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Fluorinated Pyrimidines

XL. The Reduction of 5-Fluorouridine 5′-Diphosphate by Ribonucleotide Reductase

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SUMMARY

A preparation of ribonucleotide reductase from Ehrlich ascites cells, free of thymidine kinase activity, catalyzes the formation of 5-fluorodeoxyuridine 5′-monophosphate from enzymatically synthesized 5-fluorouridine 5′-diphosphate. The product of the reaction was identified by paper chromatography before and after dephosphorylation with snake venom. The K_m for 5-fluorouridine 5′-diphosphate in the reaction is 0.072 mM. Experiments with several cell lines in culture reported here, as well as studies reported earlier, provide supporting evidence that, in some cells, the biosynthesis of 5-fluorodeoxyuridine 5′-monophosphate from 5-fluorouracil involves the initial formation of ribonucleotides, followed by reduction to the deoxyribonucleotide by ribonucleotide reductase.

INTRODUCTION

5-Fluorouracil was synthesized in 1957 by Heidelberger and co-workers (1), and the nucleosides of FUra,[2] FUrd and FdUrd were made available a short time later (2). Subsequent experiments *in vivo* and *in vitro* showed that these compounds were powerful inhibitors of DNA synthesis and that the primary site of action was the enzyme thymidylate synthetase (methylene tetrahydrofolate:dUMP *C*-methyltransferase, EC 2.1.1.b) (1, 3, 4). The inhibition of this enzyme by the 5′-deoxyribonucleotide of FUra, FdUrd-5′-P, was first demonstrated in a cell-free system by Cohen *et al.* (5), and has been extensively studied by Heidelberger and co-workers (6–8). In addition, Cohen *et al.* (5) showed that FdUrd was phosphorylated by thymidine kinase (ATP:thymidine 5′-phosphotransferase, EC 2.7.1.21) to FdUrd-5′-P. This fact, coupled with the demonstration by Sköld (9) that FUra and FdUrd were readily interconverted by uridine phosphorylase (uridine:orthophosphate ribosyltransferase, EC 2.4.2.3) from Ehrlich ascites cells, suggested one route by which FUra might be anabolized to the active inhibitor, FdUrd-5′-P (pathway A, Fig. 1). A second possible pathway for activation of FUra involves conversion of FUra to FUrd-5′-P either by consecutive reactions involving uridine phosphorylase (10) and uridine kinase (ATP:uridine 5′-phosphotransferase,

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[2] The abbreviations used are: FUra, 5-fluorouracil; FUrd, 5-fluorouridine; FUrd-5′-P, 5-fluorouridine-5′-monophosphate; FUrd-5′-PP, 5-fluorouridine 5′-diphosphate; FUrd-5′-PPP, 5-fluorouridine 5′-triphosphate; FdUrd, 5-fluoro-2′-deoxyuridine; FdUrd-5′-P, 5-fluoro-2′-deoxyuridine 5′-monophosphate; PP-ribose-P, 5-phosphoribosyl 1-pyrophosphate; deltib-1-P, deoxyribose 1-phosphate; Rib-1-P, ribose 1-phosphate.

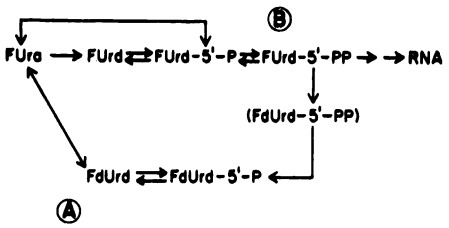

FIG. 1. *Alternative pathways for conversion of FUra to FdUrd-5'-P*

EC 2.7.1.48) (11) or directly by a phosphoribosyltransferase (12). FUrd-5'-P is then phosphorylated to FUrd-5'-PP (13), followed by reduction of FUrd-5'-PP to deoxyribonucleotide via ribonucleotide reductase (pathway B, Fig. 1). The formation of FdUrd-5'-P from FUrd-5'-PP has not been directly demonstrated, although it has been assumed (11, 14, 15) that FUrd-5'-PP reduction probably takes place, since the fluorinated pyrimidines readily undergo the same reactions as uracil and its derivatives (14).

In this paper we present evidence that FUrd-5'-PP is reduced to FdUrd-5'-P in preparations of Ehrlich ascites cells which have been freed of one of the enzymes of pathway A (Fig. 1). We have studied some of the properties of this reduction and examined reductase levels in five cell lines in culture, two of which are resistant to FdUrd.

MATERIALS AND METHODS

Chemicals. [6-^{3}H]FUra was purchased from Amersham/Searle. [5-^{3}H]CDP and [5-^{3}H]UDP were obtained from Schwarz/Mann. [2-^{14}C]FUra was prepared in this laboratory (11). Hoffmann–La Roche, Inc., generously provided [2-^{14}C]FdUrd and all unlabeled fluorinated pyrimidines. All other chemicals were obtained from commercial sources. The radiochemical purity of labeled compounds was determined to be greater than 95% by paper chromatography and subsequent analysis with a radiochromatogram scanner (Packard).

