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Pyrimidine Catabolism: Individual Characterization of the Three Sequential Enzymes with a New Assay†

Thomas W. Traut* and Steven Loechel

ABSTRACT: We have developed a one-dimensional thin-layer chromatography procedure that resolves the initial substrate uracil and its catabolic derivatives dihydrouracil, *N*-carbamoyl-β-alanine (NCBA) and β-alanine. This separation scheme also simplifies the preparation of the radioisotopes of *N*-carbamoyl-β-alanine and dihydrouracil. Combined, these methods make it possible to assay easily and unambiguously, jointly or individually, all three enzyme activities of uracil catabolism: dihydropyrimidine dehydrogenase, dihydropyrimidinase, and *N*-carbamoyl-β-alanine amidohydrolase. Earlier reports had presented data suggesting that these three enzyme activities were combined in a complex because they appeared to be controlled at a single genetic locus [Dagg, C. P., Coleman, D. L., & Fraser, G. M. (1964) *Genetics 49*, 979–989] and because they appeared able to channel metabolites [Barrett, H. W., Munavalli, S. N., & Newmark, P. (1964) *Biochim. Biophys. Acta 91*, 199–204]. Although the three enzymes from rat liver have similar sizes, with apparent molecular weights of 218 000 for dihydropyrimidine dehydrogenase, 226 000 for dihydropyrimidinase, and 234 000 for NCβA amidohydrolase, they are easily separated from each other. Kinetic studies show no evidence of substrate channeling and therefore do not support a model for an enzyme complex. The earlier reports may be explained by our studies on the amidohydrolase, which suggest that under certain conditions this enzyme may become the rate-limiting step in uracil catabolism.

The catabolism of pyrimidines proceeds in three sequential steps, as illustrated in Figure 1. The physiological importance of this pathway is indicated by whole animal studies with mice (Sonoda & Tatibana, 1978), where over 80% of orally ingested [2-^{14}C]uracil is degraded and excreted in 8 h, about 50% as $^{14}CO_2$ and the rest as dihydrouracil and *N*-carbamoyl-β-alanine (NCβA)[1] in urine. With [2-^{14}C]uracil as the initial substrate, it is easy to determine the presence of all three enzyme activities by measuring the production of $^{14}CO_2$. This approach has established the existence of this pathway in rat liver (Canellakis, 1957; Fritzson, 1957), mouse liver (Dagg et al., 1964), regenerating rat liver (Ferdinandus et al., 1971), and rat hepatomas (Weber et al., 1971) as well as in microorganisms such as *Escherichia coli* (Simaga & Kos, 1981) and *Euglena gracilis* (Wasternack & Reinbothe, 1977). Since the first enzyme of the pathway is an NADPH-dependent

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[1] Abbreviations: NCβA, *N*-carbamoyl-β-alanine; pDAB, *p*-(dimethylamino)benzaldehyde; PMSF, phenylmethanesulfonyl fluoride; TLC, thin-layer chromatography; DHU, dihydrouracil; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; UMP, uridine 5′-phosphate.

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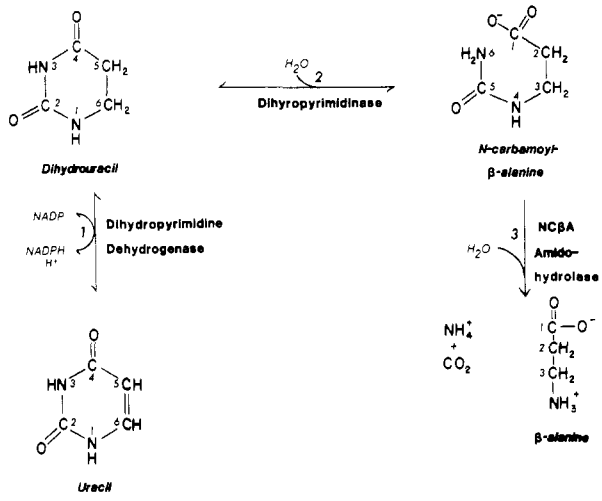

FIGURE 1: Pathway of pyrimidine catabolism. Illustrated here is the three-step sequence for the catabolism of uracil to β-alanine plus CO_2 and NH_4^+. Note that if uracil is labeled at carbon 2, then $^{14}CO_2$ will be produced; uracil tritiated at carbons 5 or 6 will produce β-[^{3}H]-alanine. A variety of trivial names are in use for each of the three enzymes: enzyme 1, dihydropyrimidine dehydrogenase, dihydrouracil dehydrogenase, dihydrothymine dehydrogenase, pyrimidine reductase, and uracil hydrogenase; enzyme 2, dihydropyrimidinase and dihydropyrimidine hydrase; enzyme 3, NCβA amidohydrolase, ureidopropionase, and β-ureidopropionic acid decarbamoylase.

