5-Iodouracil was a substrate for bovine liver dihydropyrimidine dehydrogenase (DHPDHase) and was a potent inactivator of the enzyme. NADPH increased the rate of inactivation and thymine protected against inactivation. These findings suggest that 5-iodouracil was a mechanism-based inactivator. However, dithiothreitol and excess 5-iodouracil protected the enzyme against inactivation. Thus, a reactive product, presumably 5-ido-5,6-dihydrouracil generated through the enzymatic reduction of 5-iodouracil, was released from DHPDHase during processing of 5-iodouracil. Since only 18% of [6-3H]-5-iodouracil reduced by DHPDHase was covalently bound to the enzyme and radiolabel was not lost to the solvent as tritium, the partition coefficient for inactivation was 4.5. However, the enzymatic activity was completely titrated with 1.7 mol of 5-iodouracil per mol of enzyme-bound flavin. These results indicate that there was 0.31 mol of enzyme-bound inactivator per mol of enzyme flavin. This suggests there were 3.2 flavins per active site, which is consistent with the report of multiple flavins per enzymic subunit (Podschun, B., Wahler, G., and Schnack-erz, K. D. (1989) Eur. J. Biochem. 185, 219–224). DHPDHase was inactivated by 2.1 mol of racemic 5-iodo-5,6-dihydrouracil per mol of active sites. The stoichiometry for inactivation of the enzyme by the nonenzymatically generated enantiomer of 5-iodo-5,6-dihydrouracil was calculated to be 1. Two radiolabeled fragments were isolated from a tryptic digest of DHPDHase inactivated with radiolabeled 5-iodouracil. The amino acid sequences of these peptides were Asn-Leu-Ser-X-Pro-His and Asn-Leu-Ser-X-Pro-His-Gly-Met-Gly-Glu-Arg where X was the modified amino acid containing radiolabel from [6-3H]-5-iodouracil. Fast atom bombardment mass spectral analysis of the smaller peptide yielded a protonated parent ion mass of 782 daltons that was consistent with X being a S-(hexahydro-2,4-dioxo-5-pyrimidinyl)cysteinyl residue.

Dihydropyrimidine dehydrogenase (DHPDHase, EC 1.3.1.2) catalyzes the reversible reduction of pyrimidines to 5,6-dihydropyrimidines as the first step in pyrimidine catabolism to β-amino acids (1). The enzyme has been purified to homogeneity from mouse and porcine liver and has been shown to contain multiple flavins and iron sulfur prosthetic groups per subunit (2, 3). The purified enzyme rapidly reduces the anticancer drug 5-iodouracil to 5-fluoro-5,6-dihydrouracil (2, 3). DHPDHase is estimated to degrade over 90% of an administered dose of 5-fluorouracil (4). The variability of the effectiveness of 5-fluorouracil may be due, in part, to the variability of the cellular levels of DHPDHase (5). The activity of this enzyme not only varies between individuals but also has a temporal variation in an individual with a circadian periodicity of 24 h (6). Consequently, DHPDHase has emerged as a potentially important adjunct target in fluorouracil chemotherapy. The inhibitory properties of a large number of pyrimidine analogs for DHPDHase have recently been summarized (7). Some of these analogs have submicromolar Ki values. Only 5-bromovinyluracil and 5-diazouracil have been reported to be irreversible inactivators of this enzyme (8, 9). 5-Bromovinyluracil inhibits the enzyme in vivo and causes uracil levels in the plasma of mice to increase (10). Furthermore, the half-life of 5-fluorouracil in mice increases when 5-fluorouracil is coadministered with 5-bromovinyluracil (8, 11). Thus, inhibitors of DHPDHase are useful for potentiation of the efficacy of 5-fluorouracil (8, 10–12) and for controlling the variability in 5-fluorouracil availability.

The intravenous infusion of 5-iodo-2'-deoxyuridine into human subjects generates high plasma levels of 5-iodouracil with 50–100-fold increases in plasma levels of thymine and uracil (13). The elevated uracil and thymine levels were suggested to be the result of simple competition between plasma pyrimidines and 5-iodouracil for DHPDHase. Since 5-iodouracil, an efficient substrate for DHPDHase, can compete effectively with the natural substrate thymine (2, 14), this hypothesis provides an attractive explanation for the elevated levels of pyrimidines during infusion of 5-iodo-2'-deoxyuridine. Alternatively, the product of DHPDHase-catalyzed reduction of 5-iodouracil, 5-ido-5,6-dihydrouracil, is expected to be an alkylating agent with properties similar to those of iodoacetamide (Equation 1). Consequently, the elevated plasma levels of pyrimidines in patients treated with 5-iodo-2'-deoxyuridine could be, in part, due to inactivation of DHPDHase by enzymatically generated 5-iodo-5,6-dihydrouracil. We have addressed this possibility by investigating the interaction of 5-iodouracil with purified bovine liver DHPDHase and have found that 5-iodouracil potently inactivated this enzyme.

\[ \text{DHPDHase} + \text{5-Iodouracil} \rightarrow \text{5-Iodo-5,6-Dihydrouracil} \]

