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(*R*)-5-FLUORO-5,6-DIHYDROURACIL: KINETICS OF OXIDATION BY DIHYDROPYRIMIDINE DEHYDROGENASE AND HYDROLYSIS BY DIHYDROPYRIMIDINE AMINOHYDROLASE

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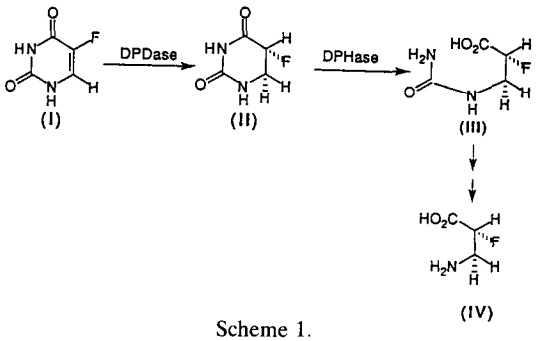
Abstract—The biologically active isomer of 5-fluoro-5,6-dihydrouracil [(*R*)-5-fluoro-5,6-dihydrouracil, *R*-FUH$_2$] was synthesized to study the kinetics of its enzymatic oxidation and hydrolysis by homogeneous dihydropyrimidine dehydrogenase (DPDase) and dihydropyrimidine aminohydrolase (DPHase), respectively. DPDase catalyzed the slow oxidation of *R*-FUH$_2$ at pH 8 and 37° with a K_m of 210 μM and a k_{cat} of 0.026 sec^{-1} at a saturating concentration of NADP$^+$. The catalytic efficiency (k_{cat}/K_m) of DPDase for *R*-FUH$_2$ was 1/14th of that for 5,6-dihydrouracil (UH$_2$). In the opposite direction, DPDase catalyzed the reduction of 5-fluorouracil (FU) with a K_m of 0.70 μM and a k_{cat} of 3 sec^{-1} at a saturating concentration of NADPH. Thus, DPDase catalyzed the reduction of FU 30,000-fold more efficiently than the oxidation of *R*-FUH$_2$. In contrast to the slow oxidation of *R*-FUH$_2$ by DPDase, *R*-FUH$_2$ was hydrolyzed very efficiently by DPHase with a K_m of 130 μM and a k_{cat} of 126 sec^{-1}. The catalytic efficiency of DPHase for the hydrolysis of *R*-FUH$_2$ was approximately twice that for the hydrolysis of UH$_2$. Because *R*-FUH$_2$ is hydrolyzed considerably more efficiently than it is oxidized and because the activity of DPHase was 250- to 500-fold greater than that of DPDase in bovine and rat liver, the hydrolytic pathway should predominate *in vivo*.

Key words: (*R*)-5-fluoro-5,6-dihydrouracil; 5-fluorouracil; dihydropyrimidine dehydrogenase; dihydropyrimidine aminohydrolase; hydrolysis; oxidation; synthesis

FU§, a widely used antineoplastic agent for the treatment of human solid tumors, is catabolized rapidly to α-fluoro-β-alanine [1–3]. DPDase (EC 1.3.1.2) catalyzes the initial step in the catabolic sequence (Scheme 1), which is the reversible reduction of FU (I) to (*R*)-FUH$_2$ (II) [4]. Subsequently, *R*-FUH$_2$ is hydrolyzed to α-fluoro-β-ureidopropionate (III) by DPHase (EC 3.5.2.2). Because *R*-FUH$_2$ has been reported to accumulate in rat hepatocytes [5] and in plasma of patients treated with FU [3], the contribution of *R*-FUH$_2$ to the efficacy and to the cytotoxicity of FU treatment is of interest. Racemic FUH$_2$ (*R,S*-FUH$_2$) is cytotoxic to Ehrlich ascites tumor cells and the human breast carcinoma cell line MCF-7 with an IC_{50} value approximately 4-fold greater than that for FU [6]. The cytotoxicity of *R,S*-FUH$_2$ was suggested to arise from FU generated by the DPDase-catalyzed oxidation of *R*-FUH$_2$ [6]. Since hydrolysis of *R*-FUH$_2$ by DPHase competes with its oxidation by DPDase, the kinetic parameters of *R*-FUH$_2$ with DPDase and DPHase are relevant for understanding *R*-FUH$_2$ cytotoxicity. *R,S*-FUH$_2$ has been synthesized chemically in low yields [7], but *R*-FUH$_2$ has not been resolved from this racemic mixture.



