containing DNA were 9200 and 27,000 cpm/μg DNA, respectively.

Protein determination. Quantitation of protein was

accomplished either by the method of Kalb and Bernlohr (29) or by the method of Bradford (30).

RESULTS

Cellular phosphorylation of FdUMP to FdUTP. Cytosols, prepared as described in Materials and Methods, were used to determine whether FdUMP could be phosphorylated to the triphosphate level. As seen in Fig. 1a, formation of FdUTP does occur, however, only in the presence of high concentrations (1 mM) of dUTP. As a preliminary determination of what enzyme(s) is responsible for the phosphorylation of FdUMP, cytosol fractions of both HeLa S3 and KB were incubated with increasing concentrations of either TMP or CMP and 1 mM dUTP. As can be seen in Fig. 1b, TMP appears not to have any effect on the phosphorylation of FdUMP. In contrast, CMP causes significant inhibition of FdUMP

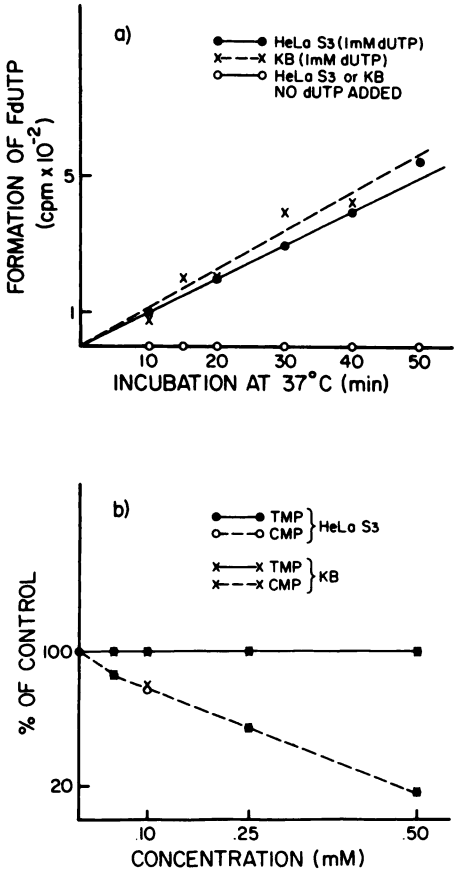

FIG. 1. *(a) Time course and (b) inhibition of FdUTP formation*
(a) Cytosols were prepared and (2.6 μg cytosol protein/time point) incubated at 37°C with FdUMP as described in Materials and Methods in the presence and absence of unlabeled dUTP (1 mM). At the times indicated, FdUTP formation was measured. No activity was detected at these time points in the absence of dUTP. The product, FdUTP, was verified as described in Materials and Methods. (b) Cytosols from KB and HeLa S3 were incubated with FdUMP reaction mixture (as described in Materials and Methods) together with 1 mM dUTP and increasing concentrations of either CMP or TMP. Inhibition is expressed as a percentage of FdUTP formation in the absence of any added CMP or TMP. Reactions contained 2.6 μg cytosol protein/assay.

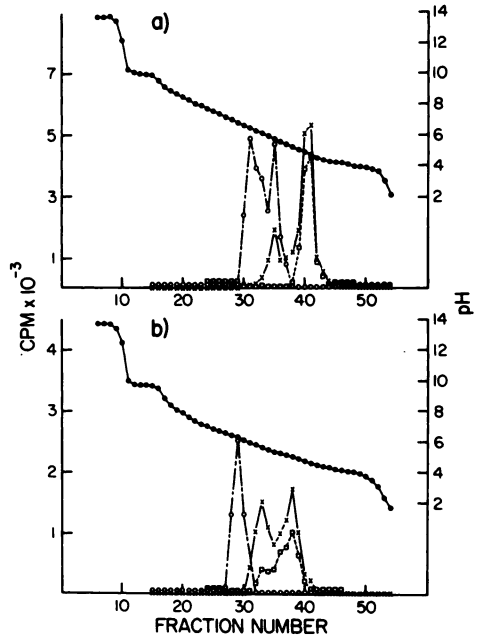

FIG. 2. *Isoelectricfocusing profiles*
Electrofocusing was performed as described in Materials and Methods. Five-tenths milligram of protein for each cytosol preparation was used for electrofocusing. (●——●) pH gradient; (○——○) dUTPase; (×——×) nucleoside monophosphate kinase; (□- - -□) FdUMP phosphorylating activity. (a) HeLa S3 cytosol preparation. Isoelectric points for dUTPase are 6.5 and 5.7, and those for NMP kinase are 5.6 and 4.8. (b) KB cytosol preparation. p*I* for dUTPase is 6.2 and for NMP kinase, 5.6 and 4.8.

