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5-ETHYNYLURACIL (776C85): INACTIVATION OF DIHYDROPYRIMIDINE DEHYDROGENASE *IN VIVO*

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Abstract—5-Ethynyluracil (776C85), a potent, mechanism-based, irreversible inactivator (Porter *et al.*, *J Biol Chem* **267**: 5236–5242, 1992) of purified dihydropyrimidine dehydrogenase (DPD, uracil reductase, EC 1.3.1.2), readily inactivated DPD *in vivo*. DPD was assayed in tissue extracts by measuring the release of $^{14}CO_2$ from [2-^{14}C]uracil with an improved method. Specific activities from 0.1 to >1000 U/mg protein were reproducibly measured. After rats were orally dosed with 20 μg/kg 5-ethynyluracil, liver, intestinal mucosa, lung, and spleen DPD were inactivated by 83–94%. The dose required to inactivate rat liver, rat brain, and mouse liver DPD by 50% was 1.8, 11, and 8.9 μg/kg, respectively. Rat liver DPD was inactivated completely within 25 min after an oral dose of 500 μg/kg 5-ethynyluracil. New DPD was synthesized with a half-time of 63 hr. We also developed an assay based on stoichiometric inactivation of DPD by 5-ethynyluracil to measure 5-ethynyluracil in plasma samples. Samples containing 5-ethynyluracil were incubated with rat liver extract for 24 hr at 12° and then assayed for DPD. DPD activity decreased linearly with the concentration of 5-ethynyluracil (between 0 and 20 nM 5-ethynyluracil). The assay could detect 5-ethynyluracil at concentrations as low as 6 nM in human plasma and was not affected by high concentrations of uracil.

Key words: dihydropyrimidine dehydrogenase tissue levels, dihydropyrimidine dehydrogenase synthesis, 5-ethynyluracil assay

5-Ethynyluracil (776C85) is a potent, mechanism-based, irreversible inactivator of purified dihydropyrimidine dehydrogenase (DPD†, uracil reductase, EC 1.3.1.2). It has a K_m of 1.6 μM and covalently modifies the enzyme in a first-order process with a rate constant of 20 min^{-1} (enzyme half-life = 2 sec) [1]. 5-Ethynyluracil also appears to inactivate DPD *in vivo* because plasma uracil and thymine, the endogenous substrates of DPD, are elevated markedly in animals treated with low doses of 5-ethynyluracil.‡ Most importantly, 5-fluorouracil (5-FU), an anticancer agent that is rapidly degraded by DPD [2–4], is not catabolized in mice and rats pretreated with 5-ethynyluracil [5]. Consequently, 5-ethynyluracil significantly potentiates the antitumor activity of 5-FU in mice [5] and rats [6]. Although 5-ethynyluracil also lowers the toxic dose of 5-FU, it potentiates toxicity less than efficacy and, therefore, increases the therapeutic index as well as the efficacy of 5-FU in these tumor-bearing animals [5, 6].

5-Ethynyluracil also improves the bioavailabilty of 5-FU [5]. It decreases the variability and increases the half-life of 5-FU in plasma of rats dosed orally with 5-FU. It promotes complete oral absorption of 5-FU and induces a linear relationship between the area under the plasma concentration–time curve (AUC) and the oral dose of 5-FU in rats. The AUC of 5-FU is normally less than expected at low doses and then increases proportionately with increasing doses in patients treated with either i.v. or oral 5-FU [7, 8]. Simulations of the pharmacokinetics of 5-FU indicate that 5-ethynyluracil should enable small doses (three per day) of oral 5-FU to replace constantly infused i.v. dosed 5-FU.§

In the present study, we developed a sensitive assay for DPD to confirm that 5-ethynyluracil inactivates the enzyme *in vivo*. We dosed rats and mice orally with 5-ethynyluracil and then determined the residual activity of DPD in several tissues and the rate of resynthesis of DPD in rat liver. We also modified this method to assay nanomolar concentrations of 5-ethynyluracil in human plasma samples.

MATERIALS AND METHODS

Chemicals. 5-Ethynyluracil was synthesized at the Burroughs Wellcome Co. [2-^{14}C]Uracil (56 Ci/mol) was purchased from Moravek Biochemicals, Brea, CA. NADPH, glucose-6-phosphate, and glucose-6-phosphate dehydrogenase were obtained from the Sigma Chemical Co., St. Louis, MO.

