The Overall Synthesis of L-5,6-Dihydroorotate by Multienzymatic Protein pyr1-3 from Hamster Cells

KINETIC STUDIES, SUBSTRATE CHANNELING, AND THE EFFECTS OF INHIBITORS

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In mammals, a trifunctional protein (ME pyr1-3) synthesizes L-5,6-dihydroorotate in three sequential reactions catalyzed by carbamyl phosphate synthetase (EC 2.7.2.9), aspartate transcarbamylase (EC 2.1.3.2), and dihydroorotase (EC 3.5.2.3). 14C-labeled HCO3- has been used as a precursor for the synthesis of L-5,6-dihydroorotate by purified ME pyr1-3, and when this product is converted enzymatically to orotidine 5'-monophosphate, the concentrations of the two intermediates of orotidine 5'-monophosphate synthetase activity to hydrolyze carbamyl phosphate. L-Cysteine, a slow acting but potent inhibitor of dihydroorotase in the absence of substrates (Christopherson, R. L., and Jones, M. E. (1980) J. Biol. Chem. 255, 3358-3370), also inactivates dihydroorotase when ME pyr1-3 is synthesizing L-5,6-dihydroorotate.

In mammals, the first three reactions of the de novo pyrimidine pathway are catalyzed by a trifunctional protein, ME pyr1-3, which contains the enzymatic activities carbamyl phosphate synthetase (EC 2.7.2.9), aspartate transcarbamylase (EC 2.1.3.2), and dihydroorotase (EC 3.5.2.3). Sequential operation of these three activities results in the synthesis of L-5,6-dihydroorotate from MgATP, HCO3-, L-glutamine, and N-carbamyl-L-aspartate. Analysis of steady state concentrations from ratios of the rate constants for the three activities. A high local concentration of inhibitors upon the overall synthesis of L-5,6-dihydroorotate by this trifunctional protein, when its three enzymatic activities are functioning in concert, has not been studied. Chen and Jones (1979) presented evidence that in vivo carbamyl phosphate synthetase is usually the rate-limiting enzyme for the de novo biosynthesis of UMP. This enzyme is subject to regulation by MgPRPP, a positive effector, and MgUTP, a negative effector (Mori et al., 1975), enabling regulation of the flux through the de novo pyrimidine pathway by modulation of carbamyl phosphate synthetase activity. Thus, characterization of the sequence of synthesis of carbamyl phosphate, N-carbamyl-L-aspartate and L-5,6-dihydroorotate by ME pyr1-3 and the effects of inhibitors upon the overall synthesis of L-5,6-dihydroorotate is important to an understanding of the regulation of the de novo pathway in vivo.

Carbamyl phosphate synthetase and aspartate transcarbamylase exist as an enzyme complex in Neurospora crassa (Williams et al., 1970), Saccharomyces cerevisiae (Lue and Kaplan, 1971) and in mammals (Hoogenraad et al., 1971) and Shoaf and Jones (1971) demonstrated that the mammalian protein also contains the third activity of the pathway, dihydroorotase. A large number of multifunctional proteins have now been characterized (Schmincke-Ott and Biswanger, 1981). The abbreviations used are: ME pyr1-3, the trifunctional protein initiating pyrimidine biosynthesis in mammals; OMP, orotidine 5'-monophosphate; PRPP, 5-phosphoribosyl-1-pyrophosphate; PALA, N-phosphonacetyl-L-aspartate; Heps, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; PEI-C, polyethyleneimine-cellulose; CAP, carbamyl phosphate; CA-asp, N-carbamyl-L-aspartate; DHO, L-5,6-dihydroorotate.
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and one of the possible advantages conferred by such an association of enzymes catalyzing sequential reactions is that an intermediate synthesized at the first catalytic site may be preferentially utilized as substrate by a subsequent site on the same protein molecule. Such "substrate channeling" (Davis, 1967) would allow synthesis of the final product of the multienzymatic protein without significant accumulation of the intermediate(s). Various degrees of substrate channeling have been demonstrated for the carbamyl phosphate synthetase-aspartate transcarbamylase of yeast (Lue and Kaplan, 1970) and for a number of other multienzymatic proteins (Gaertner et al., 1970; Fall and West, 1971; Matchett, 1974; Welch and Gaertner, 1975; Heyde, 1979) including UMP synthase (Traut and Jones, 1977), which catalyzes the fifth and sixth reactions of de novo pyrimidine biosynthesis. More quantitative aspects of the general concept of channeling have been discussed by Duggleby et al. (1978).

