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Metabolism of 5-Fluorouracil in Cultured Cells. Protection from 5-Fluorouracil Cytotoxicity by Purines

BUDDY ULLMAN[1] AND JAN KIRSCH[2]

Division of Medical Genetics, Department of Medicine and Department of Biochemistry and Biophysics, University of California, San Francisco, San Francisco, California 94143

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SUMMARY

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The metabolic reactions leading to cytotoxicity of 5-fluorouracil (FUra) were examined in cultured mouse T-cell lymphoma cells. FUra is phosphoribosylated by orotic acid phosphoribosyl transferase, the second to last enzyme in *de novo* pyrimidine biosynthesis. Mutants with altered capacities to phosphoribosylate orotic acid *in vitro* have similarly altered capacities to phosphoribosylate FUra *in vivo* and *in vitro*. The sensitivity of these mutant lymphoma cell lines to FUra is monotonically related to their capacity to phosphoribosylate FUra. This phosphoribosylation requires pyrophosphoribosyl phosphate, an intracellular metabolite whose concentration can be regulated *in vivo*. Purines, which lower the concentration of pyrophosphoribosyl phosphate in cultures of wild type cells, can protect these cells from FUra toxicity. Conversely, purines that do not affect intracellular pyrophosphoribosyl phosphate content do not affect FUra mediated growth inhibition and cytotoxicity. This protection from FUra toxicity by purines requires the presence of the appropriate purine salvage enzymes. Analogous observations with purines on FUra cytotoxicity were made in other cell lines from rat, mouse, and marmoset, indicating that the metabolic activation of FUra by phosphoribosylation may be prevalent.

INTRODUCTION

The synthetic pyrimidine analogue, 5-fluorouracil (FUra),[3] is a cancer chemotherapeutic agent whose growth inhibitory and cytotoxic effects are thought to be exerted through inhibition of thymidylate synthesis (1), or possibly, through its incorporation into RNA (2), where it might result in lethal miscoding (1, 3). In order to elicit its inhibitory effect on cell growth by

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To whom reprint requests should be addressed at University of California, San Francisco, Department of Medicine, M-1093, San Francisco, CA 94143.

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[3]The abbreviations used are: FUra, 5-fluorouracil; FUrd, 5-fluorouridine; FdUrd, 5-fluorodeoxyuridine; FUMP, 5-fluorouridine monophosphate; FdUMP, 5-fluorodeoxyuridine monophosphate; PPriboseP, pyrophosphoribosyl phosphate; HGPRTase, hypoxanthine-guanine phosphoribosyl transferase; APRTase, adenine phosphoribosyl transferase; OPRTase, orotate phosphoribosyl transferase; OMP decarboxylase, orotidylate 5-monophosphate decarboxylase; OPRTase, orotate phosphoribosyl transferase; MNNG, N-methyl-N-nitroso-N'-nitroguanidine.

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these or any other mechanisms, FUra must first be converted to a nucleotide derivative. The effect on thymidylate synthesis is thought to occur by the conversion of FUra to FdUMP (4). The FdUMP is known to inhibit thymidylate synthase (5), an enzyme whose action is essential for the *de novo* synthesis of thymidine nucleotides and thus indirectly for DNA synthesis.

The formation of FdUMP from FUra in mammalian cells could occur directly by the sequential actions of thymidine phosphorylase (6) and thymidine kinase (7) or by the intermediate formation of the ribonucleotides of FUra. The synthesis of the ribonucleotide FUMP from FUra may occur by two pathways: either by the successive catalytic actions of uridine phosphorylase and uridine kinase (8, 9) or by direct phosphoribosylation by a pyrimidine phosphoribosyl transferase (10–14). The reduction of the ribose moiety could be catalyzed by ribonucleotide reductase at the diphosphate level (15).

We tested the alternative metabolic pathways for FUra in a mouse T-cell lymphoma (S49) in continuous culture. The results indicate that FUra is not extensively metabolized in S49 cells by pyrimidine nucleoside phosphorylases and pyrimidine nucleoside kinases. Studies on S49 cell mutants have demonstrated that, instead, FUra is converted to FUMP through the action of OPRTase. This phosphoribosylation of FUra appears to be limited by the intracellular concentration of PPriboseP. It seems that purines and their nucleosides protect the cells from FUra cytotoxicity by depleting their available PPriboseP. We conclude that FUra is metabolically activated by OPRTase rather than through pyrimidine nucleoside phosphorylases. Moreover, we have observed that purines decrease FUra cytotoxicity in mouse leukemia cells (L1210), Novikoff (rat) hepatoma cells, and primate B-lymphoblasts, suggesting that phosphoribosylation of FUra may be a common mechanism of activation.