(1)
**EXPERIMENTAL PROCEDURES**

**Materials**

Sources of materials were as follows: EDTA, Tris, NADP+, NADPH, FAD, FMN, thymine, DTT, dihydrothymine, and 2',5'-ADP-Sepharose, Sigma; Bio-Gel P-6 and P-200, Bio-Rad Laboratories (Richmond, CA); 5-iodouracil, NaI, Br2, and 5,6-dihydrouracil, Aldrich Chemical Co. (Milwaukee, WI); Whatman DE-52, Bodmann Chemical Co. (Easton, PA); [6-3H]-5-iodouracil (15 Ci/mmol), Mora- vezk Biochemicals Inc. (Brea, CA); modified trypsin, Promega (Mad- ison, WI); frozen bovine liver, Pel-Freez (Rogers, AR). 5-iodo-5,6- dihydouracil was prepared from 5-bromo-5,6-dihydouracil (15) by the Finkenstein reaction as described by Rork and Pitman (16). The product was recrystallised from an acetone-H2O mixture. Analysis calculated (found) for C4H6N2O2I: C 20.00 (20.07); H 2.08 (2.10); N 11.66 (11.60). 1H NMR (200 MHz, dimethyl sulfoxide-d6) 10.25 and 7.90 (2 s, 2 H, H2, and H3). 4.75 (m, 1 H, H1), 3.45 and 3.07 (m, 2 H, H6). The mass spectrum (ci) had an M + 1 peak at 241.

**Assay of DHPDHase**

DHPDHase was routinely assayed at 37 °C in buffer A (0.05 M Tris-HCl at pH 8.0 with 1 mM DTT, 200 μM NADPH, and 200 μM thymine. The oxidation of NADPH was followed at 340 nm. 1 unit of enzyme catalyzed the oxidation of 1 μmol of NADPH per h. Protein concentration was estimated from absorbance at 274 and was expressed in terms of absorbance units at 274 nm (A274). Specific activities are expressed as activity units/A274. Enzymatic activity was also measured spectrophotometrically by following the reduction of 5-iodouracil at 283 nm, an isosbestic point between NADPH and NADP+. The ΔA274 for the reduction of 5-iodouracil to 5-iodo-5,6-dihydouracil was 5.9 mI cm⁻¹ in buffer A.

**Preparation of DHPDHase**

Purification of DHPDHase from bovine liver was based on the method of Sibbott and Weber (2). A pivotal step in this purification was the affinity purification of the enzyme with 2',5'-ADP-Sepharose. All steps were at 4 °C.

Step 1. Homogenization—Frozen bovine liver (1 kg) was quickly thawed and homogenized in 2 liters of 0.25 M sucrose in buffer B (35 mM sodium phosphate, 5 mM 2-mercaptoethanol, and 2.5 mM MgCl2 at pH 7.4) with a commercial Waring blender at high speed for 5 min. The homogenate was filtered through cheese cloth and the filtrate was centrifuged for 20 min at 10,000 × g.

Step 2. Batch DE-52—The pH of the supernatant was adjusted to 8.0 and the mixture was absorbed onto 2.5 liters of a 60% slurry of DE-52 resin equilibrated to pH 8.0. After stirring for 30 min, the enzyme was recovered by vacuum filtration and was washed with 2 liters of buffer B. After washing the resin with 1500 ml of buffer B with 50 mM KCl, enzymatic activity eluted from the resin in 650 ml of buffer.

**Ammonium Sulfate Precipitation**—The solution was brought to 40% saturated ammonium sulfate by addition of 158 g of solid ammonium sulfate. After stirring for 30 min, the enzyme was collected by centrifugation at 10,000 × g for 20 min. The supernatant was brought to 50% saturated ammonium sulfate with an additional 46 g of solid ammonium sulfate. After stirring for 30 min, the enzyme was collected by centrifugation at 10,000 × g for 20 min. The residue was dissolved in 60 ml of buffer B and dialyzed against 2 × 4 liters of buffer B for 20 h.

Step 4. 2',5'-ADP Sepharose Column—The dialyzed sample was clarified by centrifugation at 10,000 × g for 20 min and was applied (2 ml/min) to a 6-ml column of 2',5'-ADP-Sepharose equilibrated with buffer B. The column was washed with buffer B until the A280 of the eluant was below 0.1. The enzyme was eluted with 0.1 M NADPH. Fractions with enzymatic activity were pooled and precipitated with 60% ammonium sulfate.

Step 5. P-200 Column—The precipitate was collected by centrifugation at 10,000 × g for 10 min and was dissolved in 2 ml of buffer B. The enzyme was applied to a 130- × 1.5-cm column (2 ml/h) of Bio-Rad P-200 resin equilibrated in buffer B. The enzyme eluted in fractions (2 ml) 21-23 and was stored at 5 °C as a 60% ammonium sulfate precipitate.

**Determination of Enzyme-bound Flavin Concentration**

DHPDHase has multiple flavins per enzymic subunit and multiple iron sulfur prosthetic groups (2, 3). The enzyme-bound flavin of bovine liver DHPDHase was released from the protein by heating for 5 min at 100 °C. The free flavin had absorbance maxima at 447, 374, and 263 nm. The concentration of flavin released from the enzyme was calculated with an extinction coefficient for FMN at 450 nm of 12.5 mM⁻¹cm⁻¹ (17). On the basis of the flavin released from denatured enzyme, the ε274 (based on the released flavin) of purified enzyme was calculated to be 51 mM⁻¹cm⁻¹. 2.6 units/ml of DHPDHase corresponded to 1 μM enzyme-bound flavin. In most cases the concentration of DHPDHase was expressed in terms of units/ml. When necessary, the concentrations of DHPDHase in units/ml was converted to μM enzyme-bound flavin by dividing units/ml of DHPDHase by 2.