We have developed an overall radioactive assay for the first three reactions of de novo pyrimidine biosynthesis which enables simultaneous measurement of the concentrations of carbamyl phosphate, N-carbamyl-L-aspartate, and the final product of ME pyr1-3, L-5,6-dihydroorotate. A "coupling enzyme mixture" can be used to convert L-5,6-dihydroorotate to OMP to overcome the unfavorable equilibrium existing between L-5,6-dihydroorotate and N-carbamyl-L-aspartate in the physiological range of pH (Christopherson and Jones, 1979). Using this assay procedure, we find that carbamyl phosphate and N-carbamyl-L-aspartate are maintained at low steady state concentrations due to partial channeling of these intermediates and also to the favorable kinetic characteristics (Vmax/Km) of aspartate transcarbamylase and dihydroorotase relative to carbamyl phosphate synthetase. The effectiveness of PALA and L-cysteine as inhibitors of aspartate transcarbamylase and dihydroorotase, respectively, has been tested when ME pyr1-3 is synthesizing L-5,6-dihydroorotate using HCO3- as a precursor.

EXPERIMENTAL PROCEDURES

PALA was obtained from Mr. Leonard Keida of the Drug Synthesis and Chemistry Branch of the National Cancer Institute and was 79.5% pure by weight. L-Cysteine (free base), 6-azauridine 5'-monophosphate, L-glutamine, L-aspartate, dipotassium ATP, dipotassium phosphopyruvate, NAD, and PRPP were obtained from the Sigma Chemical Co. Acetaldheyde was from Mallinkrodt. All other chemicals were of the best grade commercially available. PEL-C chromatograms (20 x 20 cm) were manufactured by Machery, Nagel and Co. and obtained from Brinkmann Instruments, Inc. Chromatograms were washed (Christopherson et al., 1978) and scored into 1.5-cm strips, and the origin was marked with soft pencil 2 cm from the base.

Aqueous Na[14C]HCO3 (62.4 Ci/mol), diethyl carbamyl phosphate (10.4 Ci/mol), potassium [14C]cytate (52.0 Ci/mol), L-[14C]aspartate (230 Ci/mol), and L-[2-3H]aspartate (17.3 Ci/mmole) were obtained from New England Nuclear Co. N-[14C]Carbamyl l-aspartate (11 Ci/mol) was synthesized from penicillamine [14C]cytate and L-aspartate and purified as described by Christophersen et al. (1978). L-5,6-[14C]Dihydroorotate (220 Ci/mol) was synthesized enzymatically from the N-[14C]carbamyl-L-aspartate and purified as described by Christopherson and Jones (1980). N-Carbamyl-L-[14C]aspartate (17.3 Ci/mmole) was synthesized from L-[2-3H]aspartate and potassium cytate as described by Christophersen et al. (1978) except that two passages through a DEAE-cellulose column were required to achieve a radiochemical purity of 99.7%. Determination of [14C]-labeled compounds on PEL-C chromatograms by autoradiography was as previously described (Christophersen et al., 1978). [14C]Cytosine on PEL-C chromatograms was counted with an efficiency of 78.3% in a scintillator containing 3.0 g of 2.5-diphenyloxazole (PPO) and 0.1 g of 1,4-bis(5-phenyloxazolyl)benzene (POP0P)/liter of toluene using a Beckman LS-100C liquid scintillation system. [14C] in aqueous samples was counted with an efficiency of 89.6% in a mixture of toluene:Triton X-100 (2:1, v/v) and 5.5 g of 2,5-diphenyloxazole and 0.1 g of 1,4-bis(5-phenyloxazolyl)benzene/liter, the aqueous sample constituted 10% (v/v) of the total counting mixture.