EXPERIMENTAL PROCEDURES

Chemicals. [U-^{14}C]Glycine (99.6 mCi/mmol), [COOH-^{14}C]orotate (41.25 mCi/mmol), [COOH-^{14}C]orotidine 5′-monophosphate (35 mCi/mmol), [2-^{14}C]uracil (8 mCi/mmol), and [CH_3-^{3}H]thymidine (49.8 Ci/mmol) were purchased from New England Nuclear. [2-^{14}C]5-Fluorouracil (54 mCi/mmol) was obtained from Moravek Biochemicals. Yeast orotidylate decarboxylase was supplied by Sigma. All other chemicals, reagents, and materials have been reported previously (16, 17) and were of the highest quality commercially available.

Cultured cells. The lymphocytic properties, methods of culture, and growth characteristics of the wild type mouse T-cell lymphosarcoma line, S49, have been described (17, 18). L1210 cells, B cells, and Novikoff hepatoma cells were cultured using Dulbecco's modified Eagle medium supplemented with 10% heat inactivated horse serum and grown at 37° in 5% CO_2 in a fashion similar to S49 cells. All cells were routinely shown to be free of mycoplasma contamination by lack of cytoplasmic fluorescence after addition of the fluorescent DNA-binding benzimidole derivative, Hoeschst 33258 (19).

Mutant selections. S49 cells in Dulbecco's modified Eagle medium with 10% horse serum were mutagenized with MNNG as described previously (20, 21). Techniques for S49 clonal growth in semisolid agarose overlaying mouse embryo fibroblast feeder layers have also been described previously (21–23).

A cell line deficient in HGPRTase was selected for its resistance to 1 μM mercaptopurine riboside, while cells lacking adenine phosphoribosyl transferase (APRTase) were selected for growth in 0.1 mM diaminopurine. A thymidine kinase deficient cell line was selected for resistance to 40 μM bromodeoxyuridine. Cell lines selected by virtue of resistance to 1 μM FUra (FU1-2) or 10 μM 6-azauridine (AU13) were found to contain 50% and 300%, respectively, of wild type levels of both OPRTase and OMP decarboxylase.

Determination of cytotoxicities. The experiments to determine the rate of S49 cell growth in the presence of various chemical agents were conducted in Falcon multiwell (24 wells) tissue culture plates as described previously (20). Occasionally growth exper-

iments were performed in complete medium containing dialyzed horse serum. Growth experiments were typically begun by the addition of the chemicals to the wells containing 10^5 logarithmically growing cells in 1.00 ml. Typically, after 72 hr the number of control cells in the absence of added chemical agents had increased 16- to 25-fold (4 to 5 cell doublings). The number of cells increases logarithmically during the 72 hr period (17). The presence of dialyzed horse serum did not alter cell growth rates or the cytotoxic effects of any fluorinated pyrimidine on S49 cell lines. Less than 5% cell growth was equivalent to complete loss of all cell viability as determined by periodic monitoring by trypan blue exclusion with a hemacytometer. Experiments determining the ability of L1210 cells and primate B lymphoblasts to grow in the presence of chemical agents were performed in an identical fashion. Growth experiments with Novikoff hepatoma cells were also carried out similarly, except that prior to counting, the monolayers of cells were washed twice with phosphate-buffered saline and removed with trypsin to allow counting in suspension.

Preparation of cell extracts for enzyme assays. Cells for enzyme assays were harvested and washed with phosphate-buffered saline by centrifugation. The cell pellets (approximately 3×10^7 cells) were frozen and thawed three times in 200 μl of 50 mM potassium phosphate buffer, pH 7.4, 3 mM $MgCl_2$, 1 mM dithiothreitol and 10% (v/v) glycerol. The extract was centrifuged at 12,000 × *g* for 10 min and sieved on a column of Sephadex G25 that had been previously equilibrated with the above buffer containing $MgCl_2$, dithiothreitol, and glycerol.