Animals. Sprague–Dawley CD male rats (350–400 g) and CD1 male mice (20–25 g) were obtained from Charles River Laboratories, Wilmington, MA.

Drug dosing. Stock solutions of 0.25 mg/mL of 5-ethynyluracil were prepared in sterile saline from dry 5-ethynyluracil by adjusting the pH to 10 with NaOH and sterile filtering. Solutions of 5-ethynyluracil were stable for at least 2 weeks at 5°. Animals were dosed by gavage.

Tissue extracts. Tissues were removed from rats and mice between 8:45 and 9:30 a.m. and were

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† Abbreviations: DPD, dihydropyrimidine dehydrogenase; DTT, dithiothreitol; and 5-FU, 5-fluorouracil.

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§ Khor, SP, unpublished data; cited with permission.

placed immediately in ice-cold saline. The following operations were at 0–4°. Liver, lung, brain, or spleen was rapidly cut into sections, weighed, and suspended in 3 vol. (2 vol. for brain) of 20 mM potassium phosphate buffer, pH 7.6, 2 mM dithiothreitol (DTT) (Buffer A) containing 0.15 mM thymine (to prevent additional binding of 5-ethynyluracil). Small intestines were washed with saline and cut open. The mucosal layer was collected from the interior surface by scraping with a glass slide and was placed in 3 vol. of Buffer A. Liver and brain suspensions were homogenized in a 15-mL Wheaton glass homogenizer. Other tougher tissues (intestinal mucosa, lung, and spleen) were homogenized with a Polytron homogenizer. Homogenates were centrifuged at 105,000 *g* for 60 min. The supernatant was withdrawn with a Pasteur pipette to avoid the floating lipid layer. DPD in extracts of liver and brain was stable for at least 2 months during storage at −70°. Prior to use, extracts were thawed and small molecules were removed by centrifugation-assisted rapid gel filtration through Sephadex G-25 resin preequilibrated with Buffer A and 0.1% bovine serum albumin [9].

DPD assay. An assay [10], which measures the release of $^{14}CO_2$ from [2-^{14}C]uracil following reduction to dihydrouracil by DPD and degradation by the next two enzymes in the catabolic pathway, was modified to ensure complete conversion of dihydrouracil to CO_2. Reaction mixtures were incubated at 37° in vials sealed with a rubber stopper-sleeve containing a center well (Kontes Scientific Glassware, Vineland, NJ) that held filter paper soaked with 25 μL of 1 N NaOH. The reaction vials contained 25 μM [2-^{14}C]uracil (16 Ci/mol) and an NADPH-regenerating system consisting of 50 μM NADPH, 2.5 mM glucose-6-phosphate, and 0.7 U of glucose-6-phosphate dehydrogenase in Buffer A. Reactions were initiated with 0.3 to 5 mg of protein from the various extracts bringing the total volume to 500 μL. All subsequent additions were injected through the rubber septum. At various times, 200 μL of a 1:1 mixture of 2 mM 5-ethynyluracil and rat liver extract (see above) were added to inactivate DPD and to provide fresh enzymes to complete the conversion of radiolabeled dihydrouracil and β-ureidopropionic acid to $^{14}CO_2$, NH_3, and β-alanine. These enzymes were stable in the liver extract for at least 2 months at −70°, as fresh and stored extracts were equally effective. The mixtures were incubated for an additional 30 min at 37°. One hundred microliters of 35% percholoric acid was added, and the vials were incubated for an additional 45 min to allow the NaOH-soaked filter paper to absorb the $^{14}CO_2$. The filter papers were transferred to scintillation vials containing 0.5 mL of 0.05 M NaOH and gently shaken. Scintiverse BD (5 mL) was added, and the radioactivity was quantitated by scintillation counting. Velocities were the slope of plots of CO_2 formed versus time (at least two points in the linear region).

5-Ethynyluracil assay. Plasma samples (10–50 μL) containing 5-ethynyluracil were incubated in microfuge tubes with 30 μL rat liver extract (approximately 0.6 mg protein) and the NADPH-regenerating system (see above) in Buffer A (100 μL total volume) for 20 hr at 12°. A 50-μL sample was then removed and was added to 450 μL of reaction mixture, and assayed for DPD as described above, except that the concentration of [2-^{14}C]uracil was increased to 100 μM (2.8 Ci/mol).

