ELSEVIER

Journal of Chromatography B, 729 (1999) 307–314

JOURNAL OF CHROMATOGRAPHY B

Radiochemical assay for determination of dihydropyrimidinase activity using reversed-phase high-performance liquid chromatography

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Received 11 January 1999; received in revised form 26 March 1999; accepted 13 April 1999

Abstract

A radiochemical assay was developed to measure the activity of dihydropyrimidinase (DHP) in human liver homogenates. The method is based on the separation of radiolabeled dihydrouracil from *N*-carbamyl-β-alanine by HPLC with on-line detection of radioactivity combined with detection of $^{14}CO_2$ by liquid scintillation counting. The assay was linear with time and protein concentration. The minimum amount of radiolabeled products which could be determined proved to be 12 pmol using a purified stock solution of [2-^{14}C]-5,6-dihydrouracil. This highly sensitive assay is especially suitable to identify patients with a dihydropyrimidinase deficiency. © 1999 Elsevier Science B.V. All rights reserved.

Keywords: Enzymes; Dihydropyrimidinase

1. Introduction

In man, the catabolism of uracil and thymine consists of three consecutive steps and is primarily confined to the liver. Dihydropyrimidine dehydrogenase (DPD, EC 1.3.1.2) catalyzes the reduction of uracil and thymine to 5,6-dihydrouracil and 5,6-dihydrothymine, respectively. The second step is catalyzed by dihydropyrimidinase (DHP, EC 3.5.2.2) and consists of a reversible hydrolysis of dihydrouracil and dihydrothymine to *N*-carbamyl-β-alanine and *N*-carbamyl-β-aminoisobutyric acid, respectively. Finally, *N*-carbamyl-β-alanine and *N*-carbamyl-β-aminoisobutyric acid are converted to β-alanine and β-aminoisobutyric acid, ammonia and CO_2 by β-ureidopropionase (UP, EC 3.5.1.6).

Defects in the degradation of pyrimidines are often associated with convulsive disorders during childhood whereas defects of DPD and expectantly DHP can lead also to severe life-threatening toxicities when (partially) deficient individuals are treated with the pyrimidine analogue 5-fluorouracil (5FU) [1–5]. In patients with a DPD deficiency a large phenotypic variability was observed with convulsive disorders, motor retardation and mental retardation being the most abundant manifestations [6]. However, a clear correlation between the genotype and phenotype

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0378-4347/99/$ – see front matter
PII: S0378-4347(99)00173-5

could not be established [6]. So far, nine individuals have been reported with a deficiency of DHP with variable clinical symptoms including seizures and psychomotor retardation [7,8]. The recent identification of the human gene encoding DHP has allowed the detection of the defects at the molecular level [8].

DHP has been purified and characterized from calf liver [9], pig liver [10] and from rat liver [11]. The enzyme proved to be a homotetramer with four tightly bound zinc ions [9,11]. Besides the hydrolysis of 5,6-dihydropyrimidines, DHP is also capable of hydrolyzing a variety of other compounds such as hydantoins [12,13] and succinimides [12,13]. In order to measure the activity of DHP a radiochemical assay has been described which is based on the separation of dihydrouracil from its catabolites by thin-layer chromatography. Subsequently, extensive procedures were necessary to visualize the spots followed by quantification of the various compounds by liquid scintillation counting [14]. To date, the most frequently used assays to measure the activity of DHP are those in which the hydrolysis of the dihydropyrimidine ring is followed spectrophotometrically by a decrease in absorbance at low wavelengths (i.e. 225 nm for dihydrouracil and dihydrothymine) [9–11]. These spectrophotometric methods are very insensitive and therefore not suitable to measure the DHP activity in crude tissue homogenates, thus hampering the diagnosis of patients with a DHP deficiency at the enzyme level. Therefore, we have developed a very sensitive radiochemical assay which is based on the separation of radiolabeled dihydrouracil from *N*-carbamyl-β-alanine by HPLC with on-line detection of radioactivity combined with detection of $^{14}CO_2$ by liquid scintillation counting.