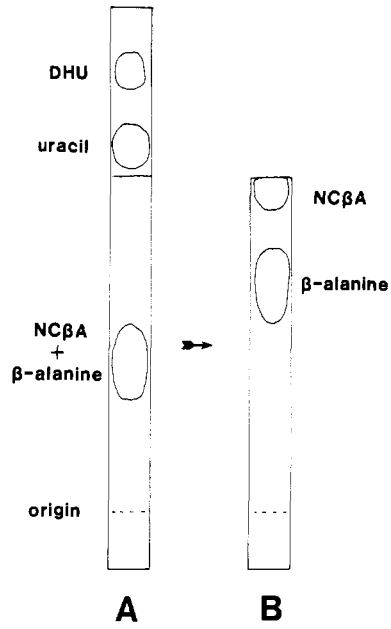

FIGURE 2: Separation of uracil and its metabolites by thin-layer chromatography on DEAE-cellulose. Lane A shows the separation obtained with solvent 1; to separate β-alanine from *N*-carbamoyl-β-alanine (NCβA), the plate is cut in half below the uracil spot, as indicated; the lower half is developed with solvent 2 to produce the separation shown in lane B. DHU is dihydrouracil.

dehydrogenase (EC 1.3.1.2), it can easily be measured spectrophotometrically and has been detected in many tissues: in the liver, thymus, intestine, spleen, kidney, brain, and muscle of rat (Queener et al., 1971) and in human leukocytes (Marsh & Perry, 1964).

Because of the lack of commercially available radioactive substrates, the other two enzymes have often been measured by less efficient colorimetric assays. Dihydropyrimidinase (EC 3.5.2.2) has been detected only in liver and kidney of rat, guinea pig, rabbit, and dog as well as in mouse liver (Dudley et al., 1974). It was not detected in lung, spleen, brain, intestine, or muscle of any of these species (Dudley et al., 1974). NCβA amidohydrolase (EC 3.5.1.6) activity has only been determined in liver of rats (Canellakis, 1956; Fritzson, 1957; Carvaca & Grisolia, 1958; Sanno et al., 1970) and mice (Dagg et al., 1964; Sanno et al., 1970). Two very comprehensive reviews on this subject have recently been published (Wasternack, 1978, 1980).

Two earlier reports suggested that all three enzyme activities might normally function in a complex (Dagg et al., 1964; Barret et al., 1964). Dagg et al. (1964) had shown that a mutation at a single locus in mice appeared to alter the activities of all three enzymes. This result could only be explained (1) if all three activities were on a single multifunctional protein (a single gene product), (2) if the enzymes functioned in a complex and a mutation in one enzyme altered the activity of the other two in the complex, or (3) if the third enzyme, the amidohydrolase, were rate limiting, since Dagg et al. (1964) used $^{14}CO_2$ production to measure each of the three enzyme activities (since this is a sequential assay, any deleterious mutation in the amidohydrolase would also produce a decrease in the observed activities of the first two enzymes). Sanno et al. (1970) disproved the first interpretation, and several reports showed that the first enzyme, dihydropyrimidine dehydrogenase, should be rate limiting (Canellakis, 1956; Fritzson, 1957, 1962). The second report suggesting an enzyme complex (Barret et al., 1964) showed that rat liver preparations could convert [2-^{14}C]uracil to $^{14}CO_2$ without any decrease if either dihydrouracil or NCβA was present at 156 μM: such channeling of metabolites implies an enzyme complex or a multifunctional protein.

In the present study, we have developed procedures for assaying each enzyme activity separately. Our thin-layer chromatography method that makes it possible to separate uracil, dihydrouracil, NCβA, and β-alanine also makes it easier to synthesize the intermediate compounds in radioactive form. Preparation of [5-^{14}C]NCβA enables the direct assay of NCβA amidohydrolase. In a similar fashion, [2-^{14}C]dihydrouracil can be used to assay dihydropyrimidinase in preparations that also contain the amidohydrolase, while [6-^{3}H]dihydrouracil makes possible the assay of dihydropyrimidinase without dependence on the amidohydrolase.

Experimental Procedures

Materials

[2-^{14}]Uracil, [6-^{3}H]uracil, K^{14}CNO, and β-[3-^{3}H]alanine were purchased from New England Nuclear. NADPH and nonradioactive substrates were from Sigma. Ultrogel AcA34 was obtained from LKB, and DEAE-cellulose plates were from Brinkmann. All other compounds were of the best reagent grade available.