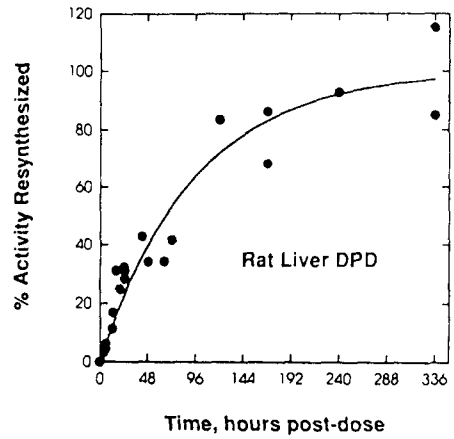

Fig. 1. Time-course for the resynthesis of DPD in rats. Liver DPD from rats dosed with 0.5 mg/kg 5-ethynyluracil was assayed at the indicated time after dosing. The data were obtained from five separate experiments. The line is the best fit for a single exponential curve.

Miscellaneous. Protein concentrations were determined by the modified [11] Coomassie Blue assay [12] with bovine serum albumin as the standard. Dose–response curves were analyzed by iteratively fitting a logistic function to the data (Sigma Plot, Jandel Scientific, Corde Madera, CA).

RESULTS

DPD assay. We measured the release of $^{14}CO_2$ from [2-^{14}C]uracil following reduction to dihydrouracil by DPD in rat liver extracts. Reactions were initiated with rat liver extract and were terminated by inactivating DPD with 100 μL of 2 mM 5-ethynyluracil. However, when the terminated reaction was incubated with fresh extract (pretreated with 5-ethynyluracil to inactivate DPD), additional CO_2 was produced. Therefore, dihydropyrimidase and/or β-alanine synthetase (β-ureidopropionase), the enzymes that convert dihydrouracil to CO_2, NH_3, and β-alanine, were apparently unstable. We found that adding 200 μL of a 1:1 mixture of 2 mM 5-ethynyluracil and rat liver extract immediately terminated the reaction by inactivating DPD and that incubating for an additional 30 min at 37° maximized the formation of $^{14}CO_2$. Greater than 95% of the radiolabel from uracil was recovered as $^{14}CO_2$ when reactions were allowed to proceed to completion and were then treated in this manner. Product formation was linear with time for at least 60% conversion of the radiolabel from 25 μM uracil to $^{14}CO_2$ and for at least 30% conversion of the radiolabel from 100 μM uracil. The rate of product formation increased linearly with protein concentration over the range tested, 0.4 to 8 mg/

Table 1. Inactivation of DPD in several rat tissues

Tissue	DPD (pmol/min/mg)	% Inhibition 5-Ethynyluracil 2 μg/kg, p.o.	% Inhibition 5-Ethynyluracil 20 μg/kg, p.o.
Liver	500 ± 20	75	94
Intestinal mucosa	410 ± 60	65	92
Lung	24.0 ± 0.9	39	83
Spleen	17 ± 2	69	91
Brain	10.3 ± 0.3	12	60

Tissues were removed from rats 1 hr after treatment with the indicated doses of 5-ethynyluracil or with no drug. DPD values are the mean ± range from two individually processed and analyzed tissues. The inactivation values were obtained from pooled tissues from two rats.

mL. Control assays with extract omitted from the first incubation produced a background level of 0.06% conversion, which was subtracted from the values obtained in complete assays. One unit of DPD catalyzed the formation of one picomole of product per minute. The sensitivity range of the assay was extended by varying the amount of protein and the assay time. Specific activities from 0.1 to >1000 U/mg protein were reproducibly measured.

Time-course for inactivation and resynthesis of DPD. Rats (2 per group) dosed orally with 0.5 mg/kg 5-ethynyluracil were killed at various times. Livers were rapidly removed, washed in cold saline, and homogenized in Buffer A containing 0.15 mM thymine to prevent additional binding of 5-ethynyluracil. Homogenates were then processed as described in Materials and Methods. DPD was >99% inactivated (specific activity decreased from 690 ± 20 to 2 ± 1 U/mg) by the first time point (15 min post-dose plus 5–10 min processing) and remained at this level for at least 1 hr.