The compositions of the flavins released from the enzyme was determined by HPLC on a LiChroCART 250-4 RP-18 column from E. Merck (Darmstadt, Federal Republic of Germany) developed in 20 mM ammonium phosphate buffer at pH 2.5 with a gradient of acetonitrile from 0 to 50% over 30 min and a flow rate of 1 ml/min. The flavins were detected at 450 nm. Under these conditions FAD had a retention time of 12.3 min and FMN had a retention time of 13.4 min.

**Preparation of DHPDHase Modified with [6-3H]-Iodouracil**

DHPDHase (350 units) in 2.0 ml of buffer A and 2 mM DTT was reacted for 10 min at 37 °C in 380 nmol of [6-3H]-iodouracil (specific activity, 5 × 10⁶ cpmmol). The inactivated enzyme retained less than 5% of its original activity. Free radiolabel was separated from bound radiolabel by size-exclusion chromatography on a 1.5- × 15-cm column of P-6 resin equilibrated in 0.05 M NH4HCO3 and the radiolabeled enzyme (35 × 10⁶ cpmmol) was lyophilized.

**Carboxymethylation and Tryptic Digestion of Radiolabeled Enzyme**

The radiolabeled enzyme was dissolved in 2 ml of 0.5 M Tris-HCl at pH 8.0 with 6 mM guanidine hydrochloride and 2.7 mM EDTA (carboxymethylazation buffer (18)). 2-Mercaptoethanol (11 μl) was added to the dissolved enzyme and the mixture was flushed with N2 for 30 min. The anaerobic solution was boiled for 2 min and allowed to equilibrate to room temperature for 90 min at which time 0.5 ml of 0.5 M iodoacetic acid in the carboxymethylation buffer was added to the reduced protein. This solution was incubated in the dark for 20 min at room temperature. The reaction was then quenched by addition of 50 μl of 2-mercaptoethanol. The mixture was dialyzed twice against 1 liter of distilled H2O. The protein suspension was lyophilized and then suspended in 4 ml of 0.2 M NH4HCO3. The protein was dissolved at 37 °C with 1.6 mg of N-tosyl-L-phenylalanine chloromethyl ketone treated trypsin that was added in five aliquots over 90 min. Tryptatin was separated from the digested enzyme by chromatography in 0.2 M NH4HCO3 on a 1.5- × 14-cm column of P-6 resin. Fractions with radiolabel were pooled and lyophilized (2.8 × 10⁶ cpmmol).

**Separation of Tryptic Fragments by HPLC**

The tryptic peptides were resolved on a Waters μBondpak C18 column (0.46 × 25 cm) and a Waters 625 LC system (Milford, MA). The peptides were eluted from the C18 column at a flow rate of 1 ml/min and detected by absorbance at 215 nm with a Waters 484 tunable absorbance detector. Two solvent systems were used to develop the column. (a) Solvent system 1: the peptides were dissolved in 2 ml of 0.1% trifluoroacetic acid and absorbed onto the C18 column that had been equilibrated in 0.1% trifluoroacetic acid. After washing the column with 5 ml, the peptides were eluted with a linear gradient of 0-30% acetonitrile in 0.1% trifluoroacetic acid over 90 min. (b) Solvent system 2: the peptides were dissolved into 2 ml of 20 mM ammonium acetate at pH 5.5 and absorbed onto the C18 column that had also been equilibrated in the ammonium acetate buffer. The column was developed isocratically with 5% acetonitrile in the ammonium acetate buffer over 50 min.

Sequence analysis was performed on a Applied Biosystems 477A protein sequencer equipped with an on-line Applied Biosystems 120A analyzer (San Jose, CA).

**Mass Spectrometry**

Fast atom bombardment mass spectrometry was used to analyze 2 μl of peptide diluted with 1 μl of glycerol/thioglycerol (1:1) matrix. A VG70-70SQ high resolution mass spectrometer was used for the analysis and ionization was produced by a cesium ion gun operating.
at 30 kV. Mass spectral data were collected over the mass range 350-1500 Da in the Multichannel Analysis data acquisition mode.

**Data Analysis**

The decelerating time courses for oxidation of NADPH or reduction of 5-iodouracil were fitted to Equation 2 as follows:

\[
[\text{Product}] = A + B e^{-kt} + C t
\]  

(2)

where \( k_{\text{app}} \) (min\(^{-1}\)) was the first order rate constant for loss of activity, \( B \) (\( \mu \)M) was the amplitude of the burst in product formation and \( t \) was time (min). Since the concentration of product in these experiments was initially zero, the value of \( A \) was equal to the negative of the value of \( B \).

The dependence of the rate constant for inactivation of DHPDHase on 5-iodouracil (IU) concentration (\( \mu \)M) was fitted to Equation 3 as follows:

\[
k_{\text{app}} = \frac{A}{B + [\text{IU}]}
\]  

(3)

where \( A \) was extrapolated rate of inactivation (\( \mu \)M/min) at zero 5-iodouracil concentration and \( B \) was the apparent \( K_i \) (\( \mu \)M) for inhibition of the inactivation process by 5-iodouracil.

The constants defined in these equations were determined by an iterative nonlinear least-squares fitting of the data to these equations (19).