Methods

Thin-Layer Chromatography. DEAE-cellulose plates (20 cm × 20 cm) were lightly scored with a thumbtack to produce 15 lanes. Samples were spotted at the origin, 2 cm from the bottom. Plates were then developed in solvent 1, composed of *tert*-butyl alcohol, methyl ethyl ketone, H_2O and NH_4OH (40:30:20:10 v/v), by ascending chromatography until the solvent reaches the top (about 4.5 h). This will separate both dihydrouracil and uracil (R_f 0.88 and 0.73, respectively) from NCβA and β-alanine (R_f 0.30 and 0.29, respectively).

When it is desirable to separate NCβA from β-alanine, the following additional steps are necessary. The uracil spot is readily visible under UV light; the plate was cut in half just below this spot. The bottom half was then developed 3–4 times in solvent 2 containing the upper phase of ethyl acetate, H_2O, and formic acid (60:35:5 v/v). This will cause NCβA to move

up the plate, away from β-alanine (Figure 2). Samples to be spotted should not be much larger than 10 μL; 5 μL works very well. Too much glycerol in the sample interferes with the resolution: a 10-μL sample must not contain more than 2% glycerol.

The separated compounds can be detected on the TLC plate as follows. Uracil is directly visible under UV light. The β-alanine spot becomes visible after spraying with ninhydrin (0.3% ninhydrin in 95% ethanol) and drying the plate at 60 °C for 5 min. Dihydrouracil must first be converted to NCβA in situ: it was sprayed with 0.5 N NaOH and allowed to dry, and this step was repeated. After the spot was allowed to dry at least 30 min, it was sprayed with pDAB. NCβA was also detected by spraying with pDAB. After spots were made visible, they were cut out of the TLC sheet and placed in scintillation vials to quantitate the product. The best visualization of all spots is obtained if the plate is first sprayed with ninhydrin, NaOH, and then pDAB.

Synthesis of Radioactive Substrates. The methods described here are adapted from the procedures of Lengfeld and Stieglitz. To synthesize [5-^{14}C]NCβA, equimolar amounts (usually 0.5 M final concentration) of β-alanine and K^{14}CNO (5 Ci/mol) were mixed in 200 μL. The solution was heated at 65 °C for several hours (usually overnight). Recrystallization of NCβA from this solution proved to be difficult; therefore, the resultant syrupy liquid was dissolved in a small amount of H_2O, applied to a DEAE-cellulose plate, and developed with solvent 2 as described above. The plate carried marker NCβA in the outside lanes to detect the position of NCβA after development; NCβA spots were cut out and put in glass vials. After 1 mL of 10 mM Tris (pH 7.4) was added, sample spots were heated at 70 °C for several minutes. The liquid was removed with a syringe. Two or three additional washes were usually necessary to extract all radioactivity.

Synthesis of [6-^{3}H]dihydrouracil started with commercial β-[3-^{3}H]alanine (30 Ci/mol) and KCNO to produce [^{3}H]-NCβA as described above, except that the solution was heated at 65 °C until dry. One hundred microliters of 0.1 N HCl was added, and the solution was heated in a water bath at 100 °C (this step should be done in a hood since cyanide will be produced). After taking to dryness, it was dissolved in a small volume of H_2O and applied to a DEAE-cellulose plate. The plate was developed with solvent 1; dihydrouracil spots were cut out and eluted with H_2O.

The purity of synthesized compounds was estimated by two procedures. Compounds were rechromatographed as described above with or without authentic standards. In all cases, the radioactive compounds had R_f values identical with those of the standards. Autoradiography of the chromatogram showed that at least 97% of the radioactivity was in the product spot. Additional tests showed essentially complete (90–96%) conversion of these compounds to appropriate products when incubated with a preparation containing all three catabolic enzymes.

Preparation of Enzymes. Freshly excised rat livers were placed in 2 volumes of ice-cold medium containing 0.25 M sucrose, 20 mM Tris-HCl, pH 7.4 at 37 °C, 10 mM β-mercaptoethanol, 0.1 mM EDTA, and the protease inhibitors α_1-antitrypsin (25 mg/I), PMSF (1 mM), and aprotinin (70 units/L). After being homogenized with a motorized homogenizer, the slurry was centrifuged at 12000*g* for 5 min. The supernatant was then centrifuged at 112000*g* for 60 min. The final supernatant was then dialyzed for 2 h against 100 volumes of buffer containing 50 mM Tris-HCl, pH 7.4 at 37 °C, 20% glycerol (v/v), and 1 mM dithiothreitol. Enzyme preparations were usually stored at >10 mg of protein/mL. Such preparations had no loss of any enzyme activity for several months when stored at −70 °C and lost <20% activity if kept at 4 °C for 2–3 days.