2. Materials and methods

2.1. Chemicals

All chemicals were of analytical grade. [2-^{14}C]-5,6-dihydrouracil (1.85–2.22 GBq/mmol) and [4-^{14}C]-thymine (45–60 mCi/mmol) were obtained from Moravek Biochemicals (CA, USA). Dithiothreitol (DTT), NADPH and Complete™ mini EDTA free tablets (protease inhibitor cocktail) were obtained from Boehringer Mannheim GmbH Biochemica (Mannheim, Germany). NaH_2PO_4, KH_2PO_4, $MgCl_2$, NaOH, $HClO_4$, CH_3CN, Tris and HCl were obtained from Merck (Darmstadt, Germany). *N*-carbamyl-β-alanine was obtained from Sigma Chemicals Co. (St. Louis, MO, USA).

2.2. Preparation of tissue homogenates

Homogenates (20%, w/v) of frozen human livers were prepared in a buffer containing 35 m*M* potassium phosphate, 2.5 m*M* $MgCl_2$ (pH 7.4), 1 m*M* dithiothreitol and Complete™ (1 tablet/10 ml) with the aid of a Teflon glass homogenizer. All homogenates were sonicated three times at 4 Watt (Vibracell Sonificator, output control 20%) for 10 s with intervals of 30 s under constant cooling in ice-water. After centrifugation (11 000 *g* at 4°C for 20 min.) the supernatants were removed and stored in liquid nitrogen until further analysis. Protein concentrations in the supernatants were determined by the copper-reduction method using bicinchoninic acid, essentially as described by Smith et al. [15].

2.3. Determination of the activity of DHP

The activity of DHP was determined in a standard assay mixture containing 100 m*M* Tris–HCl (pH 8.0), 1 m*M* dithiothreitol and 500 μ*M* [2-^{14}C]dihydrouracil. The tube containing the assay mixture was placed in a scintillation vial which contained also an Eppendorf microtube with 1 ml of 2 *M* NaOH. The scintillation vial, sealed with a rubber septum, and the supernatant were equilibrated separately at 37°C in a stirring waterbath for 2 min. The reaction was started by the injection of an amount of supernatant corresponding to 10–500 μg of protein into the reaction tube (total volume 100 μl). After an appropriate time of incubation (0.25–3 h) the reaction catalyzed by DHP was terminated by injecting 25 μl of 10% (v/v) perchloric acid through the septum into the reaction tube. After termination of the reaction, the scintillation vial was stored at 4°C for 2 h to allow the NaOH solution to trap the $^{14}CO_2$. Afterwards, the tube containing the reaction mixture was removed from the scintillation vial. The

tube containing the NaOH solution was mixed vigorously with 15 ml of scintillation liquid and the radioactivity was quantitated by scintillation counting. The reaction mixture was centrifuged in a microfuge (11 000 *g*, for 5 min) to remove the protein. An aliquot of supernatant (5 μl) was mixed with 4 ml of scintillation liquid and the radioactivity was quantitated by scintillation counting. The remaining supernatant was stored at −20°C and saved for further analysis by HPLC. A blank value was always obtained by using distilled water instead of a sample.

2.4. HPLC analysis

The separation of radiolabeled dihydrouracil and the reaction product *N*-carbamyl-β-alanine was accomplished by HPLC after injecting 70 μl of a supernatant into the HPLC system. The HPLC system consisted of two Waters 510 HPLC pumps, including a Waters 680 automated gradient controller (Waters Associates Inc. Milford, MA) and a Gilson 231 XL sample injector with a Gilson 401 diluter (Gilson Medical Electronics S.A., Villiers Le Bel, France). The radioactivity was detected on-line with a Radiomatic 525 TR Flow Scintillation Analyser with a 500 μl TR-LSC cell (Packard Intrument, Meriden, CT, USA). A scintillation fluid (Ultima Flo AP, Packard, Dowers Grove, IL, USA) was used at an effluent to scintillation fluid ratio of 1:1.