**RESULTS**

**Properties of DHPDHase Purified from Bovine Liver**—The results from an enzyme purification are summarized in Table I. The enzymic activity and protein in the initial homogenate was not assayed. The enzyme was purified 290-fold after the inactivation process by 5-iodouracil.

The ratio of reduced 5-iodouracil to inactivated DHPDHase, the ratio of reduced 5-iodouracil to inactivated enzyme (i.e. the stoichiometry for inactivation) should be independent of the reaction conditions. However, the amount of 5-iodouracil reduced by DHPDHase at the end of these reactions was increased 2lerd-fold as the concentration of DTT in the reaction mixture was increased from 2 to 20 mM (Fig. 1). Similarly, product formation increased 11-fold when the concentration of 5-iodouracil was increased from 5 to 150 \( \mu \)M (data not shown). These results suggested that the rate of release of the reactive product that inactivated the enzyme, presumably 5-iodo-5,6-dihydrouracil, was faster than the rate of inactivation of the enzyme. Consequently, the free product subsequently competed with 5-iodouracil for binding to the active site. Inactivation of the enzyme most likely occurred by covalent modification of an active site residue.

Since the reduced product should have chemical properties similar to those of iodoacetamide, it should rapidly alkylate thiolates. The reactivity of 5-iodo-5,6-dihydrouracil with DTT or glutathione in buffer A was a model for this reaction. The reaction of these thiols with 5-iodo-5,6-dihydrouracil was followed spectrophotometrically at 225 nm, which monitored the formation of iodide (20). The bimolecular rate constant for reaction of DTT with 5-iodo-5,6-dihydrouracil was 1.07 ±

### Table I

| Step                  | Volume | Protein | Activity | Specific activity |
|-----------------------|--------|---------|----------|------------------|
| 1. Homogenization     | 2500   | ND      | ND       | ND               |
| 2. Batch DE-52        | 650    | 9400    | 730      | 0.08             |
| 3. Ammonium sulfate   | 75     | 2500    | 720      | 0.29             |
| 4. 2',5'-ADP-Sepharose| 19     | 62      | 795      | 19               |
| 5. P-200 column       | 6      | 25      | 590*     | 23               |

\(^*\) ND, not determined.

\(^a\) NADPH oxidase activity was measured with 200 \( \mu \)M NADPH in buffer A at 37 °C. The activity was determined spectrophotometrically at 340 nm. One unit of NADPH oxidase oxidized 1 \( \mu \)mol of NADPH per h. This preparation of DHPDHase contained 20 units of NADPH oxidase activity.
would destroy. The enzyme was added to the reaction mixture before addition of the addition of more enzyme (this concentration of DTT When 5 mM DTT was added to this reaction mixture prior to the newly added enzyme was fully active (Fig. 2). When 5-iodouracil had built up. This possibility was examined by the product was expected to accumulate in the assay medium Drouracil was added after the concentration of 5-iodo-5,6-dihydrouracil, was released from the enzyme during the inactivation reaction. The free product either inactivated the enzyme or reacted with an exogenous nucleophile such as DTT.

Dependence of the Rate of Inactivation of DHPDHase on the Concentration of 5-iodouracil—The apparent rate constant for inactivation of DHPDHase by 5-iodouracil and 100 \( \mu M \) NADPH decreased from 10 min\(^{-1} \) to less than 0.3 min\(^{-1} \) as the concentration of 5-iodouracil was increased from 1 to 150 \( \mu M \) (Fig. 3). The maximum rate of inactivation of the enzyme at low concentration of 5-iodouracil was estimated to be 16 ± 3 min\(^{-1} \) from a fit of the data of Fig. 3 to Equation 3.

In contrast to these results, the rate constant for inactivation of an enzyme by a simple mechanism-based inactivator was expected to increase to a limiting value at high concentrations of inactivator. Since the initial velocity for reduction of 5-iodouracil by 0.14 units/ml DHPDHase decreased slightly from a value of 4.5 \( \mu M/min \) with 1 pM 5-iodouracil to 3.0 \( \mu M/min \) with 150 \( \mu M \) (data not shown), the inhibition of the rate of inactivation at high 5-iodouracil concentration is not likely due to substrate inhibition. These results suggest that 5-iodouracil and the inactivating species, enzymatically generated 5-iodo-5,6-dihydouracil, were competing for a common site.

DHPDHase was also inactivated by 5-iodouracil with DTT as the reductant. The first order rate constant for inactivation of 1.8 units/ml DHPDHase was measured at five concentrations of 5-iodouracil with 2 mM DTT as the reductant. The rate constant for inactivation decreased as the concentration of 5-iodouracil was increased (data not shown). These results were analogous to those found with NADPH as the reductant. The data were fitted to Equation 3 to yield a maximum rate constant for inactivation of 0.6 ± 0.2 min\(^{-1} \). The maximum rate constant for inactivation of the enzyme by 5-iodouracil in the presence of NADPH and DTT was 27-fold larger than that measured in the presence of DTT alone.

Effect of DTT on the Rate Constant for Inactivation of DHPDHase by 5-iodouracil—The rate constant for inactivation of DHPDHase by 20 \( \mu M \) 5-iodouracil and 100 \( \mu M \) NADPH decreased from a value of 1.3 min\(^{-1} \) at 2 mM DTT to less than 0.2 min\(^{-1} \) at 100 mM DTT. The initial velocity for enzymatic

0.02 mM\(^{-1} \) min\(^{-1} \). The analogous rate constant for the reaction with glutathione was 0.20 ± 0.01 mM\(^{-1} \) min\(^{-1} \).