Hamster ME pyr1-3 was purified by the procedure of Coleman et al. (1977) and for a mutant hamster cell line (165-23) obtained from Dr. Stark which overproduces ME pyr1-3 by more than 100-fold. The crude dialyzed enzyme was from Ehrlich ascites tumor cells. Carbamyl phosphate phosphatase activity was prepared in a solution of 20 mm Tris-HCl, pH 7.4, 2.0 mm dithiothreitol as described by Traut (1980). The following enzyme preparations were obtained from the Sigma Chemical Co.: dihydroorotate dehydrogenase (1 unit/mg) from Clostridium orbiculare, horse liver alcohol dehydrogenase (1.9 units/mg), yeast orotate phosphoribosyltransferase (182 units/mg), and yeast inorganic pyrophosphatase (9.5 units/mg). Rabbit muscle pyruvate kinase (200 units/mg) was from Boehringer Mannheim.

For determination of protein concentrations, the protein was first precipitated with 5% (v/v) trichloroacetic acid to remove interfering compounds and then assayed by a modification of the Lowry procedure described by Hartree (1972).

Assay of [14C]HCO3-—The concentration of stock solutions of Na[14C]HCO3 stored at 5°C, pH 9.3, decreases with time due to loss of [14C]CO2. Therefore, [14C]HCO3- solutions were standardized by diluting 5 μl of the stock solution (approximately 100 Ci/μl) with 4995 μl of 10 mm unlabeled KHCO3, pH 9.3. Small paper wicks were dipped in 2 mm NaOH, dried, and spotted with 2.5, 4.5, and 6 μl of the diluted [14C]HCO3-. The wicks were dried and the [14C]CO2 formed was counted at an efficiency of 66.6% in toluene scintillator. The concentration of [14C]HCO3- was calculated from the slope of a plot of micrometers of [14C]HCO3- versus counts per min determined by the method of least squares.

To measure the decreasing concentration of [14C]HCO3- in incubation mixtures, the assay for carbamyl phosphate synthetase activity was modified as follows: using K[14C]HC03 (0.1 Ci/mol) except that ME pyr3-3 was omitted. Samples of 5 μl were transferred to scintillation vials containing 500 μl of 10 mm Tris-HCl, pH 9.2, and the vials were immediately sealed with a rubber serum cap holding a plastic center well with a paper wick which had been soaked in 2 M LiOH and 0.2 M LiCl, 20 mM Tris-HCl, pH 8.25, 0.6 mm [14C]aspartate (5.0 Ci/mol), and 0.19 M LiCl, 0.75% (v/v) formic acid at room temperature and, 'C-carbamyl phosphate was separated from 14C-labeled N-carbamyl-L-aspartate by diluting 5 μl of 4 M HC10, was injected through the serum cap and all the [14C]HCO3- evolved as 'C02 to be trapped by the alkaline paper wick. [14C]CO2 was quantitated by subtracting the counts in the alkaline paper wick from the total counts in the vials and dividing by the square of the micrometers of [14C]HCO3- as described above. There was good agreement between the [14C]HCO3- concentrations determined by the two procedures indicating that virtually all 14C was volatile in HCO3-. The [14C]HCO3- concentration was calculated from the slope of a plot of micrometers of [14C]HCO3- versus counts per min determined by the method of least squares.

Assay of Carbamyl Phosphate—To standardize [14C]carbamyl phosphate directly, 2, 4, 6, and 8-μl samples of an approximately 200 μM [14C]carbamyl phosphate solution (10.4 Ci/mol) were spotted onto channels of a PEL-C chromatograph (2.5 x 20 cm) maintained at 4°C. The [14C]carbamyl phosphate was isolated by chromatography with 0.19 M LiCl, 0.1% (v/v) formic acid at 4°C, and quantitated as described elsewhere.

Unlabeled and [14C]carbamyl phosphate (10.4 Ci/mol) used for competition experiments between endogenous and exogenous carbamyl phosphate as substrates for aspartate transcarbamylase, were assayed concurrently by stoichiometric conversion to N-carbamyl-L-[14C]aspartate and N-[14C]carbamyl-L-[14C]aspartate, respectively, using L-[14C]aspartate. Assay mixtures contained in a total volume of 50 μl: 50 mm Tris-HCl, pH 8.25, 0.6 mm L-[14C]aspartate (5.0 Ci/mol), and 10% (v/v) of the total counting mixture. The concentration of [14C]carbamyl phosphate was separated from N-carbamyl-L-[14C]aspartate and L-[14C]aspartate by chromatography with 0.19 m LiCl, 0.75% (v/v) formic acid at room temperature and 'C-labeled N-carbamyl-L-aspartate and L-[14C]aspartate were cut out and quantitated by scintillation counting. The concentration of carbamyl phosphate was determined by the method of least squares from the slope of a plot of micrometers of carbamyl phosphate versus counts per min in the products. Standardized carbamyl phosphate solutions were stored as small aliquots at -70°C and used once.