In vitro enzyme assays. APRTase and HGPRTase were assayed according to the procedures described by Green and Martin (16) and by Gudas et al. (20). OPRTase and OMP decarboxylase catalytic activities were measured according to a modification of Method A of Kornberg, Lieberman and Simms (23) by measuring the evolution of $[^{14}C]O_2$ from [COOH-^{14}C]orotic acid and [COOH-^{14}C]orotidylate, respectively.

OMP decarboxylase was measured in a volume of 0.15 ml containing 100 mM Tris-HCl, pH 7.4; 5 mM $MgCl_2$; 100 μM [COOH-^{14}C]orotidylate (2 mCi/mmol); and 25 to 100 μg of protein. OPRTase activity was also assayed in a volume of 0.15 ml containing 50 mM phosphate, pH 7.4; 4 mM $MgCl_2$; 1 mM PPriboseP; 160 μM [COOH-^{14}C]orotic acid (125 mCi/mmol) and 25 to 100 μg of protein extract. The released $[^{14}C]O_2$ in either the OPRTase or the OMP decarboxylase assay was trapped on paper wicks saturated with Protosol:ethanol/1:3 and counted in a liquid scintillation system. Addition of yeast OMP decarboxylase to the OPRTase assay did not increase $[^{14}C]O_2$ release from [COOH-^{14}C]orotic acid.

The *in vitro* phosphoribosylation of FUra was measured according to the procedure of Reyes and Guganig (14). The activity was measured in a volume of 250 μl containing 45 mM glycine, pH 10.0; 2 mM $MgCl_2$; 2 mM PPriboseP; 75 μM [2-^{14}C]FUra (54 mCi/mmol); and 50 to 300 μg of protein. At 20 min intervals 50 μl aliquots were removed, placed on a PEI-cellulose strip, washed five times with 2.5 liters of H_2O, and counted in a liquid scintillation system.

The phosphoribosylation of uracil to UMP was measured under identical conditions except that 380 μM [2-^{14}C]uracil (6.2 mCi/mmol) served as the pyrimidine substrate.

In vivo measurements of enzyme activity. Phosphoribosylation of FUra was measured *in vivo* by incubating approximately 10^7 cells in 10 ml of complete medium with 1.9 μM [2-^{14}C]FUra for 16 hr. The cells were isolated by centrifugation, washed several times with phosphate-buffered saline, resuspended in 50 μl H_2O, and sonicated on a Biosonik III at 30% output. The phosphorylated FUra metabolites were isolated on PEI-cellulose strips, washed 5 times with 2.5 liters of H_2O, and counted in a liquid scintillation system. Incorporation of [2-^{14}C]FUra into phosphorylated metabolites followed a linear relationship with time for 16 hr. The relationship of incorporation to the number of cells was also linear.

Thymidine kinase was measured *in vivo* by the ability of cells to incorporate [CH_3-^{14}C]thymidine into nucleotides. Ten milliliters of cells were incubated with 10 μM

[CH_3-^{3}H]thymidine (100 mCi/mmol) for one hr, centrifuged and washed with phosphate-buffered saline. The cells were lysed and the extracts placed on DE81 Whatman filters. Filters were washed five times with 2.5 liters H_2O and counted in the liquid scintillation system. The thymidine kinase deficient mutant accumulated less than 0.01% of the metabolites of [CH_3-^{3}H]thymidine as compared to the wild type cell line. The thymidine kinase deficient variant was able to transport nucleosides and could incorporate both adenosine and uridine into nucleotides as efficiently as the wild type cell line.

Determination of cellular PPriboseP content and rates of purine biosynthesis. The PPriboseP levels in cells growing at a logarithmic rate were determined by a modification of Method A of Kornberg, Lieberman, and Simms (23) as described by Gudas et al. (20). This method measured the quantity of radioactive [^{14}C]O_2 evolved from [COOH-^{14}C]orotic acid in the presence of exogenous yeast OPRTase and OMP decarboxylase. Cells in certain flasks were treated with specific purine bases and nucleosides 90 min and 240 min before harvesting, and the PPriboseP content determined as described (20).