Enzyme Assays. All assays were done at 37 °C in a buffer containing 50 mM Tris-HCl, pH 7.4, and 2 mM dithiothreitol. Reaction volumes were 0.5 mL when the product to be measured was $^{14}CO_2$ (with the substrates [2-^{14}C]uracil or [5-^{14}C]NCβA) or 50 μL when the products were analyzed by thin-layer chromatography (with the substrates [6-^{3}H]uracil or [6-^{3}H]dihydrouracil). Reference to Figure 1 will show that the entire pathway (i.e., all three sequential enzyme activities) can be easily estimated by measuring the production of $^{14}CO_2$ from [2-^{14}C]uracil, using the procedure of Jones et al. (1978) for quantitating $^{14}CO_2$. A more accurate measure of the first enzyme, dihydropyrimidine dehydrogenase, uses [6-^{3}H]uracil, since this allows measurement of the final product and of the intermediates. For these assays, NADPH was at 1 mM, and the second substrate was either [2-^{14}C]uracil (0.5 Ci/mol) at 400 μM or [6-^{3}H]uracil (40 Ci/mol) at 200 μM.

The second enzyme, dihydropyriminidase, was assayed with the substrate [6-^{3}H]dihydrouracil (25 Ci/mol) at 200 μM. NCβA amidohydrolase was measured by the CO_2 assay with [5-^{14}C]NCβA (0.2 Ci/mol) at 400 μM. For all kinetic studies, the reaction mixture and enzyme sample were separately preincubated for 2 min at 37 °C before the addition of enzyme to start the assay.

Molecular Sieve Chromatography. Chromatography was done at 4 °C in column buffer containing 50 mM Tris-HCl (pH 7.4 at 37 °C), 2 mM dithiothreitol, glycerol at 10% (v/v), and effectors as indicated on an Ultrogel AcA34 column (1.6 cm × 90 cm). Normally 2 mL of enzyme preparation (≥10 mg of protein/mL) was applied to the column and eluted at 10 mL/h. For the calculation of the apparent molecular weight, the column was calibrated with ferritin (M_r 467000), lactate dehydrogenase (M_r 140000), and hemoglobin (M_r 64500).

Results

To explore the existence of an enzyme complex, we prepared a rat liver cytosol fraction that was minimally purified. To increase the chances for detecting the proposed complex, it was necessary that all three activities remain stable under the conditions required for sedimentation or gel filtration studies. When maintained in 50 mM Tris buffer (pH 7.4 at 37 °C) supplemented with glycerol (10% v/v) and either β-mercaptoethanol (10 mM) or dithiothreitol (2 mM), none of the enzymes lost more than 20% of their activity when maintained at 4 °C for 3 days.

The migration of the three enzyme activities on an Ultrogel AcA34 column (Figure 3) shows that the elution of all three is very similar, but not identical, suggesting that the enzymes are migrating separately. Additional studies demonstrated that each of the three enzymes could readily be separated from the others by differential precipitation with ammonium sulfate: the precipitation range as a function of the percent saturation of ammonium sulfate was 30–45% for the dehydrogenase, 35–50% for the amidohydrolase, and 50–60% for the dihydropyrimidinase.

Kinetic studies with our TLC method make it possible to monitor all three enzyme activities simultaneously. Thus, [6-^{3}H]uracil is rapidly catabolized to the final end product β-alanine (Figure 4). Under the conditions of this experiment, the first enzyme, dihydropyrimidine dehydrogenase, is clearly rate limiting since there is little accumulation of the two intermediate compounds, dihydrouracil and NCβA. The con-

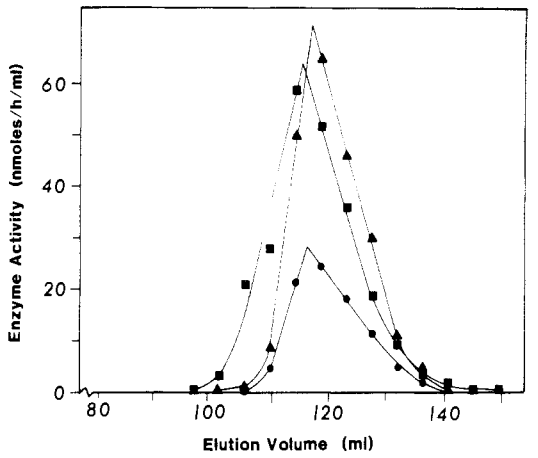

FIGURE 3: Gel filtration chromatography of the three catabolic enzymes. The enzymes were eluted from an Ultrogel AcA34 column (1.6 cm × 90 cm) and assayed separately. The following enzyme activities were recovered: dihydropyrimidine dehydrogenase (●), 90%; dihydropyrimidinase (▲), 95%; NCβA amidohydrolase (■), 76%.