FUrd-5'-PP synthesis. [6-^{3}H]FUrd-5'-PP was synthesized from [6-^{3}H]FUra by a two-step enzymatic procedure. [6-^{3}H]FUra was condensed with PP-ribose-P to give FUrd-5'-P in a reaction catalyzed by a pyrimidine phosphoribosyltransferase found in some mammalian cells (12, 16). The enzyme in this instance was derived from Ehrlich ascites cells. The method of enzyme preparation and the incubation procedure were those of Reyes (12), except that the reaction volume was increased to 1 ml and the time of incubation to 1 hr for preparative purposes. The incubation mixture was chromatographed on sheets of Whatman No. 40 paper in solvent 1 (Table 1). [6-^{3}H]FUrd-5'-P was eluted from the origin with water, and the solution was lyophilized.

A partially purified, heat-stable phosphotransferase from rat liver was used to prepare FUrd-5'-PP from FUrd-5'-P, as described by Maley *et al.* (17). The reaction products were adsorbed onto a 9 × 1.5 cm column of DEAE-cellulose (Gallard-Schlesinger), prepared according to Peterson and Sober (18), and eluted with 400 ml of a linear gradient of triethylammonium bicarbonate, pH 8.0, 0.1–0.2 M. The FUrd-5'-PP fraction was lyophilized until all traces of the volatile buffer had been removed. Both labeled and unlabeled FUrd-5'-PP

TABLE 1

R_F values of fluorinated pyrimidines in chromatography solvents

The unlabeled compounds were spotted on sheets of Whatman No. 40 paper and developed with the indicated solvents by descending chromatography. The compounds were located by ultraviolet light. Solvent 1 is the top layer of a two-phase system consisting of ethyl acetate, water, and formic acid (60:35:5). Solvent 2 is ethanol, 5 M ammonium acetate (pH 9.5), saturated sodium tetraborate, and 0.5 M EDTA (11:1:4:0.02). Solvent 3 is 1-butanol, acetone, formic acid, and 5.5% ammonium formate (35:25:15:25).

Compound	Solvent 1	Solvent 2	Solvent 3
	R_F	R_F	R_F
FUra	0.63	0.40	0.66
FUrd	0.30	0.22	0.64
FdUrd	0.73	0.35	0.69
FUrd-5'-P	0.05	0.02	0.33
FdUrd-5'-P	0.21	0.03	0.42
FUrd-5'-PP			0.19
FUrd-5'-PPP			0.13

were prepared this way. Thymidine kinase (19) and nucleoside phosphorylase (20) assays have been described.

Ribonucleotide reductase assays. The incubation mixture for all assays contained the following components, based on the procedure of Larsson (21): *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid, pH 7.6, 100 mM; ATP, 6 mM; magnesium acetate, 6 mM; dithioerythritol, 8 mM; sodium fluoride, 8 mM; and ferrous ammonium sulfate, 0.02 mM. For assays to monitor enzyme purification, the reaction volume was 0.2 ml and the substrate was 0.4 mM [5-^{3}H]CDP, 80,000 dpm/tube. Incubation was carried out at 37° for 30 min with the initial 100,000 × *g* supernatant fraction (S3), and for 10 min with fractions subsequently obtained during the purification. The reaction was stopped by the addition of 1.0 ml of 1 M perchloric acid. Products were separated on Dowex 50-H$^+$ columns according to Moore (22). One unit of enzyme is defined as the amount that will reduce 1 nmole of CDP in 30 min.

For experiments on the reduction of FUrd-5'-PP, the volume was ordinarily 0.06 ml, and [6-^{3}H]FUrd-5'-PP (165,000 dpm/tube), [5-^{3}H]UDP (107,000 dpm/tube), or [5-^{3}H]CDP (85,000 dpm/tube) was present at 0.1 mM. Incubations were stopped by immersion in a boiling water bath for 2 min. Snake venom (*Crotalus adamanteus*, Sigma), 0.2 mg in 0.04 ml, was added to dephosphorylate nucleotides. After 90 min of incubation at 37°, 0.01 ml of 65% trichloracetic acid was added, and the protein precipitate was removed by centrifugation. An aliquot of 0.05 ml was chromatographed on 1-inch strips of Whatman No. 40 paper in solvent 2 (Table 1). Unlabeled deoxyribonucleoside was added prior to chromatography. The deoxyribonucleoside spot was located under an ultraviolet lamp and cut out from the chromatogram after development, eluted with 1 ml of 0.01 N HCl, and counted in a scintillation counter (Packard) with Scintisol (Isolab, Inc.) as the counting medium.

In the double-label experiment in which [2-^{14}C] FUra or [2-^{14}C] FdUrd was present in addition to [6-^{3}H]FUrd-5'-PP, the reaction volume was 0.12 ml, and the reaction was quenched with 0.01 ml of 65% trichloracetic acid. Duplicate 0.05-ml aliquots were chromatographed in solvent 1. Nucleosides and bases migrate readily in this solvent, while nucleotides remain at the origin. Material at the origin was eluted with water, lyophilzed, dephosphorylated with snake venom, and chromatographed with solvent 2 as described above.