To measure the resynthesis of DPD, rats (3 or 4 per group) were dosed orally with 0.5 mg/kg 5-ethynyluracil at different times and then killed at the same time (within 1 hr) as untreated control animals. Livers from rats dosed as a group for a time-point were homogenized together. Livers from untreated rats were assayed separately to determine the individual variability. At 4 hr post-dosing, the specific activity of DPD from treated rats was only 3% that of untreated animals. The data of Fig. 1 show that DPD was resynthesized by approximately 2 weeks post-dosing in a first-order process. The half-time for resynthesis was 63 ± 6 hr. The half-life of 5-ethynyluracil in rat plasma is 35 min.*

Inactivation of DPD by 5-ethynyluracil. Rats (2 per group) were dosed orally with either 0 (control group), 2, or 20 μg/kg 5-ethynyluracil, and their liver, intestine, lung, spleen, and brain were removed after 1 hr and processed as described in Materials and Methods. Extracts of these tissues were assayed for DPD. Tissue extracts from animals receiving no drug were assayed separately to determine the individual variability. Pairs of organs from animals

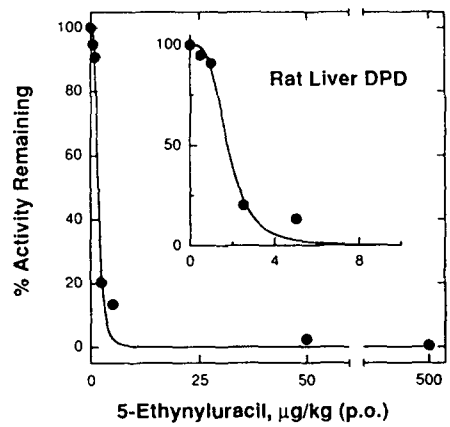

Fig. 2. Inactivation of liver DPD in rats orally dosed with 5-ethynyluracil. Livers were removed 1 hr after rats (3 per group) received the indicated oral dose of 5-ethynyluracil. DPD from livers of untreated rats had a specific activity of 620 U/mg. Inset: enlargement of the effect at lower doses.

receiving 5-ethynyluracil were homogenized together. The results, summarized in Table 1, show that 5-ethynyluracil was a very potent inactivator of DPD. Very similar degrees of inactivation were obtained in a repeat experiment.

The specific activity of DPD in spleen, lung, and brain extracts was fairly constant among individual rats from this experiment and a repeat experiment (data not shown). Although the specific activity in liver extracts was similar among individuals on a given day, it ranged from 300 to 870 pmol/min/mg in experiments performed on several different days. DPD levels in intestinal mucosa varied among individual rats on a given day as well as on different days. For example, values of 190 and 290 (average = 240) pmol/min/mg were obtained from individual rats killed on the same day at the same time. These extracts were frozen and then thawed and reassayed 8 days later to produce values of 190 and 340 (average = 265) pmol/min/mg, respectively, thus

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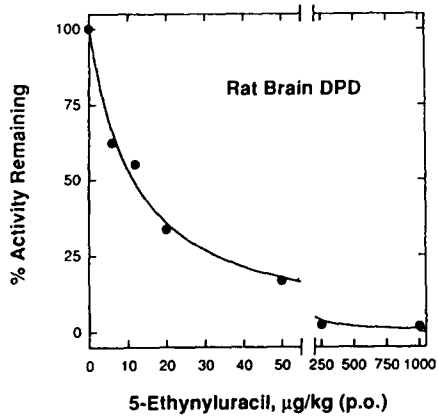

Fig. 3. Inactivation of brain DPD in rats orally dosed with 5-ethynyluracil. Brains were removed 1 hr after rats (3 per group) received the indicated oral dose of 5-ethynyluracil. DPD from brains of untreated rats had a specific activity of 9.6 U/mg.

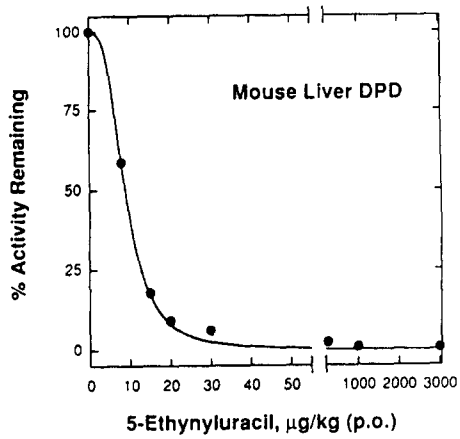

Fig. 4. Inactivation of liver DPD in mice orally dosed with 5-ethynyluracil. Livers were removed 1 hr after mice (3 per group) received the indicated oral dose of 5-ethynyluracil. DPD from livers of untreated mice had a specific activity of 1460 U/mg.

indicating that the variability was due to the specific acitivity of DPD and not to the assay.