The rates of *de novo* purine biosynthesis in the presence of FUra, FUrd, or FdUrd were measured by the silver precipitation method of Martin and Owen (24). Purine biosynthetic rates for S49 cells in regular complete medium were the same as those obtained in medium containing dialyzed horse serum.

RESULTS

Effect of FUra on wild type of S49 cells. The growth of wild type S49 cells in continuous culture is sensitive to inhibition by micromolar concentrations of FUra, as shown in the dose response curve of Figure 1. The concentration of FUra that inhibits growth 50% (EC_{50}) is 0.8 μM. The addition of 30 μM thymidine (in the presence of 10 μM deoxycytidine to circumvent inhibition of ribonucleotide reductase by thymidine) had no significant effect on S49 cell growth or the EC_{50} values for FUra (Fig. 1). However, the presence of 30 μM thymidine does

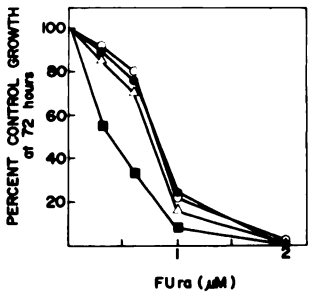

FIG. 1. *Effect of FUra on wild type and thymidine kinase deficient S49 cells*

Varying concentrations of FUra were added to cultures of wild type (●——●) or thymidine kinase deficient (○——○) cells. Wild type cells were also incubated with FUra in the presence of 50 μM uridine (■——■) or 30 μM thymidine and 10 μM deoxycytidine (△——△). Cell growth is plotted as a percentage of cell growth after 72 hr in the absence of FUra.

increase the EC_{50} of the deoxyribonucleoside of FUra, FdUrd, by at least four orders of magnitude in S49 cells (data not shown). However, uridine at a concentration of 50 μM lowers the EC_{50} value for FUra to 0.5 μM (Fig. 1).

A cell line deficient in thymidine kinase is as sensitive to FUra as the wild type cell, and these data are also shown in Figure 1. However, these thymidine kinase deficient cells are very resistant to FdUrd (data not shown). This evidence coupled with the fact that thymidine does not reverse FUra cytotoxicity suggests that FUra and FdUrd have different biochemical mechanisms of cytotoxicity in S49 cells.

It has been reported in Novikoff hepatoma cells that hydroxyurea, a potent inhibitor of ribonucleotide reductase in mammalian cells (25), eliminates FUra mediated inhibition of thymidylate synthesis (26). However, hydroxyurea does not inhibit the cytostatic or cytotoxic effects of FUra in S49 cells (data not shown), suggesting that the mechanism of activation of FUra does not involve the formation of 5-fluorinated deoxyuridine nucleotides by ribonucleotide reductase. Thus, FUra metabolites may not inhibit thymidylate synthesis in S49 cells.

Protection of S49 cells from FUra cytotoxicity. In the process of determining whether other nucleosides affected the cytotoxicity of FUra, it was noted that both