5-Iodo-5,6-dihydouracil was relatively stable in buffer A in the absence of thiols (data not shown). Consequently, this product was expected to accumulate in the assay medium during the enzymic reduction of 5-iodouracil in the absence of DTT. If 5-iodo-5,6-dihydouracil was the species that inactivated the enzyme, the rate of inactivation of enzyme added initially should be slower than the rate of inactivation of enzyme added after the concentration of 5-iodo-5,6-dihydouracil had built up. This possibility was examined by interchanging the order of addition of DTT and DHPDHase to a reaction mixture that contained enzyme that had been inactivated with 5-iodouracil in the absence of DTT (Fig. 2). When 5 mM DTT was added to this reaction mixture prior to the addition of more enzyme (this concentration of DTT would destroy 5-iodo-5,6-dihydouracil with a \( t_0 \) of 0.1 min), the newly added enzyme was fully active (Fig. 2). When enzyme was added to the reaction mixture after addition of 5 mM DTT (i.e. 5-iodo-5,6-dihydouracil was not destroyed prior to addition of the second aliquot of enzyme), the newly added enzyme had greatly reduced activity (Fig. 2). These results demonstrated that a reactive product, presumably 5-iodo-5,6-dihydouracil, was released from the enzyme during the inactivation reaction. The free product either inactivated the enzyme or reacted with an exogenous nucleophile such as DTT.

FIG. 1. Effect of DTT on the time course for inactivation of DHPDHase by 5-iodouracil. The enzymatic reduction of 50 \( \mu M \) 5-iodouracil by 200 \( \mu M \) NADPH in buffer A was followed at 283 nm. The reactions were initiated with 0.14 units/ml DHPDHase. Fresh enzyme (+E) was added to the reaction mixture with 2 mM DTT after 6 min.

FIG. 2. Release of a reactive product during the inactivation of DHPDHase by 5-iodouracil. DHPDHase was inactivated with 50 \( \mu M \) 5-iodouracil and 100 \( \mu M \) NADPH in two separate reactions. The reactions were initiated with enzyme, which was activated by storage in 5 mM DTT, by a 100-fold dilution to give a final enzyme concentration of 0.08 units/ml and a DTT concentration less than 0.05 mM. The reduction of 5-iodouracil was followed at 283 nm. To one reaction mixture 5 mM DTT was added at 8 min (—–) followed by 0.08 units/ml DHPDHase at 13 minutes. To the other reaction mixture (-----) 0.08 units/ml DHPDHase was added at 12 min followed by 5 mM DTT at 13 min. At 15 min the total additions, but not the order of additions, to the two reactions were identical.

FIG. 3. Dependence of the first order rate constant for inactivation of DHPDHase \( (k_{in}) \) on the concentration of 5-iodouracil. The time courses for reduction of 5-iodouracil by 100 \( \mu M \) NADPH and 2 mM DTT with 0.14 units/mg DHPDHase in buffer A were followed at 340 nm. These data were fitted to Equation 3 to calculate the first order rate constant describing the transition from active to inactive enzyme \( (k_{in}) \) with different concentration of 5-iodouracil. The figure shows the dependence of \( k_{in} \) on 5-iodouracil concentration and the line was calculated from a fit of this data to Equation 3 with a value for \( A \) of 35 ± 0.5 \( \mu M \) min\(^{-1} \) and a value for \( B \) of 2.2 ± 0.4 \( \mu M \). The inset is an expansion of the initial portion of the curve.
Inactivation of Dihydropyrimidine Dehydrogenase

Reduction of 5-iodouracil did not change over this concentration range of DTT. These results provide further evidence that the reactive product of 5-iodouracil reduction, 5-iodo-5,6-dihydrouracil, partitioned between inactivation of the enzyme and reaction with DTT.

**Stoichiometry for Inactivation of DHPDHase by 5-Iodouracil**—The ratio of 5-iodouracil reduced to DHPDHase inactivated was approximately 30 nmol/unit (0.14 units/ml of DHPDHase was inactivated by the reduction of 4.4 μM 5-iodouracil (Fig. 1)). However, interpretation of this result was complicated by the effects of DTT and 5-iodouracil on the inactivation reaction. For instance, the stoichiometry for inactivation was increased 2.4-fold by increasing the concentration of DTT from 2 to 20 mM (Fig. 1) and it was increased 11-fold by increasing the concentration of 5-iodouracil from 5 to 150 μM. To circumvent these problems, the enzymatic activity was titrated with 5-iodouracil at elevated concentrations of enzyme. In a typical experiment, aliquots of DHPDHase were reacted with different amounts of 5-iodouracil and the enzymatic activity was determined at the end of the inactivation reactions. Since the concentration of free 5-iodouracil in these experiments was effectively zero throughout the titration, the effects that elevated concentrations of 5-iodouracil could have on the inactivation process were minimized. Furthermore, at high enzyme concentration, 5-iodo-5,6-dihydrouracil that dissociated from the enzyme was more likely to react with enzyme than the exogenous nucleophile DTT.