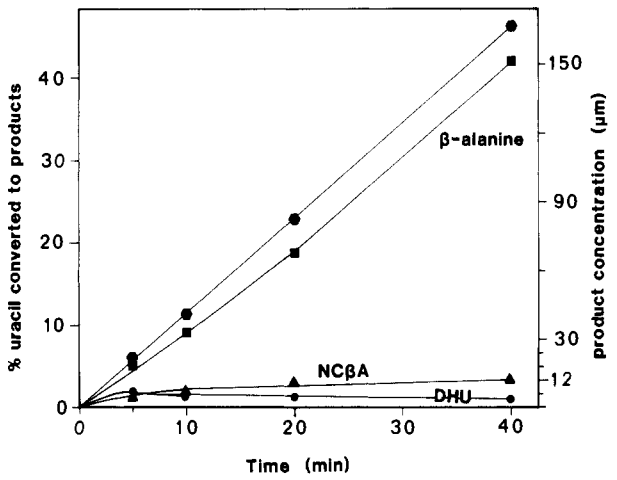

FIGURE 4: Progress curves for the complete catabolic sequence. The catabolism of [6-^{3}H]uracil leads to the formation of low levels of the intermediates, [6-^{3}H]dihydrouracil and [3-^{3}H]NCβA (steady-state concentrations are indicated on the right ordinate), and steady-state formation of the final end product, β-[3-^{3}H]alanine. The protein concentration was 7.8 mg/mL. (●) Dihydrouracil (DHU); (▲) *N*-carbamoyl-β-alanine (NCβA); (■) β-alanine; (⬢) sum of products.

centration of these two metabolites is maintained at very low steady-state levels, about 4 μM for dihydrouracil and about 11 μM for NCβA. Presumably in the intact liver the enzymes should function as well, if not better, suggesting that dihydrouracil and NCβA are maintained at equally low concentrations in vivo.

It is possible that enzymes that normally function in a complex will fall apart and migrate separately when examined by sedimentation or gel filtration chromatography. One may still be able to see evidence for a complex in kinetic studies: that is, the complex may be able to channel the initial substrate to the end product; therefore, the transient time should be fairly independent of protein concentration, and the rate of formation of CO_2 or β-alanine from uracil should not be greatly decreased in the presence of large pools of the intermediate metabolites. Extrapolation of the progress curve for the end product β-alanine to the abscissa gives the transient time for the three-step conversion of uracil to β-alanine. The intercept value is about 1.5 min (Figure 4). When the amount of protein in the reaction assay is decreased, the transient time increases significantly (Figure 5). In addition, the formation of $^{14}CO_2$

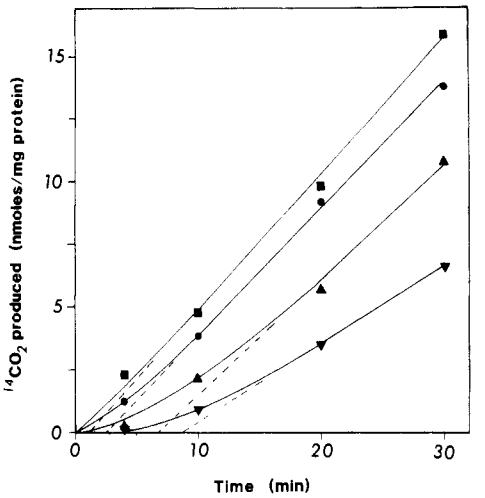

FIGURE 5: Dependence of the transient time for uracil catabolism on enzyme protein concentration. For this experiment, the transient time is the time required to produce $^{14}CO_2$ from [2-^{14}C]uracil at a constant rate and is equal to the extrapolated intercept on the abscissa. Protein concentrations (milligrams per milliliter) were the following: (■) 7.80; (●) 6.88; (▲) 1.72; (▼) 0.86.