Studies on cells in culture. The HeLa, NS, NSF, Y, and BF cells and the methods for growing them have been described previously (23), except that the HeLa cells were grown in suspension cultures with spinner minimal essential medium supplemented with 10% fetal calf serum and antibiotics.

The cells were collected during the logarithmic phase by centrifugation at 10,000 × *g*. To each gram of cells were added 1.5 volumes of 0.05 M Tris-acetate buffer, pH 7.6 (buffer A). The cells were homogenized with a Willems Polytron instrument and centrifuged at 100,000 × *g* for 1 hr. The supernatant fraction was stored at −15°.

Enzyme purification. All operations were carried out at 0–4°. Ehrlich ascites cells were harvested from Swiss mice 8 days after intraperitoneal injection of 10^6 cells. The cells were centrifuged at 10,000 × *g* for 10 min, the ascites fluid was removed by aspiration, and the cells were suspended in 1.5 volumes of buffer A. The cells were disrupted with a Polytron and centrifuged at 10,000 × *g* for 10 min. The supernatant fraction was centrifuged at 100,000 × *g* for 1 hr (S3 fraction) and was ordinarily frozen prior to purification. At this stage the reductase activity was stable indefinitely. To each 100 ml of S3 were added 15.4 mg (100 μmoles) of dithiothreitol and 11.4 g of solid ammonium sulfate (Mann, special enzyme grade) to give a 20% saturated solution. The solution was gently swirled until the salt dissolved, and was allowed to stand for 30 min with periodic agitation. The sparse precipitate was removed by centrifugation at 10,000 × *g* for 10 min. The supernatant fluid was brought to 40% saturation with ammonium sulfate by the addition of 12.3 g of the salt

to each 100 ml of solution. After the ammonium sulfate had dissolved, the solution was allowed to stand for 1 hr. The precipitate was collected by centrifugation at 10,000 × *g* for 10 min and dissolved in 20–30 ml of 0.05 M Tris-acetate, pH 7.6, 1 mM in dithiothreitol (buffer B). This solution was dialyzed for 10–12 hr in 100 volumes of buffer B, and is termed the ammonium sulfate fraction. The recovery of enzyme activity in this fraction was ordinarily 120–140%, indicating that the S3 fraction contained an inhibitor.

To each milligram of protein in the ammonium sulfate fraction was added 0.4 mg of a calcium phosphate gel (35 mg of solids per milliliter of gel) prepared as described by Keilin and Hartree (24). The resulting suspension was stirred for 1 hr, after which the gel was collected by centrifugation at 1100 × *g* for 5 min. The gel was eluted three times with 3–4 ml of 0.2 M phosphate buffer, pH 7.0, 1 mM in dithiothreitol. The eluate was dialyzed for 6 hr in 3 liters of buffer B to give the calcium phosphate fraction. Recovery of activity from this fraction often exceeded 100%, based on the S3 fraction. The dialyzed calcium phosphate fraction was concentrated to 4–5 ml by sprinkling dry Ficoll (Pharmacia, Inc.) directly onto the dialysis bag. The concentrated material was carefully placed on the top of a 100 × 4.5 cm column of Sephadex G-200 (Pharmacia) and eluted from the column with buffer B. The flow rate was 25 ml/hr, and the hydrostatic pressure never exceeded 15 cm. The void volume was approximately 500 ml, and reductase activity was eluted between 640 and 760 ml. The eluted material was concentrated by ultrafiltration, using an Amicon 410 apparatus with a PM10 filter. Prior to ultrafiltration, Pluronic F68 (Wyandotte Chemicals) was added to a final concentration of 0.1% (w/v) in order to decrease protein denaturation. The final volume of the Sephadex G-200 fraction was 4–6 ml. This step in the purification was accompanied by a substantial loss in ribonucleotide reductase activity, in terms both of units of enzyme and of specific activity. Nevertheless, because this procedure eliminated thymidine kinase, we felt it desirable to subject the enzyme preparation to Sephadex G-200 chromatography.

The purification procedure for ribonucleotide reductase from Ehrlich ascites cells described above was intended to reduce or eliminate the activities of the enzymes of pathway A (Fig. 1). The purification differs from, and is not nearly as extensive as, that attained by Larsson and Reichard (25) in *Escherichia coli* and by Moore (22) in Novikoff hepatoma.

The results of one purification are shown in Table 2, and the separation of ribonucleotide reductase from thymidine kinase and nucleoside phosphorylase activities are shown in Fig. 2.

Protein was determined as described by Lowry *et al.* (26).

RESULTS

FUrd-5′-PP reduction by purified enzyme. Preliminary experiments had shown that labeled FdUrd-5′-P could be recovered when labeled FUrd-5′-P or FUrd-5′-PP was incubated with the S3 fraction from Ehrlich ascites cells and the reductase assay mixture.[3] The possibility remained that FdUrd 5′-P was formed by initial dephosphorylation of the substrate, followed by phosphorolysis of FUrd to form FUra, formation of FdUrd by condensation of FUra with a deoxyribose donor, and phosphorylation of FdUrd to FdUrd-5′-P. Experiments on crude S3 fractions, designed to distinguish between the two possible pathways, were equivocal. Accordingly, we partially purified the reductase as described above and eliminated most of the activity of the enzymes of the competing pathway.