DHPDHase was completely inactivated by 1.7 mol of 5-iodouracil per mol of enzyme-bound flavin (Fig. 4). Titration of enzymatic activity with 5-iodouracil decreased linearly as the concentration of 5-iodouracil was increased (Fig. 4). If DTT was competing effectively with the enzyme for the 5-iodo-5,6-dihydrouracil released during the course of this titration, significant deviations from linearity should have occurred toward the end of the titration. Consequently, the trapping of 5-iodouracil by DTT was minimal.

When excess DHPDHase (12 μM enzyme-bound flavin) was reacted with 2 mM DTT and limiting [6-3H]5-iodouracil (0.7 μM), 18% of the radiolabel was bound to the protein. The bound radiolabel was not displaced by incubation with 200 μM unlabeled 5-iodouracil or by heating at 100 °C for 10 min in 0.1 N HCl. Since tritium was not released from radiolabeled 5-iodouracil during the inactivation process, the bound radiolabel provided a good estimate for the amount of inactivator bound to the enzyme. These results show that the radiolabel was covalently linked to the protein and, assuming one inactivator molecule was bound per active site, the stoichiometry for inactivation (mol of inactivator consumed per mol of enzymic activity site inactivated) was 5.5.

Since the stoichiometry for inactivation was 5.5 and 1.7 mol of inactivator was consumed per mol of enzyme-flavin (Fig. 4), the ratio of enzyme-bound flavin to covalently bound 5-iodouracil was 3.2. This result suggested that either 3.2 flavins were at each catalytic site or not all the flavins were catalytically active.

**Stoichiometry for Inactivation of DHPDHase by 5-Iodo-5,6-dihydrouracil**—The product from the enzymatic reduction of 5-iodouracil by DHPDHase is 5-iodo-5,6-dihydrouracil. Racemic 5-iodo-5,6-dihydrouracil was synthesized and was shown to inactivate the enzyme. DHPDHase activity decreased linearly with increasing concentrations of 5-iodo-5,6-dihydrouracil in 2 mM DTT (Fig. 4). The linearity of the titration demonstrated that DTT did not compete effectively with DHPDHase for reaction with 5-iodo-5,6-dihydrouracil. DHPDHase was completely inactivated under these conditions by 0.67 mol of 5-iodo-5,6-dihydrouracil per mol flavin (Fig. 4). This corresponded to a stoichiometry of 2.1 mol inactivator per active site (3.2 × 0.67). The optical spectrum of DHPDHase inactivated with 5-iodouracil was the same as that for DHPDHase inactivated with 5-iodo-5,6-dihydrouracil (Fig. 5).

**Sequence of Radiolabeled Peptides Isolated from a Tryptic Digestion of [6-3H]5-Iodouracil-inactivated DHPDHase**—Inactivation of 135 nmol of DHPDHase (based on flavin) with [6-3H]5-iodouracil as described under "Experimental Procedures" resulted in the covalent incorporation of 44 nmol of radiolabel into the enzyme. As in previous experiments the ratio of the flavin content to radiolabel bound to inactivated enzyme.

![Fig. 4. Titration of DHPDHase by either 5-iodouracil or 5-iodo-5,6-dihydrouracil with 2 mM DTT as the reducing substrate.](image)

![Fig. 5. Comparison of the optical spectra of 5-iodouracil-inactivated DHPDHase and 5-iodo-5,6-dihydrouracil-inactivated DHPDHase. The sample and reference cuvettes contained 28 units/ml DHPDHase and 10 mM DTT in buffer A. The difference spectra were recorded 10 min after incubation of the enzyme in the sample cuvette with either 100 μM 5-iodouracil (dashed line) or 25 μM 5-iodo-5,6-dihydrouracil (solid line).](image)
enzyme was 3.1. A tryptic digest of inactivated DHPDHase was prepared as described under "Experimental Procedures" and was purified by HPLC on a C18 column with solvent system 1. Fractions 33-36 were pooled as peptide 1 and fractions 37-38 were pooled as peptide 2. Peptide 1 and peptide 2 were chromatographed on the same column equilibrated in 20 mM ammonium acetate and developed with solvent system 2 (chromatogram not shown). The peptide 1, which was purified further as described in the text, and fraction 33 from peptide 2 were sequenced.

**Fig. 6. Fractionation of peptides from the tryptic digest of [6-3H]5-iodouracil-inactivated DHPDHase.** A tryptic digest of [6-3H]5-iodouracil-inactivated DHPDHase was prepared as described under "Experimental Procedures." A, one-half of the tryptic digest was separated by reversed-phase chromatography on a C18 column with solvent system 1. Fractions 33-36 were pooled as peptide 1 and fractions 37-38 were pooled as peptide 2. B, peptide 1 and peptide 2 were chromatographed on the same column equilibrated in 20 mM ammonium acetate and developed with solvent system 2 (chromatogram not shown). The fraction from each purification with the maximum amount of radiolabel was rechromatographed in this solvent system. Fraction 27 from peptide 1, which was purified further as described in the text, and fraction 33 from peptide 2 were sequenced.