HPLC was performed on a reversed-phase column (Supelcosil LC-18-S, 5 μm particle size, 250×4.6 mm, Supelco Inc., Bellefonte, PA, USA) and a guard column (Supelguard LC-18-S, 5 μm particle size, 20×4.6 mm, Supelco, Bellefonte, PA, USA). The solvents used for chromatography consisted of 50 m*M* NaH_2PO_4 (pH 4.5) (solvent A) and 50 m*M* NaH_2PO_4 (pH 4.5)–CH_3CN, (1:1) (solvent B). The elution of dihydrouracil and *N*-carbamyl-β-alanine was performed isocratically with 100% solvent A, at a flow rate of 1 ml/min. Solvent B, at a final concentration of 50%, was used for the removal of more hydrophobic impurities that were already present in the [^{14}C]-dihydrouracil-stock solution. The integration of the data was performed with a FLO-ONE integration package (Packard Instrument, Meriden, CT, USA).

2.5. Identification of the N-carbamyl-β-alanine peak

The identification of the peak representing *N*-carbamyl-β-alanine was performed by comparison of the retention time with that of authentic unlabeled *N*-carbamyl-β-alanine as well as with that of radiolabeled *N*-carbamyl-β-alanine. Radiolabeled *N*-carbamyl-β-alanine was prepared by alkaline hydrolysis of [2-^{14}C]-5,6-dihydrouracil with 0.1 *M* NaOH at 37°C for 15 min. Subsequently, the solution containing more than 98% radiolabeld *N*-carbamyl-β-alanine was neutralized by addition of 0.1 *M* HCL. The retention times of the pure standards completely matched the *N*-carbamyl-β-alanine peak produced by dihydropyrimidinase in a human liver homogenate.

2.6. Calculation of the DHP activity

The activity of DHP was calculated according to the following equation:

$$\text{DHP activity (nmol/mg/h)} = [(CPM_s - CPM_{blc}) \times \text{Eff.} \times (\text{inc.vol./inj.vol.}) + (LSC_{CO_2} - LSC_{CO_2blc})]/\text{s.a./mg/t}$$

inc. vol. = volume assay mixture (100 μl) + volume perchloric acid (25 μl)
inj. vol. = injection volume of the HPLC (usually 70 μl)
CPM_s = counts per minute of the *N*-carbamyl-β-alanine peak produced by DHP in the sample
CPM_{blc} = counts per minute of the *N*-carbamyl-β-alanine peak present in a blanc
Eff. = counting efficiency of the Radiomatic = $(LSC_m - LSC_{blc})/CPM_{tot} \times$ inj. vol/counting vol.
counting vol. = volume of perchloric acid extract analyzed by liquid scintillation counting (usually 5 or 10 μL)
LSC_m = liquid scintillation counting of a perchloric acid extract of a sample
LSC_{blc} = liquid scintillation counting of a blanc (±10 dpm)
CPM_{tot} = total counts per minute in the perchloric acid extract determined by the Radiomatic

LSC_{CO_2} = liquid scintillation counting of the CO_2 trap (2 *M* NaOH solution) of sample
LSC_{CO_2blc} = liquid scintillation counting of the CO_2 trap (2 *M* NaOH solution) of a blanc
s.a. = specific activity of [2-^{14}C]-5,6-dihydrouracil (dpm/nmol)
mg = amount of protein in the reaction mixture (mg)
t = reaction time (h).

2.7. Assay validation

The intra-assay (within-run) variation was assessed by determination of the DHP activity in five replicates of a human liver homogenate on the same day. The inter-assay (between-day) variation was determined by analyzing a human liver sample on five different days. The reproducibility of the assay is expressed as the coefficient of variation (CV):

$$\text{CV (\%)} = \frac{\text{(standard deviation)}}{\text{mean}} \times 100$$

2.8. Kinetic properties and pH optimum of DHP

The steady-state kinetics of human liver DHP were performed using the cytosolic fraction obtained after centrifugation of a human liver homogenate. The initial reaction rates were determined in 100 m*M* Tris–HCl (pH 8.0) at 37°C at various concentrations of [2-^{14}C]-dihydrouracil (3–500 μ*M*). Calculation of apparent K_m and V_{max} values was performed by fitting the data to the Michaelis-Menten equation and by analysis according to the direct-linear plot method [16].