Scheme 1.

The synthesis of *R*-FUH$_2$ with *Escherichia coli* DPDase has been described, but isolation and characterization of the product have not been reported [8]. Consequently, we developed a preparative synthesis of *R*-FUH$_2$ that uses bovine liver DPDase to catalyze the stereospecific reduction of FU to *R*-FUH$_2$. The kinetic parameters for oxidation of *R*-FUH$_2$ by DPDase and the hydrolysis by DPHase are reported herein.

MATERIALS AND METHODS

Materials. Bovine liver and rat liver were from Pel-Freez (Rogers, AR). Glucose-6-phosphate, glucose-6-phosphate dehydrogenase, 5-FU, UH$_2$, uracil, NADPH and dithiothreitol were from the Sigma Chemical Co. (St. Louis, MO). Protein concentration was determined with the Protein Assay

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§ Abbreviations: FU, 5-fluorouracil; *R*-FUH$_2$, (*R*)-5-fluoro-5,6-dihydrouracil; *R,S*-FUH$_2$, (*R,S*)-5-fluoro-5,6-dihydrouracil; UH$_2$, 5,6-dihydrouracil; DPHase, dihydropyrimidine aminohydrolase; and DPDase, dihydropyrimidine dehydrogenase.

Kit from Bio-Rad (Richmond, CA), using bovine serum albumin as the standard protein.

Purification and assay of DPDase and DPHase. DPDase was purified from bovine liver as described previously [9]. The specific activity was 0.42 μmol/min/mg with thymine as substrate [9]. DPDase active site concentration was calculated from activity and a turnover number for uracil of 1.6 sec^{-1} [10]. DPHase was purified from bovine liver by a modification of a previous procedure in which a hydroxyapatite column was substituted for the phenyl Sepharose column [11–13]. The specific activity of the homogeneous enzyme with 0.25 mM UH_2 as substrate was 5.8 μmol/min/mg at pH 8 and 37°. DPDase concentration was calculated from protein concentration and a subunit relative molecular weight of 54,000.

The activity of DPHase in rat and bovine liver was determined in homogenates that were prepared by homogenizing 5–10 g of tissue in 25 mL of 0.05 M Tris–HCl at pH 8.0 for 90 sec in a Waring blender for 3 min. Supernatants obtained by centrifugation of the homogenate for 1 hr at 40,000 *g* were assayed spectrophotometrically for hydrolysis of 500 μM *R*-FUH_2 ($\Delta\varepsilon_{230} = 1.12\ mM^{-1}\ cm^{-1}$) in 0.1 M potassium phosphate at pH 8 and 37°.

The kinetic parameters for reduction of $NADP^+$ to NADPH by *R*-FUH_2 and DPDase in 0.05 M Tris–HCl at pH 8.0 and 37° were calculated from the initial velocity of NADPH formation that was monitored by the fluorescence increase at 450 nm with an excitation wavelength of 340 nm. The kinetic parameters for reduction of FU ($\Delta\varepsilon_{266} = 5.75\ mM^{-1}\ cm^{-1}$) by DPDase were determined from the time–course of the reaction in the presence of an NADPH-regenerating system (2 mM glucose-6-phosphate and 5 U/mL glucose-6-phosphate dehydrogenase). Hydrolysis of *R*-FUH_2 and UH_2 by DPHase in 0.1 M potassium phosphate at pH 8.0 and 37° was monitored spectrophotometrically with $\Delta\varepsilon_{230} = 1.12\ mM^{-1}\ cm^{-1}$ and $\Delta A_{225} = 1.29\ mM^{-1}\ cm^{-1}$, respectively. Initial velocity or the complete time–course of the reaction data was analyzed by the Michaelis–Menten equation or the integrated rate equation [10]. Absorbance data were collected with a Kontron 860 spectrophotometer, and fluorescence data were collected with a Kontron SFM-25 spectrofluorometer.