phosphorylation. The lack of any observable inhibitory effect of TMP on FdUMP phosphorylation may indicate that: (i) Due to the liability of TMP kinase (26), the cytosol preparations may not have contained active TMP kinase even with the inclusion of ATP as a stabilizing factor; (ii) TMP kinase is unable to utilize FdUMP as a substrate; and (iii) the present assay conditions may exclude any contribution of TMP kinase while favoring FdUMP phosphorylation by the kinase which is also responsible for the phosphorylation of CMP. To eliminate the first possibility, cytosol fractions were assayed for TMP kinase activity according to Ref. 26. Under these assay conditions TMP kinase activity was detectable in significant amounts. Therefore, TMP kinase instability can be ruled out as a factor in interpreting the results in Fig. 1a. In order to determine if FdUMP is a substrate for TMP kinase, this enzyme was purified from HeLa S3 cells by affinity chromatography as described (26). Initial velocity studies with this enzyme as a function of FdUMP concentration indicate that TMP kinase will utilize FdUMP as a substrate. The affinity of FdUMP for TMP kinase appears to be sixfold lower than for TMP (K_m TMP = 40 μM and K_m FdUMP = 225 μM). Thus, the second possibility appears to be ruled out since studies with the purified TMP kinase show that FdUMP can act as a substrate for TMP kinase. It appears that the third possibility may explain the phenomenon observed in Fig. 1b. Unpublished observations[3] indicate

[3] Caradonna, S. J. and Y.-C. Cheng.

that TMP kinase is inhibited by high concentrations of dUTP (1 mM). Therefore under the present assay conditions, it would appear that TMP kinase would not contribute to the phosphorylation of FdUMP. Thus, data obtained from isolated enzyme systems indicate that both TMP kinase and the nucleoside monophosphate kinase capable of phosphorylating CMP are proficient in the phosphorylation of FdUMP to FdUDP.

Separation of dUTPase from a nucleotide kinase. Isoelectricfocusing was utilized to separate dUTPase from one of the nucleotide kinases capable of phosphorylating FdUMP. Figure 2 shows the electrofocusing profiles of cytosols prepared from HeLa S3 (Fig. 2a) and KB (Fig. 2b). As can be seen, resolution of two peaks of kinase activity occur, having p*I* values of 5.6 and 4.8 for both cell types (CMP as well as FdUMP phosphorylation was followed). What is noted is that separation of dUTPase from the nucleotide kinase allows detection of FdUMP phosphorylation in the absence of excess amounts of dUTP. dUTPase activity is also shown in Fig. 2. As indicated in Fig. 2a, two peaks of dUTPase activity occur, having isoelectric points of 6.5 and 5.7. The KB preparation (Fig. 2b) shows a single peak of dUTPase activity at a p*I* value of 6.5. An evaluation of both peaks of dUTPase activity in terms of their binding affinity for dUTP results in a K_m value of 3.7 μM for both HeLa S3 species (Fig. 3a). This value is comparable to the K_m value obtained for dUTPase purified from blast cells of patients having acute lymphoblastic leukemia (18). The binding affinity of FdUMP was also determined for the partially purified nucleotide kinase species having a p*I* of 4.8. A K_m value of 4 mM was determined. This is in agreement with the K_m values reported by Hande and Chabner (31) for a pyrimidine nucleoside monophosphate kinase derived from human cells. The nature of the heterogeneity observed in Fig. 2 for the dUTPase activity in HeLa S3 is consistent and reproducible for this cell line. The heterogeneity observed for both the dUTPase in HeLa S3 and the nucleoside monophosphate kinase in HeLa S3 and KB is currently under investigation in our laboratory.