The determination of the pH optimum of the activity of human liver DHP was studied using assay mixtures that contained either 0.2 *M* Tris–HCl (pH 6.6–9.0) or 0.2 *M* Glycine–HCl (pH 8.6–9.6). An amount of supernatant corresponding to 45 μg protein was used to determine the activity of DHP and the reaction was allowed to proceed for 2h. Blank values were also obtained for each data point.

2.9. Determination of the activity of DPD

The activity of DPD was determined in a reaction mixture containing 35 m*M* potassium phosphate (pH 7.4), 2.5 m*M* $MgCl_2$, 1 m*M* dithiothreitol, 2.5 m*M* NADPH and 50 μ*M* [4-^{14}C]-thymine, essentially as described before [17].

3. Results

3.1. HPLC procedure

Analysis of a particular stock solution of [2-^{14}C]-5,6-dihydrouracil by HPLC showed that the radiolabeled dihydrouracil was essentially pure (>97%) and that no significant amount of radiolabeled *N*-carbamyl-β-alanine was present. A complete baseline separation was achieved within 7 min for *N*-carbamyl-β-alanine and dihydrouracil with retention times of 4.2 and 5.4 min, respectively (Fig. 1B). The detection limit, defined as the baseline noise plus three times the standard deviation, of radiolabeled *N*-carbamyl-β-alanine by HPLC combined with quantification of $^{14}CO_2$ by liquid scintillation counting was approximately 60 pmol. The small amount of impurities present in the stock solution of radiolabeled dihydrouracil eluted partly in the dead volume of the separation and after cleaning the column with 25% acetonitrile, which was routinely performed after each run. During the time of incubation of a blanc (i.e. radiolabeled dihydrouracil in the absence of a sample) a slight increase of radioactivity was detected in the $^{14}CO_2$ trap solution, whereas this was not observed after purification of the stock solution of radiolabeled dihydrouracil by HPLC. In that case, the detection limit of the entire procedure could be lowered to approximately 12 pmol.

3.2. Reaction conditions

Maximal DHP activity was observed at pH 8.8 (Fig. 2A). However, the analysis of the stability of radiolabeled dihydrouracil at various pH values showed that above pH 8.2 an increased hydrolysis of dihydrouracil towards *N*-carbamyl-β-alanine occurred (Fig. 2B). At pH 9.6 the blanc value contributed approximately 10% to the DHP specific activity as determined in a human liver preparation. No significant hydrolysis of dihydrouracil was observed between pH 6.6 and 8.2. Therefore, DHP activity was routinely performed at pH 8.0.