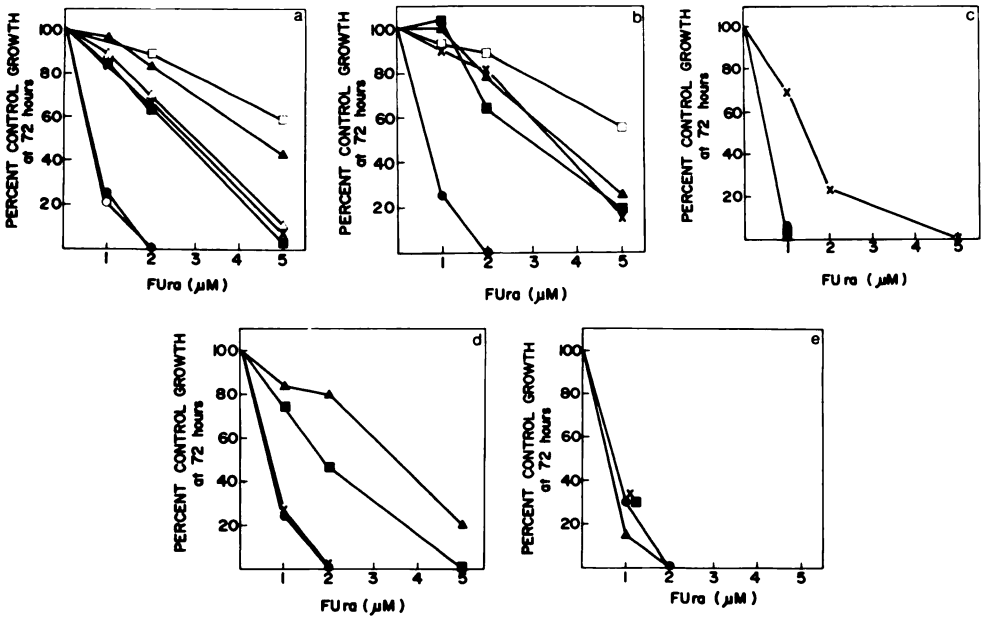

FIG. 2. *Effect of purines of FUra toxicity in wild type and in mutant S49 cells*

S49 cells in culture were incubated with varying concentrations of FUra in the absence of purines (●——●), or in the presence of either 0.1 mM adenine (×——×), 0.1 mM inosine (▲——▲), 0.1 mM deoxyinosine (△——△), 0.1 mM hypoxanthine (■——■), 0.5 mM hypoxanthine (□——□), or 0.05 mM guanosine (○——○). Purines and FUra were administered to cultures of wild type cells, Panel A; thymidine kinase deficient cells, Panel B; HGPRTase deficient cells, Panel C; APRTase deficient cells, Panel D; and cells deficient in both HGPRTase and APRTase, Panel E. Each growth condition in every cell type was repeated at least three times, and the results reported are those of a single typical experiment.

inosine and 2′-deoxyinosine protected the wild type S49 cells from FUra toxicity (Fig. 2A). In addition, the presence of hypoxanthine or adenine protected the cells from FUra toxicity (Fig. 2A). However, the purine nucleoside, guanosine, at a concentration of 50 μM, had no effects on the sensitivity of cells to FUra (Fig. 2A). The concentration of guanosine employed in these experiments was lower than the concentrations of other purines, since guanosine at higher concentrations is itself growth inhibitory (unpublished observations). Similar protective effects were seen in the thymidine kinase deficient cells (Fig. 2B). None of the above purines or purine nucleosides at the concentrations tested had any effects themselves on S49 cell growth rates nor on the toxic effects of the riboside (FUrd) or deoxyriboside (FdUrd) of FUra on S49 cells (data not shown).

It seemed important to know whether the purine bases and purine nucleosides exerted their protective effects directly or required specific metabolism. To answer this question we utilized S49 cell mutants deficient in specific purine salvage enzymes. Since inosine is converted to hypoxanthine by purine nucleoside phosphorylase, we tested the effects on these purines on FUra toxicity in HGPRTase deficient cells. HGPRTase deficient cells were not protected from FUra cytotoxicity by inosine or hypoxanthine, but were still protected by adenine (Fig. 2C). APRTase deficient cells were protected from FUra cytotoxicity by inosine and hypoxanthine, but not by adenine (Fig. 2D). A double mutant, deficient in both HGPRTase and APRTase, was not protected from FUra cytotoxicity by inosine, hypoxanthine, or adenine (Fig. 2E). These data with S49 cell mutants indicate that the protection from FUra cytotoxicity by purines requires the metabolism of these purines to their respective nucleotides.

These data are consistent with the hy-

pothesis that FUra or its metabolites inhibit *de novo* purine synthesis. We tested this hypothesis by measuring the incorporation of [^{14}C]glycine into purines in the presence and absence of the fluorinated pyrimidines. Neither FUra (1-100 μM), FUrd (100 nM), nor FdUrd (100 nM) inhibited the rate of *de novo* purine synthesis in complete medium containing either dialyzed or undialyzed horse serum. Similarly, these three fluorinated compounds did not affect intracellular concentrations of PPriboseP, an early substrate of *de novo* purine synthesis.