Synthesis of R-*FUH*$_2$. Initially we attempted to synthesize *R,S*-FUH_2 chemically. Thus, a suspension of FU (2.00 g, 15.4 mmol) and palladium(II) chloride (0.62 g, 3.5 mmol) in 1:1 ethanol:H_2O (200 mL) in a Parr bottle was degassed for 15 min and then pressurized with hydrogen gas (35 psi). The bottle was agitated for 20 hr, degassed, and filtered through a bed of Celite. The bed was washed with 1:1 ethanol:water (50 mL), and the combined filtrates were concentrated *in vacuo* to an off-white solid that was washed with acetone (20 mL) to give a white powder (1.94 g, 95%). A proton NMR spectrum indicated two compounds present in a 88:12 ratio with the major component being identified as *R,S*-FUH_2. Attempts at isolating pure *R,S*-FUH_2 from the mixture by recrystallization or chromatographic techniques proved unsuccessful. Furthermore, synthesis of a racemic product would necessitate a chiral resolution to obtain *R*-FUH_2. These obstacles prompted us to abandon a chemical synthesis in favor of an enzymatic synthesis.

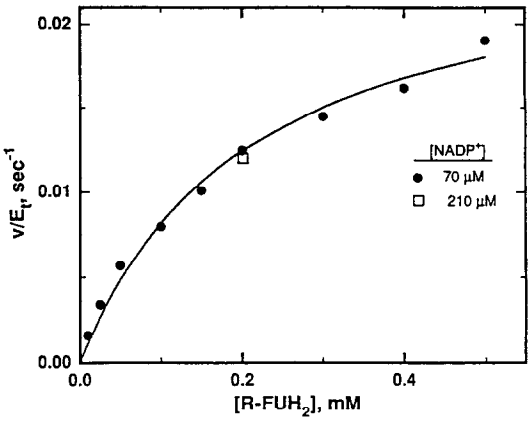

Fig. 1. Oxidation of *R*-FUH_2 by DPDase. Initial velocity data were determined for the reduction of 70 and 210 μM $NADP^+$ by 0.16 μM DPDase as a function of *R*-FUH_2 concentration. The solid line was calculated from the Michaelis–Menten equation with $k_{cat} = 0.026\ sec^{-1}$ and $K_m = 210$ μM.

Enzymatic reduction of FU was initiated with 21 U (1 μmol/min/U) of bovine DPDase in 1 L of 50 mM NH_4HCO_3 at pH 8.0 that contained 10 mmol glucose-6-phosphate (Na^+), 5 μmol NADPH, 2000 U glucose-6-phosphate dehydrogenase, 4 mmol FU and 5 mmol dithiothreitol. After 270 min at 37°, the reduction of FU was complete as judged by A_{260}. The reaction was terminated by freezing, and the solid was lyophilized for 36 hr. The resulting powder was extracted twice with 100 mL of boiling 95% ethanol. The volume of the extract was reduced by rotary evaporation at 40° to 20 mL, which resulted in product precipitation. The product was redissolved by heating and the solution was filtered. The product crystallized as a white powder to yield 278 mg (55% yield) that was recrystallized from 90% ethanol. ^1H NMR (300 MHz, DMSO-d_6): δ 10.4 (s, 1H), 7.70 (s, 1H), 5.14 (ddd, J = 49, 8.1, 5.4 Hz, 1H), 3.54 (m, 1H), 3.38 (m, 1H). MS (CI) for $C_4H_5N_2O_2F$, m/z (relative intensity):133 (100), 113 (8.9). Analysis calculated (found) for $C_4H_5N_2O_2F$: C, 36.36 (36.28); H, 3.82 (3.82); N, 21.21 (21.14). Optical rotation: $[\alpha]_{489}^{20} = +24.5°$(c 1, H_2O).