Metabolism of FdUTP. Once FdUTP is formed it can proceed along two primary metabolic paths. dUTPase can hydrolyze the triphosphate back to FdUMP or α DNA polymerase can incorporate FdUTP into DNA in place of TTP. Figure 3b is a Lineweaver–Burke plot of dUTPase reaction velocity as a function of FdUTP concentration. The K_m of FdUTP is 1.2 μM. Figure 4 analyzes the incorporation of FdUTP as well as dUTP into DNA by α DNA polymerase in terms of a Lineweaver–Burke plot. The K_m for both FdUTP and dUTP is 4.8 μM. The V_{max} for FdUTP is 21 pmol/min/ml, whereas the V_{max} for dUTP is 91 pmol/min/ml.

Fate of incorporated FdUMP residues in DNA. Figure 5 evaluates the removal of fluorouracil from DNA by uracil-DNA glycosylase. As can be seen, the affinity of the enzyme is about twofold greater for dUMP than for FdUMP residues (K_m of dUMP is 0.6 μM and K_m of FdUMP is 1.4 μM). Of particular interest is that the maximal rate of removal of fluorouracil (V_{max} of uracil is 1.8 nmol/min/ml, whereas V_{max} for FdUMP is 0.06 nmol/min/ml).

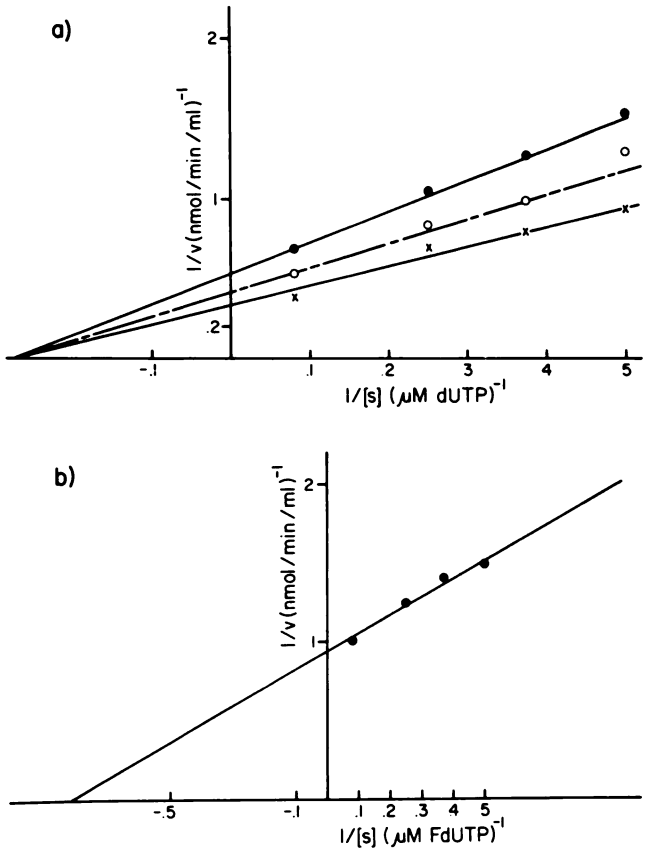

FIG. 3. *Lineweaver–Burke plots for dUTPase*

(a) Double reciprocal plots comparing the kinetic parameters of the dUTPase enzyme (●——●) purified from blast cells of patients having acute lymphoblastic leukemia (ALL) with those of the enzymes from HeLa cytosol after electrofocusing. (○——○) dUTPase species having a p*I* of 6.5; (×——×) dUTPase species having a p*I* of 5.7. Protein concentrations used for each enzyme assay are: 15 ng/ml for ALL enzyme, 1.1 μg/ml for 6.5 (p*I*) species, and 0.94 μg/ml for 5.7 (p*I*) species. Enzyme assays have been described previously (18). (b) Double reciprocal plot of dUTPase purified from ALL as a function of FdUTP concentration. Protein concentration used is 15 ng/ml for each assay.