The phosphoribosylation of FUra. The protection of S49 cells from FUra cytotoxicity by purines requires their metabolism to purine nucleotides, a process which involves the consumption of PPriboseP. It has been reported that a pyrimidine phosphoribosyl transferase in mammalian cells is capable of phosphoribosylating FUra (10–14). Thus, we attempted to measure the phosphoribosylation of FUra in extracts of wild type cells as described in EXPERIMENTAL PROCEDURES. The *in vitro* phosphoribosylation of 75 μM [2-^{14}C]FUra can be demonstrated in these extracts to be linear with time and protein, and the reaction is dependent on PPriboseP. The activity can be abolished by the addition of 0.5 mM orotic acid and is inhibited about 50% by 0.5 mM uracil. These observations suggest that the enzyme, OPRTase, is responsible for the phosphoribosylation of FUra. However, it appears that the phosphoribosylation of FUra (Table 1) does not occur sufficiently rapidly to deplete the intracellular PPriboseP levels.

Accordingly, we examined the phosphoribosylation of FUra by extracts of S49 mutants possessing different levels of OPRTase activity. An S49 mutant, AU13, with 300% wild type levels of OPRTase, phosphoribosylated FUra at a rate 3.5 times higher than did wild type extracts (Table 1). Conversely, extracts of a cell line (FU1-2) containing 50% of the wild type level of OPRTase, phosphoribosylated FUra *in vitro* at 65% of the wild type rate (Table 1). The phosphoribosylation of FUra in all cell lines was dependent upon addition of PPriboseP. In addition, extracts of the

TABLE 1

Phosphoribosylation of pyrimidine bases in wild type and mutant S49 cell extracts

Phosphoribosylation in mutant cell extracts is expressed as a percentage of phosphoribosyl transferase activity in wild type cells. Wild type cell extracts phosphoribosylated 160 μM orotic acid at a rate of 37.4 nmol/hr/mg protein, 75 μM FUra at a rate of 0.4 nmol/hr/mg protein, and 380 μM uracil at a rate of 0.7 nmol/hr/mg protein. These data are those from one of several identical experiments, all of which gave similar results.

Pyrimidine base	Percentage of wild type activity	
	Cell type	
	AU13	FU1-2
Orotate	303	49
FUra	346	65
Uracil	247	55

AU13 and FU1-2 cells, respectively, had 2.5 and 0.55 times the capacity of wild type cell extracts to phosphoribosylate uracil (Table 1). Although incorporation of labeled uracil is the basis of a technique for detection of mycoplasma contamination, the ratios of [^{3}H]uridine/[^{3}H]uracil uptake into RNA (300–1000/liter) (27) in all S49 cells tested were consistent with those found in mycoplasma-free cultures. Furthermore, the cells were shown to be free of mycoplasma by lack of cytoplasmic fluorescence after staining with Hoechst 33258 (19).

The altered levels of OPRTase in the AU13 and FU1-2 cell lines suggest that these cells *in vivo* would have an increased and decreased capacity, respectively, to metabolize FUra. The ability of wild type, AU13, and FU1-2 cells to phosphoribosylate FUra *in vivo* is shown in Table 2. The greater the ability of each line to phosphoribosylate FUra *in vivo* the greater the amount of OPRTase catalytic activity measured *in vitro*.

If OPRTase is a limiting factor in the metabolic activation of FUra, then these mutants with different levels of OPRTase activity should exhibit sensitivities to FUra cytotoxicity proportional to their enzyme levels. We examined the effects of varying doses of FUra on the growth rates of wild type, AU13, and FU1-2 cells and showed

TABLE 2

Incorporation of [2-^{14}C]FUra into nucleotides in wild type and mutant S49 cells

Cells were incubated for 16 hr with 1.9 μM [2-^{14}C]FUra and incorporation measured as described in EXPERIMENTAL PROCEDURES. This experiment was repeated three times with the same results.

Cell type	Addition	Concentration (μM)	FUra incorporation (pmol/hr/10^9 cells)
AU13	—	—	1260
FU1-2	—	—	95
Wild type	—	—	382
Wild type	Uridine	50	683
Wild type	Thymidine	20	417
Wild type	Inosine	250	67
Wild type	Guanosine	100	408
Wild type	Hypoxanthine	500	73
Wild type	Adenine	100	115

that the sensitivity of all three cell lines was related to their ability to phosphoribosylate FUra *in vitro*, and thus to their OPRTase levels. The EC_{50} values for FUra in the AU13 and in the FU1-2 cells were 0.15 μM and 8.0 μM, respectively (Fig. 3), while that for wild cells is 0.8 μM (Figs. 1 and 3). As it does for wild type cells, 0.5 mM hypoxanthine protected against FUra mediated cytotoxicity in both mutant cell lines (Fig. 3). These data correlating rate of growth in FUra and rate of phosphoribosylation of FUra *in vivo* and *in vitro* provide strong support for the hypothesis that phosphoribosylation by OPRTase is the rate-limiting process in the metabolic activation of FUra.