The data in Table 3 show that FUrd-5′-PP is directly reduced to FdUrd-5′-P and is not dependent on the fate of FUra. This is most clearly seen in the data from the Sephadex G-200 fraction, where the level of [^{3}H]FdUrd-5′-P, i.e., FdUrd-5′-P derived from FUrd-5′-PP, exceeded in every case the level of [^{14}C]FdUrd-5′-P derived from FUra or FdUrd. Similarly, at any stage of purification of the enzyme,

[3] Unpublished observations.

conversion of FUrd-5'-PP to FdUrd-5'-P was greater than conversion of FUra to FdUrd-5'-P when FUra and FUrd-5'-PP were incubated together in the absence of a deoxyribosyl donor. These data preclude a mode of conversion of FUrd-5'-PP to FdUrd-5'-P which involves FUra as an intermediate. It is also seen from the data that crude

TABLE 2

Partial purification of ribonucleotide reductase from Ehrlich ascites cells

The enzyme purification and enzyme assays are described under MATERIALS AND METHODS. In the reductase assay 0.083 ml of each protein fraction was used. The total volume was 0.2 ml. The corresponding volumes were 0.025 ml of protein fraction in a total volume of 0.125 ml for the thymidine kinase assay and 0.2 ml in a total volume of 1.5 ml for the FdUrd phosphorylase assay. The substrates were 0.4 mM [5-^{3}H]CDP for the reductase assay, 0.4 mM [6-^{3}H]FdUrd for thymidine kinase, and 0.4 mM FdUrd for the phosphorylase assay. In all assays 1 unit is that amount of enzyme which will produce 1 nmole of product in 30 min. Specific activity is units per milligram of protein. All incubation times were 10 min, except for S3 reductase and Sephadex G-200 kinase and phosphorylase assays, which were 30 min long. All incubations were carried out at 37°.

Fraction	Volume	Protein	Reductase		Kinase		Phosphorylase	
			Units	Specific activity	Units	Specific activity	Units	Specific activity
	ml	*mg*						
S3	190	4,920	9,750	2.0	21,000	4.3	675,000	137
$(NH_4)_2SO_4$, 20–40%	49	1,940	9,750	4.9	7,400	3.8	256,000	132
Calcium phosphate gel	16	460	4,900	10.7	1,700	3.6	40,000	87
Sephadex G-200	5.5	80	250	3.1	—[a]	—[a]	1,700	22

[a] Not detectable.

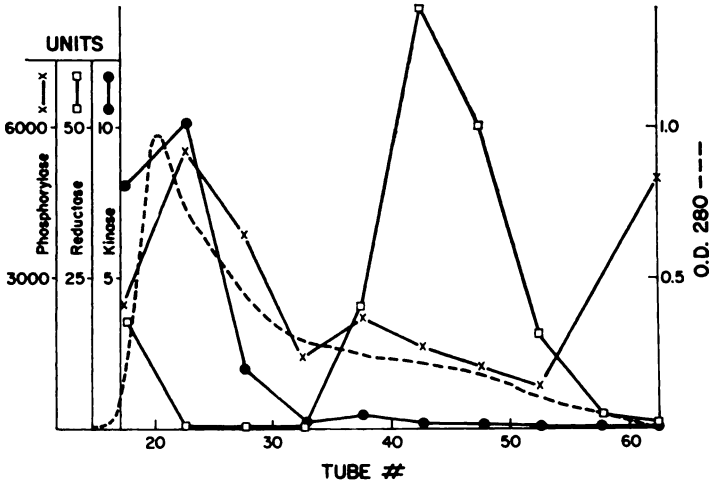

FIG. 2. *Separation of ribonucleotide reductase from thymidine kinase and nucleoside phosphorylase on Sephadex G-200*

The calcium phosphate gel fraction was concentrated with Ficoll to 5 ml, (protein 45 mg/ml), and layered on a 4.5 × 100 cm column of Sephadex G-200 equilibrated with buffer B. Enzymes were eluted by downward flow with buffer B at 25–30 ml/hr and a hydrostatic pressure of 13–15 cm. Groups of four tubes, 10 ml/tube, were pooled, and solid ammonium sulfate was added to 40% saturation. The precipitated protein was concentrated to a pellet by centrifugation, dissolved in buffer B, and analyzed for the three enzymes (see MATERIALS AND METHODS). One unit for each of the three enzymes is that amount which will form 1 nmole of product in 30 min.

TABLE 3

Conversion of labeled precursors to FdUrd-5'-P

The assay procedure and isolation of FdUrd-5'-P are described under MATERIALS AND METHODS. The assay was performed at 37° for 30 min with the S3 fraction, and for 10 min with the calcium phosphate and Sephadex G-200 fractions. There was 0.05 ml of protein fraction in a total volume of 0.12 ml.