Profiles of enzyme activities in various cell types. Table 1 outlines uracil-DNA glycosylase activity in various cultured cell lines and cell samples obtained from patients with different forms of leukemia. As can be seen, glysosylase activity varies widely in different types. Table 1 also contains a profile of dUTPase activity in different cultured cells and clinic samples.

dUTPase and glycosylase activity through the cell cycle. Figure 6 represents the activities of both the dUTPase and the glycosylase activities through the cell cycle. Glycosylase activity varies proportionately with thymidine incorporation, whereas dUTPase activity increases and plateaus at the point where thymidine incorporation decreases.

DISCUSSION

Cell-free extracts prepared from HeLa S3 and KB cultured cells demonstrate that FdUMP can be phosphorylated to FdUTP. This occurs only in the presence of high concentrations (1 mM) of dUTP. What can be inferred from these data is that in the absence of any added

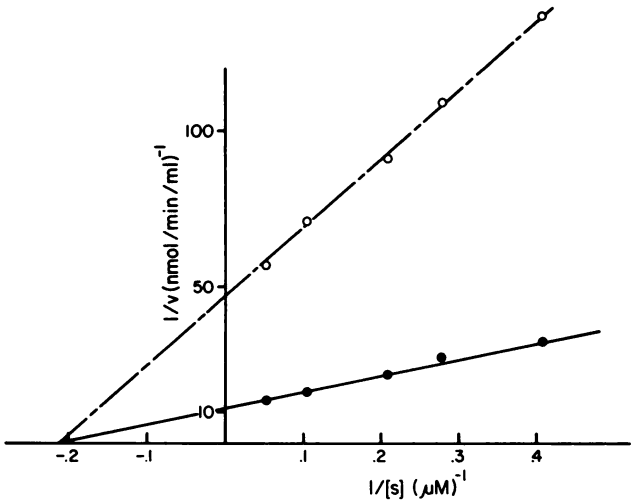

FIG. 4. *Lineweaver–Burke plot for α-DNA polymerase*

Double reciprocal plots comparing the kinetic parameters of FdUTP (○——○) and dUTP (●——●) incorporation by purified α-DNA polymerase. Assays were conducted as described in Materials and Methods. The concentration of purified enzyme used per assay is 0.50 μg/ml.

dUTP, dUTPase will hydrolyze FdUTP back to the monophosphate level. When exogenous dUTP is supplied, saturation of the dUTPase occurs, sparing FdUTP and allowing measurement of FdUTP formation. Separation of the dUTPase activity from the nucleoside monophosphate kinase activity, as seen in Fig. 2, allows detection of FdUTP in the absence of any exogenously added dUTP. What can be concluded from these data is that phosphorylation of FdUMP to the triphosphate level can occur, but it is then hydrolyzed by the dUTPase enzyme back to FdUMP. A firm conclusion as to which nucleoside monophosphate kinase is responsible for the phosphorylation of FdUMP to the diphosphate cannot be drawn from these data. Evidence generated from crude extracts and purified enzymes indicate that TMP kinase as well as CMP kinase will phosphorylate

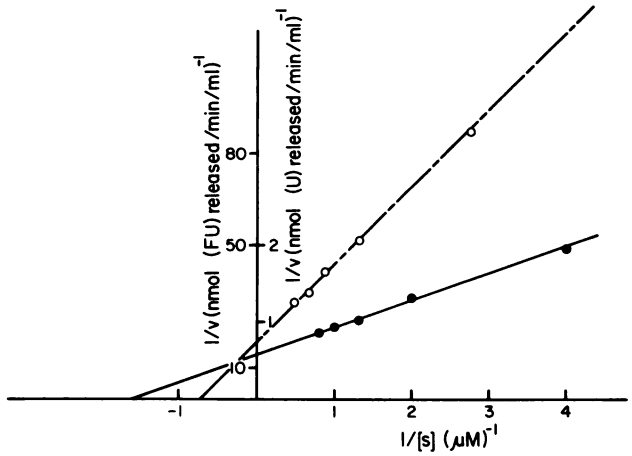

FIG. 5. *Lineweaver–Burke plot for uracil-DNA glycosylase*

Double reciprocal plots comparing the removal of fluorouracil from FdUMP-containing DNA (○——○) to the removal of uracil from dUMP-containing DNA (●——●). Substrate concentration is expressed as either micromolar quantities of FdUMP in DNA or micromolar quantities of dUMP in DNA. The concentration of purified enzyme used per assay is 0.6 μg/ml.