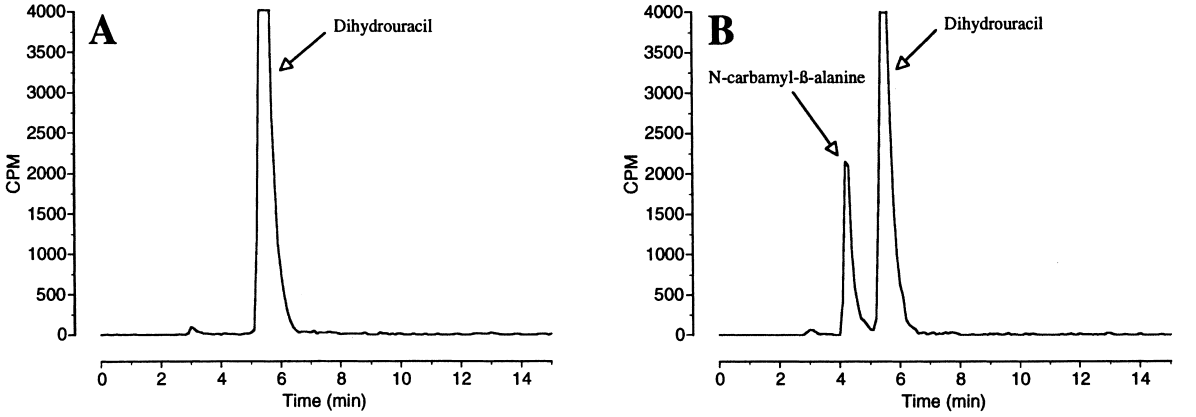

Fig. 1. HPLC elution profile of radiolabeled dihydrouracil and its catabolite *N*-carbamyl-β-alanine. Panel A shows the elution profile of [2-^{14}C]-dihydrouracil stock solution (500 μ*M*). Panel B shows the elution profile of [2-^{14}C]-dihydrouracil and the reaction product *N*-carbamyl-β-alanine obtained after incubation of 500 μ*M* [2-^{14}C]-dihydrouracil with a cytosolic fraction containing human DHP.

The reaction catalyzed by DHP proved to be linear with protein concentration up to at least 4.6 mg/ml (Fig. 3). With respect to the time dependence of the reaction catalyzed by DHP, a linear increase in product formation was observed between 0.17 and 3 h (Fig. 4). Comparable DHP activities were detected in human liver supernatants that had additionally been frozen and subsequently stored for 5 days at −20°C (recovery = 102% ± 4%, $n = 5$). Thus, no profound effects of freeze-thawing were observed for DHP. The intra-assay variation ($n = 5$) and inter-assay variation ($n = 5$) for the determination of the DHP activity did not exceed 10%.

Fig. 5 shows the steady-state kinetics of human liver DHP with dihydrouracil. Under these conditions, a linear Lineweaver-Burk plot was obtained with an apparent K_m for dihydrouracil of 7 μ*M*, which is comparable to that observed for the purified

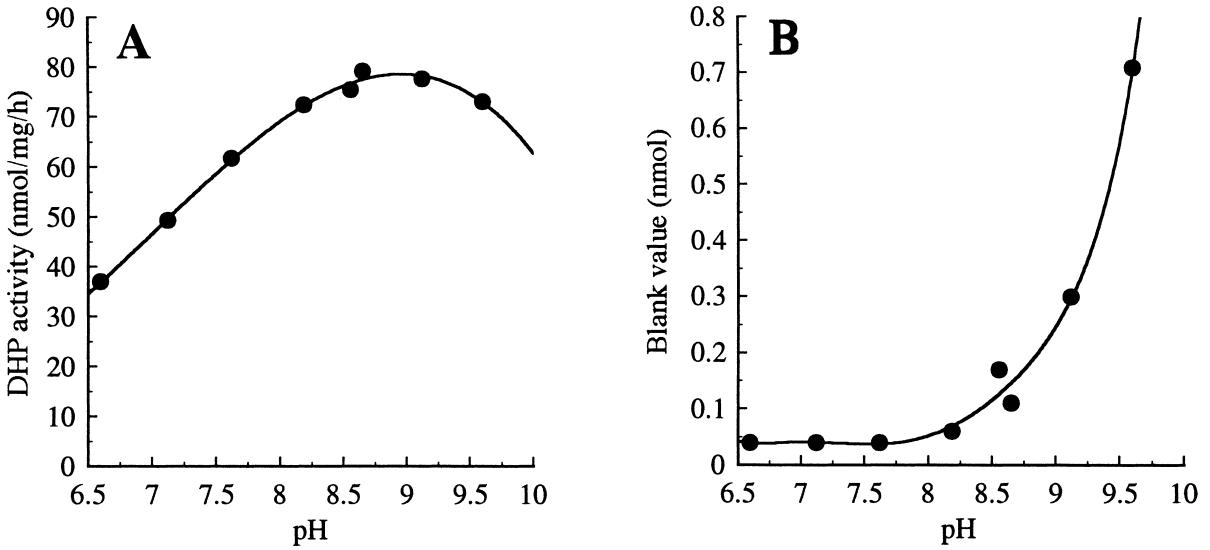

Fig. 2. The pH optimum of the DHP activity is shown by panel A. The DHP activity was measured at a protein concentration of 0.45 mg/ml for 2 h at 37°C. Panel B shows the pH depended hydrolysis of 5,6-dihydrouracil in a blanc.