Data analysis. The Michaelis–Menten equation was fitted to initial velocity data by a nonlinear least squares fitting routine to yield estimates for K_m and V_{max} values. The integrated rate equation was fitted to the complete time–course for substrate consumption to yield estimates for K_m and V_{max} values. The time–course predicted from these estimates for K_m and V_{max} was calculated by numerical integration of equation 1 [10].

$$\frac{d[S]}{dt} = -\frac{V_{max}[S]}{[S] + K_m} \quad (1)$$

Equation 2 was fitted to the time–course for absorbance changes at 225 nm ($A(t)$) resulting from the hydrolysis of *R*-FUH_2. The k_{obs} is the observed first-order rate constant for hydrolysis, A_0 is the initial absorbance, and A_∞ is the absorbance at the

Table 1. Steady-state kinetic parameters for DPDase and DPHase

	DPDase			DPHase		
	k_{cat} (sec^{-1})	K_m (μM)	k_{cat}/K_m (μM^{-1} sec^{-1})	k_{cat} (sec^{-1})	K_m (μM)	k_{cat}/K_m ($\mu M^{-1} sec^{-1}$)
R-FUH_2	0.026 ± 0.002	210 ± 30	1.2×10^{-4}	126 ± 4	130 ± 10	1.0
FU	3.0 ± 0.1	0.70 ± 0.05	4.3	NA*	NA	NA
UH_2	0.4 ± 0.02†	240 ± 40†	1.7×10^{-3}	4.3 ± 0.1	9 ± 1	0.47
Uracil	1.6 ± 0.1†	0.8 ± 0.1†	2.0	NA	NA	NA

Values are means ± SE, except for FU where values are means ± range, N = 2.
* Not applicable.
† Data are from Ref. 10.

end of the reaction.

$$A(t) = A_\infty + (A_0 - A_\infty)\exp(-k_{obs} \cdot t) \quad (2)$$

Equation 3 was fitted to titration data

$$A([H^+]) = A_0 + \frac{A_f[H^+]}{K_a + [H^+]} \quad (3)$$

where A_f is the absorbance of the solution at pH values much greater than the pK_a and A_0 is the absorbance of the solution at pH values much less than the pK_a. Nonlinear fitting routines were performed with SigmaPlot (Corte Madera, CA). Parameters are reported either as the value ± SE, or as the mean ± the range when the indicated number of determinations (N) is two and the value ± SD when N > 2. The latter two parameters are identified by N values in parentheses.

RESULTS

Properties of R-*FUH*$_2$. *R*-FUH_2 was synthesized on a preparative scale by stereospecific reduction of FU by bovine liver DPDase. The product of reduction of FU by bovine liver DPDase is *R*-FUH_2 [6]. *R*-FUH_2 was isolated in 50% yield and was readily crystallized from 90% ethanol-10% H_2O. The product had a specific rotation $[\alpha]_{489}^{20} = +24.5°$ (c 1, H_2O). The pK_a for ionization of *R*-FUH_2 was determined by spectrophotometic titration (λ = 225 nm) to be 10.2 ± 0.2.

R-FUH_2 was readily hydrolyzed nonenzymatically to α-fluoro-β-ureidopropionic acid, which was confirmed by ^1H NMR. The first-order rate constant for hydrolysis (ΔA_{225}) was linearly dependent on the hydroxide concentration with a proportionality constant of 0.96 ± 0.04 and was not dependent on buffer concentration. The value of the first-order rate constant at pH 8 was 6×10^{-5} sec^{-1}, which corresponded to a half-life of 3.2 hr. The first-order rate constant for hydrolysis of 5,6-dihydrouracil increased linearly with hydroxide ion concentration with a proportionality constant of 1.14 ± 0.02, which was similar to that found in a detailed study of the base-catalyzed hydrolysis of UH_2 [14, 15]. The rate constant for hydrolysis of *R*-FUH_2 at pH 8 was 250-fold greater than that of UH_2.