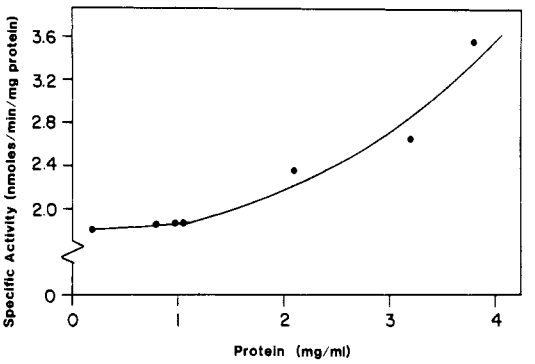

FIGURE 6: Dependence of the specific activity of NCβA amidohydrolase on protein concentration. NCβA amidohydrolase activity was measured by the release of $^{14}CO_2$ from [5-^{14}C]NCβA.

from [2-^{14}C]uracil was greatly decreased in the presence of either dihydrouracil at 100 μM or NCβA at 100 μM (data not shown). Therefore, our kinetic studies give no evidence of metabolite channeling and do not support the model that these three enzymes function in a complex.

The decreasing slopes of the progress curves in Figure 5, as a function of protein concentration, indicate that for at least one of the three enzymes, its specific activity varies with enzyme concentration. When progress curves were obtained for the individual enzymes at different concentrations of proteins, it was quite apparent that the observed specific activity of NCβA amidohydrolase is very dependent on protein concentration (Figure 6). For the amidohydrolase, the specific activity becomes almost constant at protein concentrations ≤1 mg/mL; an increase in protein concentration from 1 to 4 mg/mL produces a 100% increase in specific activity (Figure 6). Results of this type are often indicative of self-association of enzyme subunits (where the larger species has greater catalytic activity) since increasing protein concentration favors association of subunits (Frieden, 1971; Klotz et al., 1975; Cann, 1978; Nichol, 1981). By comparison, there is no change in the specific activity of dihydropyrimidine dehydrogenase with protein concentration (Figure 7). For enzymes that readily change their degree of subunit association, this process of association/dissociation is often regulated by substrates or products. This was specifically tested by examining the elution of all three enzymes on an Ultrogel column equilibrated with

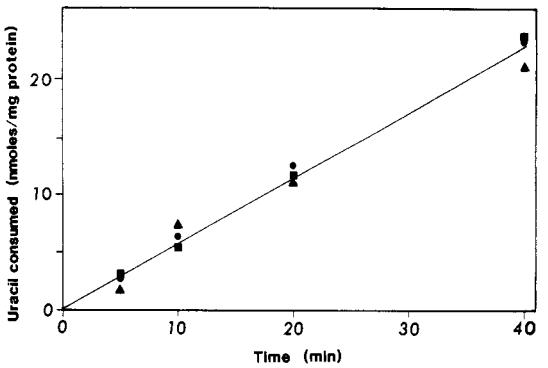

FIGURE 7: Progress curves for dihydropyrimidine dehydrogenase at different protein concentrations. Enzyme activity was measured as the sum of products formed from [6-^{3}H]uracil with protein concentrations of (■) 7.8, (▲) 4.0, and (●) 1.0 mg/mL.

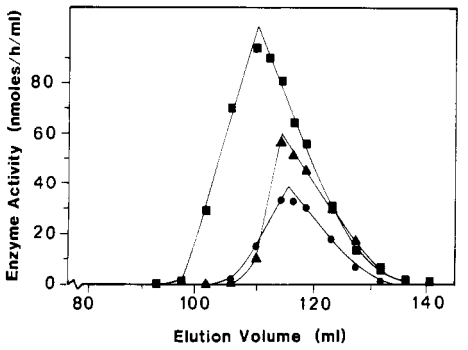

FIGURE 8: Effect of the substrate *N*-carbamoyl-β-alanine on the molecular size of NCβA amidohydrolase. All three enzymes were eluted through an Ultrogel AcA34 column (1.6 cm × 90 cm) that was equilibrated with NCβA at 300 μM. The following enzyme activities were recovered: dihydropyrimidine dehydrogenase (●), 135%; dihydropyrimidinase (▲), 63%; NCβA amidohydrolase (■), 122%.

300 μM NCβA, the substrate for the amidohydrolase. As expected, the presence of NCβA had no significant effect on the elution of dihydropyrimidine dehydrogenase or dihydropyrimidinase (Figure 8); however, NCβA amidohydrolase eluted more rapidly, with an apparent molecular weight of 340 000 (Figure 8). For comparison, see the elution curves in Figure 3.

The general properties of the three enzyme activities are summarized in Table I. Although other workers have reported that rat liver preparations comparable to the ones used in this study contained dehydrogenase activities that could reduce uracil with either NADH or NADPH (Canellakis, 1956; Smith & Yamada, 1971), our enzyme preparation was highly dependent on NADPH as the cofactor for the first enzyme.