Effect of purines on intracellular PPriboseP levels. Since hypoxanthine, inosine and adenine increase cellular resistance to FUra toxicity, and since PPriboseP appears to be necessary for FUra metabolism via OPRTase, we examined the effects of these purines on PPriboseP levels. As shown in Figure 4, hypoxanthine, inosine, and adenine all significantly decreased intracellular PPriboseP content of wild type cells. Conversely, guanosine, which does not affect FUra sensitivity in wild type S49 cells, does not decrease PPriboseP concentrations (Fig. 4). Moreover, the *in vivo* phosphoribosylation of FUra in wild type cells is severely diminished by the presence

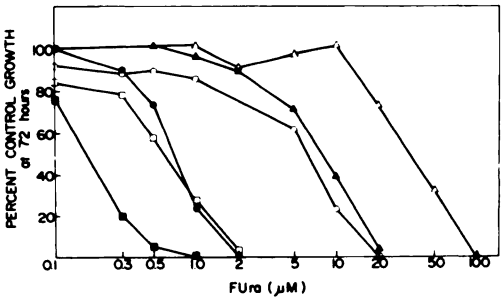

FIG. 3. *Comparison of FUra toxicity on wild type cells and cells with altered OPRTase levels*

The growth inhibition and cytotoxicity of various concentrations of FUra were determined in the absence of purines for wild type cells (●——●), for AU13 cells (■——■), and for FU1-2 cells (▲——▲). Wild type (○——○), AU13 (□——□), and FU1-2 (△——△) cells were also incubated with varying FUra concentrations in the presence of 0.5 mM hypoxanthine. The identical experiment with these same cell lines has been repeated three other times with similar results.

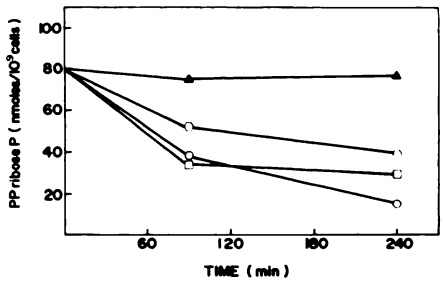

FIG. 4. *Effect of purine bases and nucleosides on intracellular concentrations of PPriboseP*

Wild type S49 cells were incubated with 0.5 mM hypoxanthine (○——○), 0.1 mM guanosine (▲——▲), 0.25 mM inosine (□——□), or 0.1 mM adenine (◯——◯) for 90 min and 240 min, and the PPriboseP levels determined as described previously (20, 21). These results are those of a single experiment, which has been repeated two other times with similar results.

of inosine, hypoxanthine, or adenine in the culture medium; guanosine and thymidine have no effects, while uridine increases the *in vivo* phosphoribosylation slightly (Table 2). These data are consistent with the hypothesis that those purines which decrease FUra cytotoxicity do so by diminishing intracellular PPriboseP levels, thereby impairing the ability of the S49 cell to phosphoribosylate FUra.

Effects of purines on FUra cytotoxicity

TABLE 3

Comparison of the effect of hypoxanthine on FUra toxicity in S49 cells and other cell lines

EC_{50} values for FUra in the absence and presence of 0.5 mM hypoxanthine were determined as described in EXPERIMENTAL PROCEDURES. Values were determined at least three times for each cell type, and the values reported are those of a single typical experiment.

Cell line	EC_{50} for FUra	
	No hypoxanthine	+0.5 mM hypoxanthine
	(μM)	
S49 (wild type)	0.7	6.5
S49 (HGPRTase deficient)	0.56	0.52
L1210 (wild type)	0.4	1.2
L1210 (HGPRTase deficient)	0.36	0.36
Primate B Lymphocytes (wild type)	10.2	34.0
Novikoff Hepatoma (wild type)	1.8	32.0

in other cell lines. The addition of exogenous purines reversed FUra cytotoxicity in three other cell lines: mouse leukemia cells (L1210), Novikoff hepatoma cells, and marmoset B cells. The addition of 0.5 mM hypoxanthine to the cell cultures raised the EC_{50} values for FUra from 0.4 μM to 1.2 μM in L1210 cells, from 11 μM to 34 μM in primate lymphoblasts, and from 1.8 μM to 32 μM in Novikoff hepatoma cells. In HGPRTase deficient L1210 cells hypoxanthine did not decrease cellular sensitivity to FUra as indicated in Table 3.