Precursor(s)	Label	FdUrd-5'-P formation			
		S3, 0 min	S3	Calcium phosphate	Sephadex G-200
				pmoles/30 min	
0.1 mM [^{3}H]FUrd-PP	^{3}H	<6	16	250	220
	^{14}C	<7	<7	<7	<7
	^{14}C:^{3}H				
0.1 mM [^{3}H]FUrd-PP + 0.1 mM [^{14}C]FUra	^{3}H	<6	16	240	280
	^{14}C	<7	<7	19	15
	^{14}C:^{3}H			0.08	0.05
0.1 mM [^{3}H]FUrd-5'-PP + 0.1 mM [^{14}C]FUra + 1 mM deRib-1-P	^{3}H		20	245	240
	^{14}C		160	270	72
	^{14}C:^{3}H		8	1.1	0.3
0.1 mM [^{3}H]FUrd-5'-PP + 0.1 mM [^{14}C]FdUrd	^{3}H	<6	22	260	250
	^{14}C	<7	320	1160	24
	^{14}C:^{3}H		15	4.5	0.1

extracts of Ehrlich ascites cells have the capacity to synthesize FdUrd-5'-P from FUra when the deoxyribosyl donor deRib-1-P is present. This point is discussed later. We have also noted in preliminary experiments that thymidine was completely ineffective as a deoxyribose donor, in accordance with earlier observations (27) that Ehrlich ascites cells have no deoxyribosyltransferase activity.

Identity of product of FUrd-5'-PP reduction. Figure 3 presents chromatographic evidence on the identity of the product of the reductase reaction. The radioactive product migrated with marker FdUrd-5'-P in solvent 3 (Table 1), which readily separates FdUrd-5'-P and FUra ribonucleotides. After dephosphorylation with snake venom the product migrated with marker FdUrd in solvent 1, which separates FUra nucleotides, FUrd, FdUrd, and FUra. It was concluded, therefore, that FdUrd-5'-P was the product of the reductase reaction.

Time course of FUrd-5'-PP reduction. Figure 4 shows the amount of FdUrd-5'-P formed with time. The reaction velocity was linear for 15 min but deviated considerably from linearity by 30 min. All assays for which linearity was required were limited to 10 or 15 min of incubation.

Kinetics of FUrd-5'-PP, UDP, and CDP reduction. In order to obtain some measure of the ability of ribonucleotide reductase to reduce FUrd-5'-PP, we performed a substrate saturation experiment with FUrd-5'-PP and, for comparison, the natural pyrimidine nucleotide substrates UDP and CDP. Figure 5 is a Lineweaver-Burk (28) plot of the data for the three substrates. The K_m values obtained were 0.072, 0.095, and 0.03 mM for FUrd-5'-PP, UDP, and CDP, respectively. The corresponding V_{max} values for the experiment shown in Fig. 5 were 0.48, 3.7, and 3.7 nmoles of substrate reduced per 30 min for FUrd-5'-PP, UDP, and CDP. Larsson and Reichard (29) obtained K_m values of 0.22 and 0.04 mM for UDP and CDP with reductase from *E. coli*. Moore and Hurlbert, in studies of the enzyme from Novikoff hepatoma, reported K_m values of 0.023 and 0.067 mM for CDP and UDP in the presence of 2.1 mM ATP (30). It is perhaps noteworthy that the K_m values reported here are of roughly

the same order of magnitude for all three substrates but that the V_{max} of FUrd-5'-PP reduction is only one-eighth that of either CDP or UDP reduction. It would be hazardous to offer an interpretation of this observation without a thorough kinetic analysis of the enzyme.