Discussion

Previously the separation of all four compounds in the catabolism of uracil required two-dimensional chromatography (Fink et al., 1956). One-dimensional procedures failed to resolve uracil from dihydrouracil (Fritzson, 1962; Ikenaka et al., 1979) or NCβA from β-alanine (Fritzon, 1962; Sanno et al., 1970; Bols et al., 1980). A recent report describes the complete separation of the four fluoro analogues of these uracil metabolites by high-pressure liquid chromatography (Sommadossi et al., 1982).

A real benefit of our TLC procedure is that it facilitates the preparation of radioactive dihydrouracil and NCβA. With these two radioactive substrates, it is now a simple matter to assay, unambiguously and with high sensitivity, the dihydropyrimidinase or amidohydrolase activities, which previously were measured with colorimetric procedures (Wallach & Grisolia, 1957; Sanno et al., 1970; Maguire & Dudley, 1978).

Table I: Properties of the Enzymes of Pyrimidine Catabolism from Rat Liver

enzyme	substrate	K_m (μM)	activity [nmol min^{-1} (mg of protein)$^{-1}$]	M_r[a]
dihydropyrimidine dehydrogenase	NADPH	11[b]	0.6	218 000
	uracil	1.8[b]		
dihydropyrimidinase	dihydrouracil	11	2.5	226 000
NCβA amidohydrolase	NCβA	23.5	1.9	234 000

[a] Average of three determinations. [b] Values taken from Shiotani & Weber (1981).

The three enzymes are remarkably similar in size, and the sedimentation coefficient (data not shown) of about 10.5 S for all three activities is in reasonable agreement with a value of 9.2 S determined by Shiotani & Weber (1981) for the dihydropyrimidine dehydrogenase from rat liver. Wallach & Grisolia (1957) obtained a value of 6.99 S for the dihydropyrimidinase from calf liver. This value appears too low, corresponding to a molecular weight of ~140 000. Maguire & Dudley (1978) have shown that the dihydropyrimidinase from both calf liver and rat liver exists as a tetramer. Perhaps under the conditions used by Wallach & Grisolia (1957) the enzyme dissociated to a dimer, for which a value of 6.99 S would be more appropriate. Compared to the molecular weight value listed in Table I, Shiotani & Weber (1981) reported a very similar value of 220 000 for homogeneous dihydropyrimidine dehydrogenase, while the value of 266 000 reported by Maguire & Dudley (1978) for the dihydropyrimidinase is 15% larger than our molecular weight for this enzyme. No previous sedimentation coefficients or molecular weights have been reported for the amidohydrolase.

Previously published K_m values for dihydrouracil with the dihydropyrimidinase and for NCβA with the amidohydrolase were larger than ours by more than 1 order of magnitude, 117 mM for dihydrouracil (Wallach & Grisolia, 1957) and 390 μM (Sanno et al., 1970) or 500 μM (Caravaca & Grisolia, 1958) for NCβA. Like our own value, the earlier values were obtained with enzymes prepared from rat liver. We feel that our K_m values in Table I may be more accurate, since they are consistent with the steady-state values for the entire pathway in Figure 4.

Easterby (1981) has shown that for the following coupled reaction sequence:

$$S \xrightarrow{v_o} I_j \xrightarrow{V_j} P \qquad (1)$$

where S, I_j, and P are the initial substrate, the intermediate, and the final product, respectively, v_o is the observed velocity, and V_j is the maximum velocity for the second enzyme, if $V_j >> v_o$ then the steady-state concentration, $[I_j]$, is

$$[I_j]_{ss} \simeq (v_o/V_j)K_j \qquad (2)$$

where K_j is the Michaelis constant. For the enzyme sequence in our studies, the V's for the second and third enzyme are only 4–5-fold greater than v_o, but using eq 2 plus the K_m values from Table I produces estimates of the steady-state values of 2.7 μM for dihydrouracil and 7.3 μM for NCβA. These values are in reasonable agreement with our measured steady-state values of 4 and 11 μM (Figure 4) and therefore support our K_m values.

Neither our physical studies nor our kinetic studies support a model of the three enzymes functioning as a complex in vitro. We found no evidence for metabolite channeling when we measured the conversion of [2-^{14}C]uracil to $^{14}CO_2$ in the presence of the intermediate metabolites. Also, the increasing lag times or transient times for the production of CO_2 as total protein concentration was lowered are not indicative of channeling. In earlier studies with UMP synthase, a bifunctional protein that contains on one polypeptide chain the two catalytic centers for converting orotate → OMP → UMP (orotate phosphoribosyltransferase and OMP decarboxylase), we showed that UMP synthase could clearly channel radioactive orotate to UMP in the presence of unlabeled OMP pools (Traut & Jones, 1979) and that the transient time for this two-step pathway was generally not dependent on enzyme protein concentration (Traut & Payne, 1980). It is still possible that the enzymes of pyrimidine catabolism could function as a complex in vivo, but the present studies have no data on that possibility.