Oxidation of R-*FUH*$_2$ *by DPDase.* DPDase catalyzes the reversible oxidation of dihydropyrimidines to pyrimidines [16]. The oxidation of *R*-FUH_2 was monitored by the fluorescence increase resulting from the reduction of $NADP^+$ to NADPH. Initial velocity data for the oxidation of *R*-FUH_2 by DPDase and 70 μM $NADP^+$ were analyzed by the Michaelis–Menten equation to give a K_m for *R*-FUH_2 of 210 ± 30 μM and a k_{cat} of 0.026 ± 0.002 sec^{-1} (Fig. 1). Because the initial velocities for reduction of 70 and 210 μM $NADP^+$ by 200 μM *R*-FUH_2 were similar, the K_m of DPDase for $NADP^+$ was significantly less than 70 μM. The K_m and k_{cat} values for oxidation of UH_2 by DPDase under similar conditions are 240 ± 40 μM and 0.40 ± 0.02 sec^{-1}, respectively [10]. Thus, the catalytic efficiency of DPDase (k_{cat}/K_m) for oxidation of *R*-FUH_2 was 1.2×10^{-4} $\mu M^{-1}sec^{-1}$, which is $1/14^{th}$ that for oxidation of UH_2 by DPHase (Table 1).

Reduction of FU by DPDase. The K_m of DPDase for pyrimidines in the reductive reaction is sufficiently small that determination of initial velocities by monitoring the absorbance decrease due to NADPH oxidation may be inaccurate. To circumvent this problem, the complete time–course for reduction of FU was monitored by the absorbance decrease associated with FU reduction in the presence of an NADPH-regenerating system. These data were analyzed by the integrated rate equation as described for uracil [10]. The kinetic parameters for the reduction of 5 μM FU by 10 μM NADPH were a K_m of 0.70 ± 0.05 μM (N = 2) and a k_{cat} of 3.0 ± 0.1 sec^{-1} (N = 2), which are comparable to the values of 1.8 μM and 1.9 sec^{-1} reported for pH 7.4 [17]. The catalytic efficiency of DPDase for reduction of FU was 4.3 μM^{-1} sec^{-1}, which was twice that for reduction of uracil (Table 1).

Hydrolysis of R-*FUH*$_2$ *by DPHase.* The kinetic parameters for the hydrolysis of *R*-FUH_2 by homogeneous bovine liver DPHase were determined from the complete time–course of the reaction. The integrated rate equation was fitted to these data to give a k_{cat} of 106 ± 8 sec^{-1} and a K_m of 98 ± 4 μM (Fig. 2). Initial velocity data for the enzymatic hydrolysis of *R*-FUH_2 (10 concentrations of *R*-FUH_2) gave similar values with a k_{cat} of 126 ± 4 sec^{-1} and a K_m of 130 ± 10 μM (Table 1). The integrated rate equation was fitted to the complete time–course for hydrolysis of UH_2 (ΔA_{225} = 1.29 $mM^{-1}cm^{-1}$ at pH 8.0) to give a k_{cat} of 4.3 ± 0.1 sec^{-1} and a K_m of 9 ± 1 μM (Fig. 2). This K_m value was similar to previously reported values for UH_2 [11–13]. The catalytic efficiency of DPHase for hydrolysis of *R*-FUH_2 was approximately twice that for hydrolysis of UH_2 (Table 1).

Activity of DPHase in liver homogenates. The

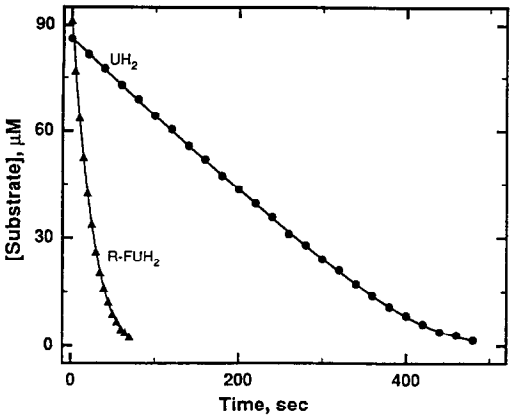

Fig. 2. Time–courses for enzymatic hydrolysis of R-FUH_2 and UH_2. The time–courses for the hydrolysis of 86 μM UH_2 and 91 μM R-FUH_2 were monitored at 225 nm in 0.1 M potassium phosphate at pH 8.0 and 37°. The reaction was initiated with 3.1 μg/mL of bovine liver DPHase. The solid lines were calculated by numerical integration of equation 1 with $V_{max} = 6\ \mu M\ sec^{-1}$ and $K_m = 98\ \mu M$ for R-FUH_2, and $V_{max} = 0.24\ \mu M\ sec^{-1}$ and $K_m = 9\ \mu M$ for UH_2.