DISCUSSION

To ascertain the relative importance of thymidine phosphorylase and uridine phosphorylase in initiating sequential metabolic reactions leading to FUra cytotoxicity, known ribose and deoxyribose donors were administered to FUra treated mouse lymphoid cells grown in continuous culture. Thymidine has no effect on FUra cytotoxicity or growth inhibition, whereas uridine increased FUra toxicity slightly. When, however, inosine, a ribose donor, or deoxyinosine, a deoxyribose donor, was administered to FUra treated cells, we observed that both of these purine nucleosides protected them from the cytotoxicity of FUra; however, hypoxanthine and adenine also protected cells from FUra cytotoxicity. Experiments with S49 cell mutants deficient in the specific enzymes which salvage these purines (HGPRTase and APRTase) suggested that the formation of the nucleotides of these purines was necessary for their protective effects.

Having demonstrated a PPriboseP dependent *in vitro* phosphoribosylation of FUra, we examined the *in vitro* and *in vivo* phosphoribosylation of FUra and the *in vivo* FUra sensitivity of two mutants with altered activities of OPRTase. The results of these experiments with the mutant S49 cells (AU13 and FU1-2) confirmed the hypothesis that the rate-limiting step in the metabolic activation of FUra is its phosphoribosylation by OPRTase. The phosphoribosylation of FUra via OPRTase suggests a possible mechanism by which uridine potentiates FUra toxicity and increases incorporation of labeled FUra into nucleotides. Since uridine phosphorylase activity is negligible in S49 cells (unpublished observations), uridine is primarily phosphorylated to uridine nucleotides. Uridine 5′-triphosphate is a potent inhibitor of carbamyl phosphate synthetase, the rate-limiting enzyme in *de novo* pyrimidine biosynthesis (28–31). In S49 cells the addition of exogenous uridine inhibits *de novo* pyrimidine synthesis and depletes intracellular orotic acid levels (B. Levinson, unpublished observations). Since orotic acid inhibits FUra phosphoribosylation, this depletion of orotic acid levels in S49 cells allows FUra to be phosphoribosylated more rapidly by OPRTase. However, it is still possible that the potentiation of FUra toxicity by uridine is due to increased metabolism of FUra via uridine phosphorylase and uridine kinase.

Since FUra is metabolized by OPRTase, for which PPriboseP is an obligatory substrate, we proposed that the protective effects of purines might be mediated by depletion of intracellular PPriboseP. Adenine, hypoxanthine, and inosine, all of which reverse FUra toxicity, significantly depleted the cellular PPriboseP content, whereas guanosine, which cannot protect cells from

FUra toxicity, did not affect PPriboseP levels. Thus, it is likely that purine bases and their nucleosides affect FUra toxicity by regulating the availability of PPriboseP, necessary for FUra metabolism. Data from purine protection experiments in other cell lines from different species are consistent with those obtained in S49 cells and suggest that the requirement for PPriboseP in the metabolic activation of FUra may be common to many cell types.

FUra is widely used as an antineoplastic agent for the treatment of breast, gastrointestinal and a variety of other carcinomas (32–34). An understanding of the metabolism of FUra in different tissues might provide a biochemical basis for the design of more efficacious clinical protocols for 5-fluorinated uracil analogues.

Although no drug is known to reverse FUra cytotoxicity, the demonstrated protection by purines may provide a clinical means to manipulate FUra metabolism in humans. FUra may be metabolically activated either by pyrimidine nucleoside phosphorylases and pyrimidine nucleoside kinases or by OPRTase, the *in vivo* activity of the latter being dependent on intracellular PPriboseP content. Since the activities of these enzymes vary widely among different tissues (35–39), the administration of purines or even allopurinol might diminish the metabolism of FUra in tissues dependent on OPRTase activity for FUra metabolism while not affecting its metabolism in those tissues dependent on pyrimidine nucleoside phosphorylase activity.

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