Dose titration. Livers or brains were removed from rats and mice (3 per group) 1 hr after orally dosing with different amounts of 5-ethynyluracil. Tissues from individual animals within each group were processed and assayed together. The dose–response curves are shown in Figs. 2–4. DPD from rat liver, rat brain, and mouse liver was 50% inactivated (ED_{50}) by 1.8 ± 0.1, 11 ± 1 and 8.9 ± 0.2 μg/kg, respectively. Repeat experiments measuring liver DPD at 1 hr or brain DPD at 2 hr post-dosing gave similar results.

DPD in human liver. Samples from ten transplantable human livers were processed and assayed

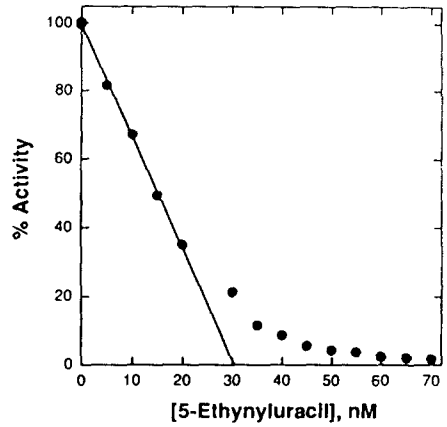

Fig. 5. Assay of 5-ethynyluracil in plasma. Samples of fresh human plasma containing various amounts of 5-ethynyluracil were diluted 10-fold into tubes containing rat liver extract, NADPH, and Buffer A as described in Materials and Methods. After incubating for 24 hr at 12°, an aliquot was removed and the residual DPD was assayed. The concentration of 5-ethynyluracil in the incubation mixture is indicated. The specific activity of DPD from eight mixtures containing plasma samples with no 5-ethynyluracil was 506 ± 7 U/mg.

for DPD. The average specific activity was 250 ± 80 (range 145 ± 374) pmol/min/mg.

5-Ethynyluracil assay. An assay based on the stoichiometric inactivation of DPD by 5-ethynyluracil [1] was developed to measure 5-ethynyluracil in plasma samples. We incubated various concentrations of 5-ethynyluracil and rat liver extract for different times and at different temperatures to determine the proper conditions. NADPH and its regenerating systeme were included to enable DPD to catalyze both the reduction of substrates present in plasma samples and its mechanism-based inactivation by 5-ethynyluracil. Liver extract was used to provide dihydropyrimidase and β-alanine synthetase to catalyze the degradation of dihydrouracil and dihydrothymine, which could compete with 5-ethynyluracil for binding to DPD.

We determined that 12° was a satisfactory temperature for inactivating DPD by low concentrations of 5-ethynyluracil. Maximum inactivation occurred by 20 hr with no significant loss of control DPD activity. At lower temperatures, binding of 5-ethynyluracil and degradation of uracil were considerably slower. At higher temperatures, control DPD lost activity before maximum inactivation was achieved. Thirty microliters of rat liver extract per 100 μL of incubation mixture was selected to provide an appropriate assay range. It also provided adequate pyrimidine catabolic enzymes to degrade large amounts of substrates during the incubation. 5-Ethynyluracil (10 nM) produced $45 \pm 2\%$ inactivation of DPD in incubation mixtures that contained 0, 5, 10, 20, 40, or 80 μM uracil.

5-Ethynyluracil was added to fresh human plasma samples to final concentrations of 50–700 nM. Ten microliters of these samples was mixed with 90 μL

of a mixture containing Buffer A, rat liver extract, NADPH, and an NADPH-regenerating system, as described in Materials and Methods, and was incubated for 24 hr at 12°. This 100-μL incubation mixture could accommodate up to 50 μL of plasma without affecting the subsequent assays. A 50-μL aliquot of each incubation sample was then assayed for DPD. The titration of DPD activity was linear between 0 and 20 nM 5-ethynyluracil with a slope of -3.27 ± 0.06 and an X-axis intercept (DPD concentration) of 30.4 ± 0.4 μM (Fig. 5). The ratio of molar equivalents of DPD to the units of activity (3200 U/mL) indicates that one activity unit was equivalent to 0.01 pmol DPD. Very similar values were obtained in repeat experiments.