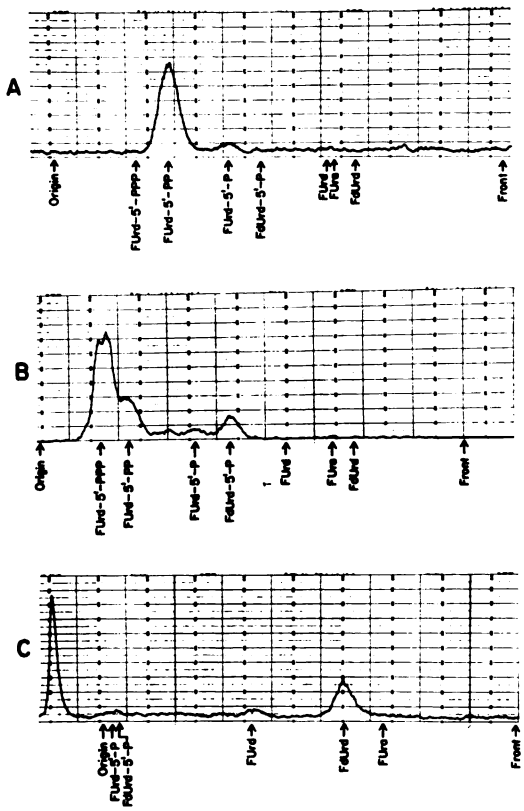

FIG. 3. *Identity of product of FUrd-5'-PP reduction*

The usual reductase assay was used, except that the volume per tube was 0.12 ml and the enzyme was the Ficoll-concentrated calcium phosphate gel fraction (54 units/tube, 24 units/mg of protein). Tubes were incubated for 0 or 15 min and quenched with 0.01 ml of 65% trichloracetic acid. The entire content of each tube was chromatographed on strips of Whatman No. 40 paper in solvent 3 (Table 1). Unlabeled compounds were added as markers. The spot corresponding to FdUrd-5'-P in panel B was cut out, eluted with water, lyophilized, and dephosphorylated with snake venom in 0.1 ml of buffer A. The dephosphorylation reaction was quenched with 0.01 ml of 65% trichloracetic acid, and the supernatant fraction was chromatographed in solvent 1 with unlabeled fluorinated pyrimidines.

A. No incubation, solvent 3. B. 15-min incubation, solvent 3. C. FdUrd-5'-P spot from panel B, dephosphorylated and chromatographed in solvent 1.

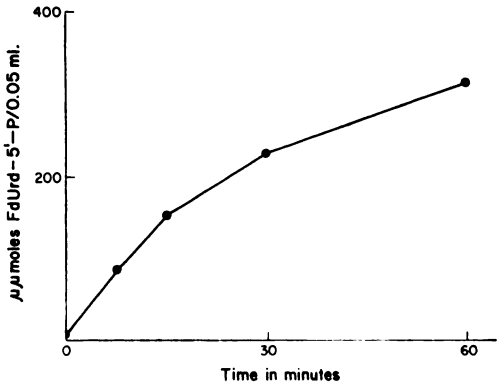

FIG. 4. *Time course of FdUrd-5'-P formation*

The assay is described under MATERIALS AND METHODS. A calcium phosphate gel fraction was used for this assay (6.5 units/0.05-ml tube, 15 units/mg of protein).

Effect of pH on FUrd-5'-PP reduction. Some of the biological effects of the fluorinated pyrimidines have been attributed to the increased acidity of the proton on nitrogen 3 in the pyrimidine ring of fluorouracil (pK_a 8.15), compared to uracil (pK_a 9.45) (14). In order to determine whether the difference in acidities between FUra and uracil affects the ability of FUrd-5'-PP to serve as a substrate for ribonucleotide reductase, the effect of pH on reduction of FUrd-5'-PP, UDP, and CDP was determined. The pH curves in Fig. 6 show that although there is a large difference in reaction velocities among the three substrates, the velocities of FUrd-5'-PP and UDP reduction are roughly proportional throughout the pH range examined, which suggests that the difference in acidity of the N-3 protons is not important with regard to ribonucleotide reduction.

Metabolism of FUra in extracts of tissue culture cells. In their work on the effects of fluorinated pyrimidines on various cell lines in culture, Umeda and Heidelberger (23) provided evidence that in some cells FUra was metabolized via a pathway that included ribonucleotide formation and subsequent reduction. They found that L5178Y-BF (BF) cells, which are resistant to FdUrd because of a deletion of thymidine