TABLE 1

Deoxyuridine triphosphate nucleotidohydrolase activity[a] and uracil DNA-glycosylase activity[b]; in various cell types

Cell type	dUTPase[c]	Uracil DNA-glycosylase[c]
	nmol dUMP formed mg protein	*nmol uracil released mg protein*
Cultured		
B35	8.13	2.96
Molt 4F	10.39	8.55
KB	6.00	6.88
HeLa S3	8.41	2.11
HeLa BU	3.63	9.31
HeLa BU T-I	—	20.26
HeLa BU T-II	5.20	42.05
RAJI	6.25	4.41
RAJI TK⁻	6.08	4.16
D98/HR-I	14.17	8.20
CV-I	1.86	3.05
Clinic[d]		
AML 149732	3.47	0.49
AML 147958	3.89	1.09
AML 149257	4.90	2.10
AML 141007	2.22	0.30
AML 147022	7.09	3.11
AML 151731	8.10	1.14
ALL 097212	11.96	1.51
ALL 145979	7.69	1.50
CLL 140061	5.20	1.50

[a] dUTPase extraction was preformed as described by Williams and Cheng (18).

[b] Uracil DNA-glycosylase extraction was preformed as described by Caradonna and Cheng (24).

[c] Enzyme assays were preformed as described in Materials and Methods.

[d] Leukophoresis samples were provided by the Department of Medicine A, Roswell Park Memorial Institute.

FdUMP. The reason for the phenomenon observed in Fig. 1b is not clear at the moment. Inhibition of TMP kinase by high concentrations of dUTP (≥1 mM) may be one reason for the lack of effect of TMP on FdUMP phosphorylation. Lee and Cheng (26) noted that TTP and IdUTP are competitive inhibitors with respect to both TMP and ATP. The possibility exists that dUTP may effect TMP kinase in a similar manner. This possibility is being pursued in our laboratory. A major pitfall in utilizing crude enzyme preparations in this manner (Fig. 1b) is the number of variables one has to deal with in attempting to determine the metabolic fate of a labeled precursor. Various alternative pathways may come into play as one manipulates concentrations of exogenously added nucleotides. In addition, as yet unknown associations between proteins may contribute to discrepancies in results obtained between crude enzyme preparations and highly purified enzymes.

Studies with the purified dUTPase, α DNA polymerase and uracil-DNA glycosylase enzymes allude to the possible fate of FdUTP. One distinct possibility is its hydrolysis by dUTPase back to FdUMP. As can be seen in Fig. 3b, FdUTP is comparable to, if not better than, dUTP in its affinity for dUTPase. The K_m value of FdUTP for the *ALL* purified enzyme is 1.2 μM, whereas the K_m of dUTP for this enzyme is 3.7 μM. The V_{max} value

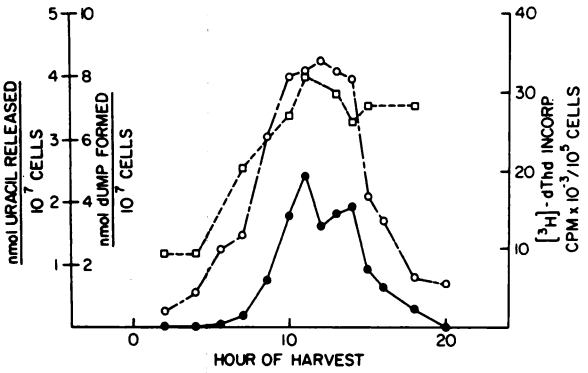

FIG. 6. *Fluctuation of dUTPase and uracil-DNA glycosylase through the cell cycle*

Enzyme assays were performed as described in Materials and Methods. (●——●) [^{3}H]TdR incorporation; (○——○) uracil-DNA glycosylase activity; (□——□) dUTPase activity.

for FdUTP hydrolysis is approximately twofold lower than for dUTP hydrolysis. Another possible pathway for FdUTP is its incorporation into DNA. Figure 4 compares the rate of incorporation of FdUTP to that of dUTP by α DNA polymerase. The K_m value of FdUTP and dUTP for the purified HeLa α DNA polymerase is 4.8 μM. This value is in close agreement with the K_m values reported for the typical deoxynucleoside triphosphates (32). If FdUTP escapes hydrolysis by dUTPase and is incorporated into DNA, the enzyme uracil-DNA glycosylase is capable of excising the fluorouracil moiety. As seen in Fig. 5, the K_m values are 1.4 and 0.6 μM for FdUMP-containing DNA and dUMP-containing DNA, respectively. These data also reveal that the maximal rate of removal of fluorouracil is about 30-fold less than the maximal rate of removal of uracil.