**Peptide 1**

| Cycle | Amino Acid | pmol | cpm |
|-------|------------|------|-----|
| 1     | Asn        | 21   | 0   |
| 2     | Leu        | 35   | 6   |
| 3     | Ser        | 9    | 15  |
| 4     | X          | 705  |     |
| 5     | Pro        | 16   | 132 |
| 6     | His        | 2    | 57  |

**Peptide 2**

| Cycle | Amino Acid | pmol | cpm |
|-------|------------|------|-----|
| 1     | Asn        | 58   | 0   |
| 2     | Leu        | 77   | 0   |
| 3     | Ser        | 27   | 30  |
| 4     | X          | 2680 |     |
| 5     | Pro        | 56   | 180 |
| 6     | His        | 12   | 87  |
| 7     | Gly        | 42   | 33  |
| 8     | Met        | 7    | 45  |
| 9     | Gly        | 40   | 27  |
| 10    | Glu        | 8    | 15  |
| 11    | Arg        | 10   | 21  |

* The peptide sequenced contained 12 pmol of radiolabel based upon the specific radioactivity of 5-iodouracil (8 × 10⁵ cpm/nmol) and the total radioactivity recovered in cycles 3-6.

**DISCUSSION**

Cooper and Greer (12) have shown that rat liver supernatants catalyze the reduction of 5-iodouracil by NADPH. They also noted that the rate of product formation decelerates to zero prior to depletion of the substrate. Addition of a second aliquot of the rat liver supernatant to this reaction mixture causes a second burst of product formation. Since this activity is stable to prolonged dialysis, these observations suggest that 5-iodouracil is an inactivator of this activity. Other investigators have reported that 5-iodouracil is a substrate for DHPDHase but have not commented on inactivation of the enzyme by this substrate (2, 21). The present work has demonstrated unequivocally that 5-iodouracil was a substrate and a time-dependent inactivator of bovine liver DHPDHase. Inactivation of the enzyme by 5-iodouracil required a reducing substrate, but the rate constant for inactivation decreased with increasing concentrations of 5-iodouracil and DTT concentrations greater than 2 mM. These results demonstrate that the enzyme was inactivated by a reactive product that could dissociate from the enzyme. The product that inactivated the enzyme was 5-iodo-5,6-dihydrouracil.

DHPDHase catalyzed the oxidation of NADPH by 100 μM thymine with a Km of 1.5 μM and a kcat (based on enzyme-bound flavin) of 33 min⁻¹. DTT was an alternative substrate for NADPH in this reaction with a Km of 2.2 mM and a kcat (based on enzyme-bound flavin) of 4.3 min⁻¹. Furthermore, DHPDHase was inactivated by 5-iodouracil and DTT in the absence of NADPH. Presumably, DTT served as the reducing substrate in the inactivation reaction. However, the rate constant for inactivation of DHPDHase by 5-iodouracil was 27-fold larger with 100 μM NADPH as the reductant than with 2 mM DTT as the reductant. By comparison, the enzyme catalyzed the reduction of thymine 17-fold faster with 100 μM thymine.
NADPH as the reductant than with 2 mM DTT. This correlation suggested that reduction of enzyme was the rate-limiting step in the reduction of thymine and 5-iodouracil.

The active site concentration of DHPDHase was initially assumed to be equal to the concentration of flavin that was released by heat denaturation of the enzyme. However, comparison of the titration data for 5-iodouracil with the fraction of 5-iodouracil bound to the enzyme indicated that there were 3.2 mol of flavin per mol of radiolabeled product covalently linked to the enzyme. This implied that there were multiple flavins per active site or some of the enzyme-bound flavin was catalytically inactive. Since the enzyme contained FAD and FMN in nearly equal amounts, it was probable that there were multiple flavins per active site. Porcine liver DHPDHase also contains an iron-sulfur center and multiple flavins per subunit (3).

DHPDHase was completely inactivated by 0.67 mol of racemic 5-iodo-5,6-dihydouracil per mol of enzyme-bound flavin. This was equivalent to 2.1 (0.67 × 3.2) mol of racemic inactivator per mol of active site. Since DHPDHase probably catalyzed the stereospecific reduction of 5-iodouracil to 5-iodo-5,6-dihydouracil, the finding that the stoichiometry for inactivation of DHPDHase by racemic 5-iodo-5,6-dihydouracil (2.1) was less than that for the enzymatically generated enantiomer (5.5) suggested that the nonenzymatically generated enantiomer was a more potent inactivator of the enzyme.

The stoichiometry for the nonenzymatically generated enantiomer was calculated to be 1.3.3 Since the stoichiometry for inactivation of DHPDHase by the enzymatically generated enantiomer was 5.5, there was partitioning of this enantiomer between inactivation of the enzyme and conversion to a product that did not inactivate the enzyme. Possible mechanisms for deactivation of 5-iodo-5,6-dihydouracil are: 1) the reductive dehalogenation to 5,6-dihydouracil that is analogous to the reductive debromination of 6-(bromo-methyl)purine to 6 methylpurine catalyzed by xanthine oxidase (22) or the reductive deiodination of 5-iodouracil catalyzed by thymidylate synthetase (23), and 2) the elimination of HI to form uracil (24).

Preliminary studies have shown that uracil and rhodouracil were products of the reaction of racemic 5-iodo-5,6-dihydouracil with DHPDHase. UV difference spectra data (Fig. 5) indicate that the enzymatically generated isomer of 5-iodo-5,6-dihydouracil and the chemically synthesized racemic inactivator modified the same active site residue. This result suggests that the enantiomers either bind as mirror images or they bind similarly and displacement of the iod group occurred from different sides of the molecule. Since high concentration of DTT or 5-iodouracil afforded nearly complete protection against inactivation by 5-iodouracil, it appeared that dissociation of the enzymatically generated enantiomer of 5-iodo-5,6-dihydouracil might be required for inactivation. If this were the case, rebinding of this isomer to DHPDHase might have correctly positioned the iod group for nucleophilic attack.