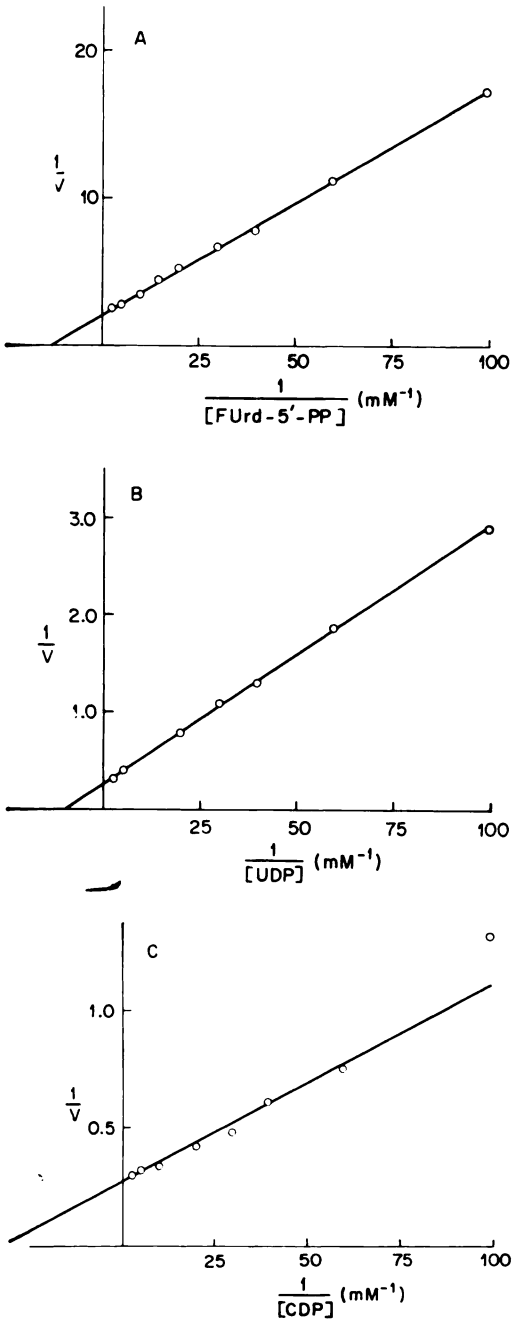

FIG. 5. *Kinetics of FUrd-5'-PP reduction*
The assay was performed as described under MATERIALS AND METHODS, except that the concentrations of FUrd-5'-PP, UDP, and CDP were varied as indicated. The incubation volume was 0.06 ml. Enzyme from the Sephadex G-200 fraction was used (0.75 unit/tube, 2.5 units/mg). Reciprocal velocity was plotted against reciprocal concentration according to Lineweaver and Burk (28). Michaelis constants were calculated from the slope and intercepts.

A. FUrd-5'-PP: K_m = 0.072 mM; V_{max} = 0.48 nmole/30 min. B. UDP: K_m = 0.095 mM; V_{max} = 3.7 nmoles/30 min. C. CDP: K_m = 0.030 mM; V_{max} = 3.7 nmoles/30 min.

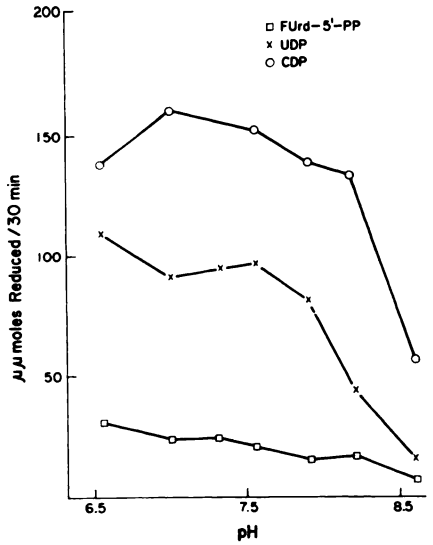

FIG. 6. *Effect of pH on reduction of FUrd-5'-PP*
The standard assay was employed, except that the buffer pH was varied as shown. The incubation volume was 0.06 ml. The Sephadex G-200 fraction was used (0.1 unit/tube, 1.5 units/mg).

kinase, were as sensitive to FUra as the parent L5178Y (Y) cells, which contained the kinase (23). Furthermore, the toxicity of FUra to Y cells was abolished by thymidine, whereas in the BF cells thymidine had no such effect. The conclusion was that in Y and BF cells FdUrd-5'-P is formed by reduction of a FUra ribonucleotide.

By contrast, cultured Novikoff hepatoma cells resistant to FdUrd, also because of a thymidine kinase deficiency (NSF cells), were partially cross-resistant to FUra (23), in confirmation of the observations of Morse and Potter (31). This observation suggests that in the parent hepatoma line (NS cells) FdUrd must be an intermediate when FUra is converted to FdUrd-5'-P. Alternatively, a deficiency of one of the enzymes of pathway B (Fig. 1), in addition to the thymidine kinase deletion, could also explain the cross-resistance of NSF cells to FUra.

In an attempt to distinguish between these possibilities, we incubated labeled FUra with the reductase assay mixture, the S3 fractions prepared from the various cell lines, and either deRib-1-P, Rib-1-P, or PP-ribose-P. In this way we could compare the activities of FdUrd phosphorylase, FUrd phosphorylase, FUra phosphoribosyltransferase, and possibly ribonucleotide reductase. In preliminary experiments we had verified that the resistant NSF and BF cells still lacked significant thymidine kinase activity. The results of the experiment are shown in Table 4.

We could not detect FdUrd-5′-P formation unless FUra and deRib-1-P were present together in the assay mixture. Therefore ribonucleotide reductase activity was not detectable by this method. We also could not detect significant amounts of FUrd or FdUrd on the chromatograms. The most significant finding is that FUra is less readily converted to ribonucleotides by FUra phosphoribosyltransferase in resistant NSF cells than in NS cells. In Y and BF cells the activities of the phosphoribosyltransferase are nearly the same. The decreased level of phosphoribosyltransferase in NSF cells explains why NSF cells are cross-resistant to FUra and is further evidence for the importance of FUrd-5′-PP reduction for FdUrd-5′-P formation.

Reductase levels in tissue culture cells. Because ribonucleotide reductase activity could not be detected in the previous experiment the S3 fractions of the various tissue culture cells were assayed directly for the enzyme (Table 5). Reductase levels for all cell lines are low in comparison to Ehrlich ascites cells. The cells resistant to FdUrd (NSF and BF) contain higher reductase activity than the

TABLE 4

FUra metabolism in extracts of cultured cells

Preparation of the enzyme is described under MATERIALS AND METHODS. [6-^{3}H]FUra at 0.1 mM and 500,000 dpm/tube was incubated for 60 min with the indicated pentose donor (1 mM) in the standard reductase assay mixture. There was 0.05 ml of enzyme in a total volume of 0.12 ml. The reaction was quenched with 0.01 ml of 65% trichloracetic acid, and the products were chromatographed in solvent 1. The compounds were located by ultraviolet visualization of unlabeled markers, and the radioactivity was determined by a strip scanner (Packard).