An unexpected but very interesting result is that the activity of NCβA amidohydrolase appears to be regulated both by enzyme concentration and by substrate concentration. The effects of protein concentration on enzyme activity are seen in Figure 6, where it is clear that the specific activity is different at high and low protein concentrations, and Figure 8 shows that the apparent molecular weight of the amidohydrolase is significantly greater in the presence of NCβA. The simplest interpretation for these results is that the amidohydrolase readily undergoes association and dissociation and can exist in several aggregation states. Both increased protein concentration and the presence of substrate as a positive effector could promote aggregation.

The larger enzyme aggregate(s) appear(s) to have a greater catalytic rate. This is supported by Figure 8, where the larger molecular weight species has more recovered activity than the smaller species in Figure 3. The decreased recovery of dihydropyrimidinase (Figure 8) in the presence of NCβA most likely reflects inhibition by NCβA added with the enzyme aliquot from the column fraction (final concentration of NCβA would be 60 μM in the assay mixture). While we have not measured the molecular size of the amidohydrolase as a function of protein concentration, we feel that Figure 6 also supports the interpretation that larger aggregates of the enzyme are more active since aggregation is favored at higher enzyme concentration, and Figure 6 shows increased specific activity as the protein concentration is increased. By comparison, none of these features were observed with the first enzyme, dihydropyrimidine dehydrogenase (Figure 7). Such regulatory properties are more customarily observed with enzymes at the beginning of a metabolic pathway, rather than at the end of a pathway.

At high protein concentrations (presumably more similar to in vivo conditions), the first enzyme, dihydropyrimidine dehydrogenase, is clearly the rate-limiting enzyme (Figure 4, Table I), and intermediates do not accumulate significantly. In the catabolism of 5-fluorouracil by rat hepatocytes, it was recently shown that 5-fluorodihydrouracil was the major intracellular compound (Sommadossi et al., 1982). This means that in vivo either the second enzyme becomes rate limiting, or, more likely, 5-fluorodihydrouracil is a less effective substrate than dihydrouracil for the dihydropyrimidinase. At low protein concentrations, it is quite possible that the third enzyme of this pathway, NCβA amidohydrolase, becomes rate limiting. The values in Table I were determined at intermediate protein concentrations (4.0 mg/mL) and probably reflect activity ratios in vivo. However, at protein concentrations ≤1 mg/mL, the specific activity of the amidohydrolase diminishes (Figure 6) to become comparable with the rate of the dehydrogenase. Since such rates are determined at saturating substrate concentrations, then under conditions where the overall pathway is assayed by starting with uracil, the amidohydrolase activity would be rate limiting until NCβA concentrations had increased sufficiently to activate the enzyme. Evidence for this is seen in Figure 5, where the time required to produce $^{14}CO_2$ from uracil increases as protein concentration is lowered.

It may be asked why the amidohydrolase exists at all. It has been shown that in mice almost half of dietary uracil is readily excreted as dihydrouracil and NCβA (Sonoda & Tatibana, 1978). While the results are certainly biased by the previously existing difficulty in assaying the second and third enzymes, a survey of the literature shows that the first enzyme, dihydropyrimidine dehydrogenase, is found in most tissues, the dihydropyrimidinase in liver and kidney, and the amidohydrolase only in liver. It could be suggested that the amidohydrolase is properly confined to the liver since ammonia is one of its products, and this can more readily be disposed by a tissue that also has the urea cycle.

Added in Proof

In more recent studies, β-alanine has tended to smear more when developed in solvent 1; we have not yet determined if the problem is in the DEAE plates, or the reagents in the solvent. An alternative solvent, collidine, first used by Fritzson & Pihl (1957), keeps both β-alanine and NCβA at the origin (R_f 0.02) while permitting easy migration of uracil (R_f 0.69) and dihydrouracil (R_f 0.55).

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Registry No. NCβA, 462-88-4; DHU, 504-07-4; [5-^{14}C]NCβA, 89509-92-2; [6-^{3}H]DHU, 89509-93-3; NCβA amidohydrolase, 9027-27-4; β-alanine, 107-95-9; uracil, 66-22-8; dihydropyrimidine dehydrogenase, 9026-89-5; dihydropyrimidinase, 9030-74-4.

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