activity of DPHase in liver homogenates was sufficient to determine the kinetic parameters for hydrolysis of R-FUH_2 and UH_2 by spectrophotometric assay. The K_m values of DPHase in bovine and rat liver homogenates for R-FUH_2 were 140 ± 20 μM (N = 2) and 110 ± 20 μM, respectively. The K_m for DPHase in bovine liver homogenate was similar to that for homogeneous enzyme (126 μM). Since the R-FUH_2 hydrolase activity in bovine liver homogenate could be accounted for by the activity of homogeneous DPHase purified from liver homogenates, R-FUH_2 hydrolase activity in liver homogenates under these assay conditions was due to DPHase and not to the high K_m imidase from rat liver [15]. Bovine and rat liver homogenates hydrolyzed 500 μM R-FUH_2 with an initial velocity of 50 ± 6 (N = 3) and 17 ± 2 (N = 3) μmol/min/g of tissue, respectively.

DISCUSSION

R-FUH_2 is the first intermediate in the catabolism of the antineoplastic agent FU. By analogy with UH_2, DPDase catalyzed the reversible oxidation of R-FUH_2 to FU and DPHase catalyzed the hydrolysis of R-FUH_2 to α-fluoro-β-ureidopropionic acid. However, the kinetic parameters for the oxidation and hydrolysis of R-FUH_2 by DPDase and DPHase, respectively, have not been determined previously. We report herein the preparative synthesis and isolation of the biologically active isomer, R-FUH_2, in sufficient quantities (>200 mg) to determine the kinetic parameters for R-FUH_2 oxidation by DPDase and hydrolysis by DPHase.

DPDase catalyzed the slow oxidation of R-FUH_2 by $NADP^+$ with a catalytic efficiency of $1.2 \times 10^{-4}\ \mu M^{-1}\ sec^{-1}$ at a saturating concentration of $NADP^+$ (Table 1). The reduction of FU by DPDase had a catalytic efficiency of $4.3\ \mu M^{-1}\ sec^{-1}$ at a saturating concentration of NADPH. Thus, DPDase catalyzed the reduction of FU approximately 30,000-fold more efficiently than the oxidation of R-FUH_2 under optimal conditions. If the cellular ratio of $NADP^+$ to NADPH is not extremely large, the *in vivo* oxidation of R-FUH_2 to FU by DPDase is predicted to be very inefficient.

In contrast to the slow rate of oxidation of R-FUH_2 by DPDase, DPHase catalyzed the rapid rate of hydrolysis of R-FUH_2. The catalytic efficiency of DPHase with R-FUH_2 was $1\ \mu M^{-1}\ sec^{-1}$, which was 2-fold larger than that for UH_2. R-FUH_2 was also slowly hydrolyzed nonenzymatically with a $T_{1/2}$ = 3.2 hr at pH 8. The rate of nonenzymatic hydrolysis of R-FUH_2 was approximately 250-fold greater than that for UH_2. The 250-fold increased lability of R-FUH_2 relative to UH_2 to nonenzymatic hydrolysis was reflected in only a 25-fold increase in k_{cat} and a 2-fold increase in substrate efficiency of R-FUH_2 over UH_2 in the DPHase reaction.