Table 1 reports the activities of dUTPase and uracil-DNA glycosylase for various cell types. There are large variations in enzyme activity between cell types, possibly reflecting the ability of each cell type to efficiently prevent dUTP from entering into the DNA synthetic process. Figure 6 demonstrates the cyclic nature of uracil-DNA glycosylase through the cell cycle. Peak activity of the glycosylase occurs at the point of peak incorporation of thymidine into DNA. In contrast, dUTPase reaches its peak activity during thymidine incorporation and then remains at a high level while DNA synthesis declines.

In "normal" circumstances (in which cells are not perturbed by agents such as 5-FU) it would appear that incorporation of dUMP into DNA is a very rare event. This is due to the action of the dUTPase enzyme. Goulian and co-workers (33), analyzing a line of human lymphoid cells, determined that intracellular pools of dUTP are at or less than 0.3 fmol/10^6 cells. At this level it was determined that less than 1 dUMP enters DNA per 10^5 TMP residues. Therefore under normal physiological conditions it would appear that dUTPase is mainly responsible for the absence of dUMP residues in DNA by its action on dUTP and that uracil-DNA glycosylase functions mainly in the monitoring of deaminated dCMP residues (34) and very occasionally for dUMP residues resulting from its incorporation via dUTP.

In contrast, when cells are exposed to 5-fluorouracil, large increases in intracellular dUMP pools occur (7–11) due to the inhibition of thymidylate synthetase by FdUMP (4–6). It is conceivable that large increases in dUMP levels would ultimately lead to increases in dUTP levels. In turn, the increase in dUTP concentration would tend to overwhelm the hydrolyzing capacity of the dUTPase enzyme. In effect, what could result would be similar to what is seen in Fig. 1a. Saturation of the dUTPase would allow formation of FdUTP and an increased probability of FdUTP incorporation into DNA. Evidence supporting the idea of increased dUTP pools subsequent to expansion of dUMP pools has come from Goulian *et al.* (33). They have indicated that cells exposed to the dihydrofolate reductase inhibitor methotrexate have increased levels of dUTP up to 1000-fold over controls. Inhibition of dihydrofolate reductase will deplete cells of reduced folate, a necessary cofactor in the conversion of dUMP to TMP by thymidylate synthetase. The action of methotrexate will cause depletion of TMP pools and an expansion of dUMP, leading to increased levels of dUTP. These investigators report significant levels of dUMP incorporated into DNA of cells exposed to methotrexate. As stated earlier, a similar phenomenon occurs when cells are exposed to 5-FU, except in this situation (when saturation of dUTPase occurs) FdUMP as well as dUMP can be phosphorylated to the triphosphate level and become incorporated into DNA. Once FdUMP and dUMP are incorporated into DNA, uracil-DNA glycosylase would function in the removal of both uracil and fluorouracil. Therefore under conditions of expanded dUMP pools (such as with the exposure of cells to the chemotherapeutic agents MTX or 5-FU) where the capacity of dUTPase is exceeded, uracil-DNA glycosylase would participate to a much greater extent in the removal of uracil (and fluorouracil) from DNA resulting from incorporation of dUMP (and FdUMP). Through this reasoning an additional mode of action has been evoked for agents such as MTX and 5-FU (33). It is postulated that a self-defeating cyclic repair process of dUMP removal, reincorporation, and reremoval may result in extensive degradation of DNA and possible toxicity to the cell. The possible association between 5-FU incorporation into DNA and cellular toxicity remain, for the moment at least, in the speculative stages. However, according to the kinetic data presented here, removal of fluorouracil is about 30 times slower than the removal of uracil. Cellular toxicity could arise at this point, due to the possible interference of FdUMP residues with the DNA repair process.

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