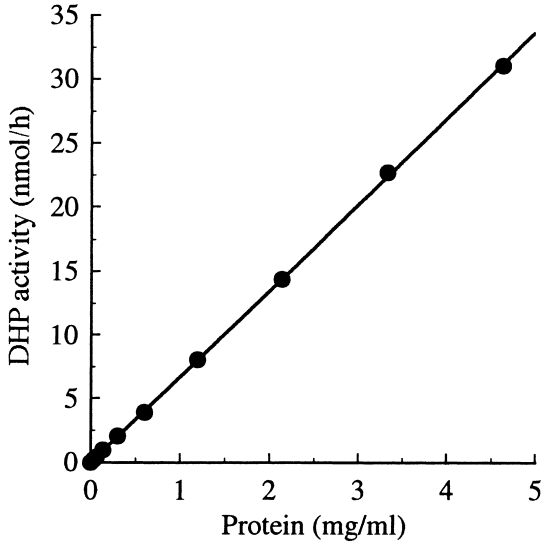

Fig. 3. Protein dependence of the DHP activity. The reaction was allowed to proceed for 77 min at 37°C.

enzyme of rat liver [11,18] and calf liver [9]. The activity of DHP in control livers ranged from 20 to 74 nmol/mg/h with a mean activity of 56±19 nmol/mg/h ($n=8$). On average, 61±15% (range 44%–92%) of the total amount of radiolabeled products was encountered in *N*-carbamyl-β-alanine with the remaining part being $^{14}CO_2$. The DPD activity in these control livers proved to be much lower with a mean specific activity of 9.3±2.4 nmol/mg/h ($n=8$).

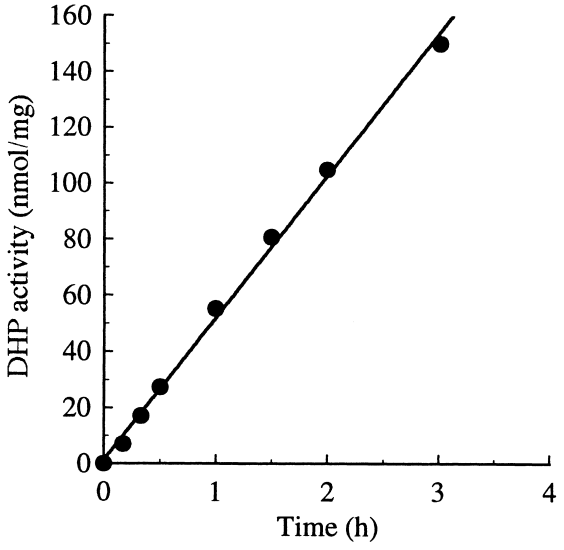

Fig. 4. Time dependence of the DHP activity. The DHP activity was measured at a protein concentration of 0.36 mg/ml at 37°C.

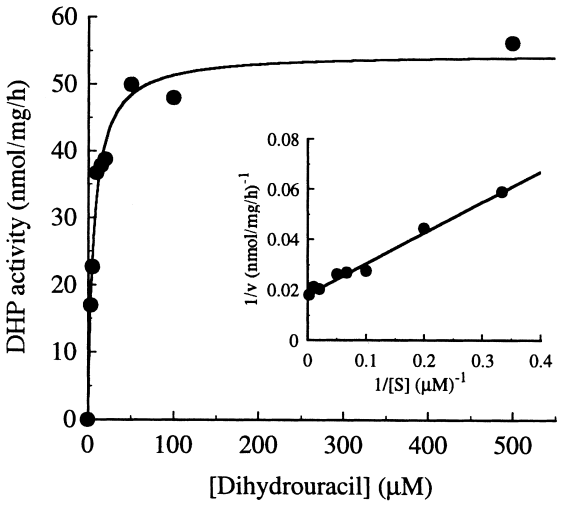

Fig. 5. Steady-state kinetics of human DHP with 5,6-dihydrouracil. The data points were fitted according to the Michaelis-Menten equation. The insert shows the double-reciprocal plot of the reaction velocity versus the concentration of dihydrouracil.