Our kinetic parameters for purified DPDase and DPHase and the relative activity of these enzymes in liver homogenates can be used to estimate the steady-state concentration of R-FUH_2 during the catabolism of FU in liver homogenates. The maximal velocity for reduction of FU in bovine liver homogenates was estimated from the data of Lu *et al.* [17] to be 0.03 μmol/min/g of tissue. The K_m and V_{max} of DPHase for R-FUH_2 were 140 μM and 50 μmol/min/g of tissue, respectively. Thus, the maximal steady-state concentration of R-FUH_2 during the catabolism of FU by bovine liver homogenates is calculated to be less than 0.1 μM (equation 5 in (*) footnote below). The maximal velocity of reduction of FU by rat liver homogenates was estimated from the data of Shiotani and Weber [16] to be 0.07 μmol/min/g of tissue. The K_m and V_{max} for DPHase in rat liver homogenates were 110 μM and 17 μmol/min/g of tissue, respectively. Consequently, the maximal steady-state concentration of R-FUH_2 during the catabolism of R-FUH_2 by rat liver homogenates is calculated to be 0.45 μM (equation 5 in (*) footnote below). This

* Because the rate of oxidation of R-FUH_2 by DPDase and the rate of nonenzymatic hydrolysis of R-FUH_2 are slow, the steady-state concentration of R-FUH_2 during the catabolism of FU is determined by the rate of reduction of FU by DPDase and the rate of hydrolysis of R-FUH_2 by DPHase (Scheme I). The steady-state velocities of FU reduction by DPDase (V_{DPDase}) and of R-FUH_2 hydrolysis by DPHase (V_{DPHase}) are given by equations 4a and 4b, respectively, where V_{max1} is the maximal velocity of DPDase and V_{max2} is the maximal velocity of DPHase.

$$V_{DPDase} = \frac{V_{max1}[FU]}{[FU] + K_{m1}} \quad (4a)$$

$$V_{DPHase} = \frac{V_{max2}[R\text{-}FUH_2]}{[R\text{-}FUH_2] + K_{m2}}. \quad (4b)$$

If V_{max2} is greater than V_{max1}, R-FUH_2 will reach a steady-state concentration such that V_{DPDase} (equation 4a) equals V_{DPHase} (equation 4b). The maximal steady-state concentration of R-FUH_2 ($[R\text{-}FUH_2]_{ss}$), which occurs where FU concentration is much greater than its K_m for DPDase, is given by equation 5.

$$[R\text{-}FUH_2]_{ss} = \frac{V_{max1}K_{m2}}{V_{max2} - V_{max1}}. \quad (5)$$

estimate for the steady-state concentration of *R*-FUH_2 is in agreement with the finding of Naguib *et al.* [18] that *R*-FUH_2 does not accumulate during the catabolism of FU by rat liver homogenates. However, after treatment of rat hepatocytes with 30 μM FU for 10 min the intracellular concentration of *R*-FUH_2 was reported to approach 800 μM [5], which is 2000-fold higher than the value predicted from the *in vitro* kinetic parameters for DPHase and DPDase in liver homogenates. Some possible explanations for this discrepancy include: (1) the specific activity of DPDase determined for liver homogenates underestimates the specific activity of DPDase in hepatocytes, (2) the specific activity of DPHase determined for liver homogenates overestimates the activity of DPHase in hepatocytes, and (3) a large fraction of the *R*-FUH_2 is sequestered in hepatocytes in a form that is not available for hydrolysis by DPHase. Further studies are required to evaluate these possibilities. The observation that detectable amounts of *R*-FUH_2 are found in the plasma of patients treated with FU [3] is consistent with the observation that extrahepatic tissue, in general, has very low levels of DPHase and significant levels of DPDase [18].

In summary, we have developed an efficient method to synthesize and purify significant amounts (>200 mg) of *R*-FUH_2 and have shown that DPDase catalyzed the reduction of FU approximately 30,000-fold more efficiently than the oxidation of *R*-FUH_2 under optimal conditions. Furthermore, DPHase catalyzed the hydrolysis of *R*-FUH_2 with a catalytic efficiency 2-fold larger than for UH_2. Based on the activities of DPDase and DPHase in liver homogenates and the kinetic parameters for these enzymes, the maximal steady-state concentration of *R*-FUH_2 in liver tissue was estimated to be less than 0.5 μM. If the kinetic parameters of DPDase for oxidation of *R*-FUH_2 are applicable to the *in vivo* environment, significant oxidation of *R*-FUH_2 by DPDase is unlikely.

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