Because the data points from incubation mixtures containing >20 nM 5-ethynyluracil deviated from the linear titration expected for an irreversible inactivator, we tested whether 5-ethynyluracil was partially depleted by chemical or enzymatic degradation during the 24-hr incubation. Excess 5-ethynyluracil (200 nM) was incubated for 24 hr with rat liver extract containing 30 nM DPD as in the experiments described above. The resultant mixture was expected to contain 30 nM covalently modified DPD and 170 nM 5-ethynyluracil if no extraneous degradation had occurred. We assayed this mixture by the above method and determined that the concentration of 5-ethynyluracil was, within experimental error, equal to 170 nM, thereby eliminating degradation as an explanation for the titration's deviation from linearity.

DISCUSSION

A sensitive and convenient assay was developed to measure DPD in animal tissue extracts. We used this assay to assess the effects on tissue DPD from animals orally dosed with 5-ethynyluracil. Liver, the predominant source of DPD [13, 14, and references cited therein], and several other tissues were studied. The studies confirmed that 5-ethynyluracil is a very potent inactivator of DPD *in vivo*. The enzyme in rat liver was >99% inactivated within minutes of dosing and was resynthesized slowly with a half-time of 63 hr. Rat and mouse liver enzymes were 50% inactivated by a dose of 1.8 and 8.9 μg/kg, respectively. These doses appear more similar when they were converted [15] to μg/m^2: 15 μg/m^2 for the rat and to 28 μg/m^2 for the mouse.

Uracil plasma levels rise from about 2 μM to a maximum of 75 μM in rats and to 60 μM in mice dosed with 5-ethynyluracil.* The dose that induces 1/2 maximal elevation of uracil, 10 μg/kg in rats and 250 μg/kg in mice, inactivated approximately 90% of DPD in livers of the corresponding animals. Moreover, rat plasma uracil concentrations approached normal levels by 24 hr post-dosing,* a time when approximately 70% of liver DPD remained inactivated. Therefore, DPD must be inactivated by >95% to completely preserve elevated plasma uracil. We calculated by extrapolation [15] that a dose of approximately 100 μg/kg would achieve >95% inactivation of DPD in humans.

DPD was present and was readily inactivated by 5-ethynyluracil in all tissues studied including rat brain. However, the ability of 5-ethynyluracil to cross the blood–brain barrier in the rat appeared to be impaired as the ED_{50} of 5-ethynyluracil for brain DPD was 6-fold larger than that for liver DPD.

DPD specific activity was very high, but variable, in liver and intestinal mucosa. The variability in the latter was particularly striking and could account for the variable bioavailability of 5-fluorouracil given orally to rats [5] and to human cancer patients [7, 16, 17]. Accordingly, 5-ethynyluracil, which essentially eliminates DPD, markedly reduces the variability of oral bioavailability in rats and should have a similar effect in humans.

The DPD assay was used as an integral part of an assay to measure 5-ethynyluracil in human plasma. Samples were incubated for 24 hr with rat liver extract, and DPD was assayed. The per cent DPD activity remaining decreased linearly between 0 and 20 nM 5-ethynyluracil. The lowest concentrations of 5-ethynyluracil that produced reliable inactivation was 3 nM (10% inactivation). It sets the lowest detectable concentration of 5-ethynyluracil in plasma at 6 nM when 50 μL plasma are incubated in the 100-μL mixture. There was no upper limit because the plasma can be diluted to yield 5-ethynyluracil concentrations within the 3–20 nM range in the incubation mixture. We do not know why the assay deviated from linearity at concentrations above this range.

The assay for 5-ethynyluracil (Fig. 5) revealed that the concentration of DPD was 30 nM in the incubation mixture and that 1 pmol of enzyme catalyzed the reduction of 100 pmol uracil/min (turnover number = 100 per min). A similar value, 96 per min, was determined for DPD purified from bovine liver (data not shown). Therefore, DPD can be quantitated by activity measurements, as one unit of DPD corresponds to 0.01 pmol of enzyme.

In conclusion, we have developed an assay for DPD and confirmed that 5-ethynyluracil was a potent inactivator of DPD *in vivo*. Very low oral doses inactivated DPD in all tissues studied. Liver DPD was inactivated within minutes and was resynthesized over several days.

We anticipate that very low doses of 5-ethynyluracil will completely inactivate DPD in cancer patients and will thereby preserve 5-fluorouracil in body fluids and tissues. We hope that 5-ethynyluracil will improve 5-fluorouracil therapy in these patients by increasing its efficacy and therapeutic index as it has done in laboratory animals [5, 6].

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