4. Discussion

In this study, we developed a sensitive assay to measure the activity of DHP, which is based on the separation of radiolabeled dihydrouracil from *N*-carbamyl-β-alanine by HPLC with on-line detection of radioactivity combined with detection of $^{14}CO_2$ by liquid scintillation counting. The [^{14}C]-*N*-carbamyl-β-alanine produced by DHP from [2-^{14}C]-5,6-dihydrouracil is subsequently converted into β-alanine, NH_3 and $^{14}CO_2$ by UP which is also present in human liver:

$$[2\text{-}^{14}C]\text{-5,6-dihydrouracil} \xrightarrow{DHP} [^{14}C]\text{-}N\text{-carbamyl-}\beta\text{-alanine} \xrightarrow{UP} {}^{14}CO_2 + \beta\text{-alanine} + NH_3$$

The assay proved to be linear with time indicating that under our conditions no inhibition occurred due to the accumulation of *N*-carbamyl-β-alanine and

β-alanine. In this respect, it has been shown that *N*-carbamyl-β-alanine and *N*-carbamyl-β-amino-isobutyric acid are mixed-type inhibitors of dihydropyrimidinase [10,11]. Furthermore, no variation in specific activity of DHP with protein concentration was observed indicating that there is no slow association-dissociation equilibrium between the four subunits of the tetrameric enzyme [14]. The optimal pH proved to be 8.8, which is in line with that observed for DHP from bovine liver [9] and rat liver [11]. We and others observed that above pH 8.5 significant hydrolysis of dihydrouracil occurred resulting in high blank rates [18].

Previously, it has been suggested that the activity of DHP in crude homogenates was stimulated by Mg^{2+} at a concentration of 5 m*M* [19]. In our hands, no effect of Mg^{2+} on the DHP activity was observed which is in agreement with the results obtained for the purified rat liver enzyme [11]. In this respect, it should be noted that an imidase has been purified to homogeneity from rat liver which catalyzed the hydrolytic cleavage of imides including hydantoins, dihydropyrimidines and phtalimide [20]. The affinity of this imidase towards dihydrouracil was low (K_m = 2.2 m*M*) but the activity was stimulated in the presence of $MgCl_2$ [20]. Thus, it is tempting to speculate that in the study of the pyrimidine degradation pathway during rat liver regeneration the imidase activity has been measured instead of dihydropyrimidinase [21]. In human liver homogenates the affinity of dihydropyrimidinase towards dihydrouracil proved to be high with a K_m value of 7 μ*M* which is comparable to previously reported values for dihydrouracil [9,11,18].

The minimum amount of radiolabeled products which could be determined proved to be 12 pmol using a purified stock solution of [2-^{14}C]-5,6-dihydrouracil. Thus, this highly sensitive assay is especially suitable to identify patients with a dihydropyrimidinase deficiency [7,22]. Patients with a deficiency of dihydropyrimidinase excrete strongly elevated levels of dihydropyrimidines and moderately elevated levels of uracil and thymine [23,24]. In the Japanese population a relative high frequency (0.1%) for dihydropyrimidinuria has been observed [24]. Since 5FU is also degraded by the three-step degradation pathway of the pyrimidine bases patients with a DHP deficiency are probably at risk of developing severe 5FU associated toxicities. To date the DHP activity has not been measured directly in human liver tissue. Our study demonstrates that the mean DHP activity is approximately five-fold higher than that of DPD. Moreover, in bovine and rat liver the DHP activity was 250–500 fold greater than that of DPD which might explain the observation that hydrolysis of (*R*)-5-fluoro-5,6-dihydrouracil is strongly favored over the oxidation of (*R*)-5-fluoro-5,6-dihydrouracil to 5FU by DPD [18].

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