Addition	Fraction of total radioactivity	
	FdUrd-5′-P	FUra ribonucleotides
HeLa cells		
None		
deRib-1-P	0.34	
Rib-1-P		0.04
PP-ribose-P		0.20
NS cells		
None		0.13
deRib-1-P	0.17	0.23
Rib-1-P		0.45
PP-ribose-P		0.93
NSF cells		
None		
deRib-1-P		0.08
Rib-1-P		0.25
PP-ribose-P		0.20
Y cells		
None		0.05
deRib-1-P		0.08
Rib-1-P		0.09
PP-ribose-P		0.72
BF cells		
None		0.05
deRib-1-P		0.06
Rib-1-P		0.02
PP-ribose-P		0.89

TABLE 5

Ribonucleotide reductase activity in cultured cells

The preparation of the enzymes and assay procedure are described under MATERIALS AND METHODS. The assay was performed with 0.025 ml of enzyme in a total volume of 0.06 ml. The substrate was 0.1 mM FUrd-5′-PP. All assays were done in duplicate, and scintillation counting of the product was repeated several times. The assay time was 30 min.

Cells	FdUrd-5′-P formation	Concentration required for 0% growth[a]	
		FUra	FdUrd
	pmoles/30 min	*M*	*M*
HeLa	0.75	10^{-5}	5×10^{-7}
NS	2.1	5×10^{-4}	2×10^{-7}
NSF	8.1	5×10^{-3}	2×10^{-3}
Y	4.2	5×10^{-6}	5×10^{-10}
BF	7.8	10^{-7}	5×10^{-7}

[a] Data from Umeda and Heidelberger (23).

sensitive parent lines (NS and Y). This is not surprising, since the resistant cell lines have lost one or more of the salvage enzymes of nucleic acid biosynthesis. The inhibition of growth of these cells produced by FUra and FdUrd is also shown in Table 5.

DISCUSSION

We have shown that FUrd-5′-PP is reduced to FdUrd-5′-P by ribonucleotide reductase from Ehrlich ascites cells. This is the first direct demonstration of FdUrd-5′-P formation by reduction of FUra ribonucleotides, but there are instances in the literature which imply that FUrd-5′-PP reduction must occur. The work of Umeda and Heidelberger (23) provides good evidence that in Y and BF cells FUra is activated to FdUrd-5′-P through the reductase. Hiraga *et al.* (32) reported similar findings with a thymidine kinase-deficient mutant of *E. coli* and concluded that reduction of FUra ribonucleotides to FdUrd-5′-P probably takes place.

Kessel *et al.* (33) have found a correlation between response to FUra and the amount of FUra ribonucleotides formed in a series of mouse leukemias. Reyes and Hall (16) were further able to correlate the survival of FUra-treated leukemic mice with the level of the enzyme FUra phosphoribosyltransferase, which converts FUra to FUrd-5′-P. Kessel and Hall (34) succeeded in increasing FUra ribonucleotide formation by adding exogenous ribose donors, but found no potentiation of FUra antitumor action. They concluded that ribonucleotide formation was a major factor, but not the sole determinant, for response to FUra (34, 35).

The relative contributions of the two alternative pathways (Fig. 1) for FdUrd-5′-P formation in intact cells have not yet been determined. The phosphorylase responsible for FUra and FdUrd interconversion is known to be reversible (9), and FdUrd is readily phosphorylated by thymidine kinase (5, 9). Therefore, pathway A (Fig. 1) is feasible thermodynamically. Nevertheless, present evidence indicates that endogenous deoxyribosyl donors required for anabolism of FUra to FdUrd are in short supply within the cell, in both bacterial (36–38) and mammalian systems (39, 40). Alternatively, the endogenous level in Ehrlich ascites cells of the ribose donor, PP-ribose-P (41), was considered sufficient to support the synthesis of FUrd-5′-P from FUra (12). Also, it has been shown that FUra is converted primarily to ribonucleotides in intact Ehrlich ascites cells (11). On the basis of these studies and the work reported in this paper, we feel that the pathway for the formation of FdUrd-5′-P from FUra by reduction of FUrd-5′-PP is important physiologically, whereas the significance of the alternative pathway is unknown.

The question of alternative pathways for activation of FUra has obvious clinical significance. If in certain tumors FUra is activated primarily via ribonucleotides, followed by subsequent reduction to FdUrd-5′-P, one would predict that the combination of FUra and a reductase inhibitor such as hydroxyurea or thiosemicarbazones would be a poor choice for chemotherapy. On the other hand, the combination of FdUrd and a reductase inhibitor might be beneficial. Results presented in this paper show that reductase levels are elevated in cells resistant to FdUrd. Thus a reductase inhibitor may prove to be effective against tumors that have become resistant to FdUrd or FUra because of a deletion of one or more salvage enzymes.

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