Inactivation of DHPDHase by radiolabeled 5-iodouracil resulted in covalent binding of radiolabel to inactivated enzyme. The amino acid sequence of the smaller tryptic fragment was Asn-Leu-Ser-X-Pro-His—in which X was the radiolabeled amino acid. Data from fast atom bombardment mass spectrophotometric analysis of this tryptic fragment were consistent with the unknown amino acid residue, X, being 5-(hexahydro-2,4-dioxo-5-pyrimidinyl)cysteinyl residue. Since the pyrimidine ring was reduced in this adduct, it was reasonable to suppose that enzymatic reduction of the pyrimidine ring preceded displacement of the iod group and subsequent inactivation of the enzyme (Equation 1).

In conclusion, 5-iodouracil was an effective inactivator of DHPDHase at low concentrations. Thus, the elevated plasma levels of uracil and thymine that occurred during infusion of 5-iodo-2′-deoxyuridine into human subjects (13) could have resulted, at least in part, from inactivation of the enzyme by enzymatically generated 5-iodo-5,6-dihydouracil.

Acknowledgments—We thank Dr. J. Dev and B. Yates for advice on the tryptic digestion of the enzyme, and Dr. John Bearden and Dr. Robert Morrison for helpful discussions during the course of this work.

REFERENCES

1. Wasternack, C. (1980) Pharmacol. Ther. 8, 629-651
2. Shiotani, T., and Weber, G. (1981) J. Biol. Chem. 256, 219-224
3. Podschun, B., Wahler, G., and Schnackerz, K. D. (1980) Eur. J. Biochem. 185, 219-224
4. Woodcock, T. M., Martin, D. S., and Damin, L. E. M. (1980) Cancer 45, 1135-1143
5. Diario, R., Beavers, T. L., and Carpenter, J. J. (1988) J. Clin. Invest. 81, 47-51
6. Harris, B. E., Song, R., Soong, S., and Diasi, R. B. (1990) Cancer Res. 50, 197-201
7. Tatsumi, K., Fukushima, M., Shirasaka, T., and Fujii, S. (1987) Jpn. Cancer Res. 78, 748-755
8. Iigo, M., Araki, E., Nakajima, Y., Hoshi, A., and De Clercq, E. (1988) Biochem. Pharmacol. 37, 1609-1613
9. Iigo, M., Nishihata, K., Nakajima, Y., Hoshi, A., Okudaira, N., Odagiri, H., and De Clercq, E. (1989) Biochem. Pharmacol. 38, 1885-1889
10. Degranges, C., Razaga, G., de Clercq, E., Herdevijio, P., Balzarini, J., Droullet, E., and Brack, H. (1986) Cancer Res. 45, 1094-1101
11. Naguib, F. N. M., el Kouni, M. H., and Cha, S. (1989) Biochem. Pharmacol. 38, 1471-1480
12. Cooper, G. M., and Greer, E. (1970) Cancer Res. 30, 2937-2941
13. Klecker, R. W., Jr., Jenkins, J. F., Kinsella, T. J., Fine, R. L., Strong, J. M., and Collins, J. M. (1982) Clin. Pharmacol. Ther. 38, 45-51
14. Barrett, W. H., Munavalli, S. N., and Newman, P. (1964) Biochem. Biophys. Acta 91, 199-204
15. Zee-Cheng, K., Robins, R. K., and Cheng, C. C. (1961) J. Org. Chem. 26, 1877-1884
16. Rok, G. S., and Pitman, I. H. (1975) J. Am. Chem. Soc. 97, 5566-5572
17. Gunasekera, S., Massey, V., Lhoste, J. M., and Mayhew, S. G. (1974) Biochemistry 13, 589-597
18. Dev, I. K., Yates, B. B., Atashi, J., and Dallas, W. (1989) J. Biol. Chem. 264, 19132-19137
19. Bevington, P. R. (1969) Data Reduction and Error Analysis for the Physical Sciences, pp. 204-246, McGraw-Hill, New York
20. Sander, E. G., Sedor, M., and Young, E. (1976) Bioorg. Chem. 5, 231-239
21. Dorsett, M. T., Morse, P. A., Jr., and Gentry, G. A. (1969) Cancer Res. 29, 79-82
22. Porter, D. J. T. (1990) J. Biol. Chem. 265, 13540-13546
23. Wataha, Y., and Santi, D. V. (1975) Biochem. Biophys. Res. Commun. 67, 818-823
24. Barrett, W. H., and West, R. A. (1986) J. Am. Chem. Soc. 108, 1612-1615

1 The stoichiometry was calculated as follows. Since synthesized 5-iodo-5,6-dihydouracil was an equal mixture of the two isomers, the enantiomer corresponding to enzymatically generated 5-iodo-5,6-dihydouracil (1.05 mol (2.1/2)) inactivated 0.19 mol of enzyme (1.05/5.5). This meant that 0.81 mol (1.00 - 0.19) of enzyme was inactivated by 1.05 mol (2.1/2) of the opposite enantiomer to yield a stoichiometry of 1.3 (1.05/0.81).
2 D. J. T. Porter, unpublished observations.