Assay of Carbamyl Phosphate Synthetase—To determine the
maximal activity of carbamyl phosphate synthetase, endogenous aspartate transcarbamylase activity was used to convert carbamyl phosphate synthesized to N-carbamyl-L-aspartate (Coleman et al., 1977), some of which was cyclized to L-5,6-dihydroorotate (see "Results").

Assay mixtures contained in a total volume of 25 μl: 50 mM KHepes, pH 7.4, 10% (v/v) glycerol, 2.0 mM dithiothreitol, 10 mM ATP, 25 mM MgCl₂, 4.0 mM L-glutamate, 0.1 mM PRPP, 150 milliunits of rabbit pyruvate kinase, 5.0 mM L-aspartate, 3.26 mM Na[14C]HCO₃ (52.4 Ci/mmol) and the assay was initiated by addition of appropriate dilutions of ME pyrl-3 to incubation mixtures. Samples of 5 μl were spotted into PEI-C at 4°C, 2, 4, and 6 min and chromatograms were developed with 0.19 M LiCl, 0.1% (v/v) formic acid at 4°C and 14C-labeled N-carbamyl-L-aspartate and L-5,6-dihydroorotate were detected by autoradiography and quantitated by scintillation counting. Under these conditions of assay, [14C]carbamyl phosphate was present at relatively low concentrations (see "Results") and was not quantitated.

For determination of the K₅₀ value of HCO₃⁻, assay mixtures for carbamyl phosphate synthetase were made up as described above with appropriate concentrations of [14C]HCO₃⁻ (52.4 Ci/mmol) standardized as described above. The reaction was initiated by addition of 1.85 μg of hamster ME pyrl-3 and 5-μl samples were spotted onto PEI-C chromatograms maintained at 4°C at 2, 4, and 6 min. Isolation and quantitation of [14C]labeled N-carbamyl-L-aspartate and L-5,6-dihydroorotate were described as above.

Carbamyl phosphate synthetase activity was calculated by the method of least squares from plots of product formed versus time. The specific activity of aspartate transcarbamylase was calculated by the method of least squares from the slope of a plot of pico moles/min of product synthesized versus the concentration of ME pyrl-3. These calculation procedures were also used for determination of aspartate transcarbamylase and dihydroorotate activities.

Assay of Aspartate Transcarbamylase—To determine the maximal activity of aspartate transcarbamylase, a modification of the procedure of Bethell et al. (1968) was used. Assay mixtures contained in a total volume of 80 μl: 50 mM KHepes, pH 7.4, 10% (v/v) glycerol, 2.0 mM dithiothreitol, 5.0 mM L-aspartate, appropriate concentrations of [14C]carbamyl phosphate (10.4 Ci/mol), and 5.0 mM L-aspartate. The assay was initiated by addition of four different dilutions of ME pyrl-3 to incubation mixtures and 20-μl samples were transferred into 180 μl of 0.37 M HClO₃ in a Biovial (Beckman Instruments Inc.) at 2, 4, and 6 min. Samples were processed and the specific activity of aspartate transcarbamylase was calculated as described above for carbamyl phosphate synthetase.

For determination of the K₅₀ value of carbamyl phosphate, assay mixtures for aspartate transcarbamylase contained in a total volume of 6.2 ml: 50 mM KHepes, pH 7.4, 10% (v/v) glycerol, 2.0 mM dithiothreitol, 5.0 mM L-aspartate, appropriate concentrations of [14C]carbamyl phosphate (10.4 Ci/mol) standardized directly by chromatography, and the reaction was initiated by addition of 23 ng of hamster ME pyrl-3. Samples of 2.0 ml of the assay mixture were transferred into 58.0 μl of 11.7 M HClO₃ in scintillation vials at 2, 4, and 6 min. The samples were heated in a boiling water bath for 10 min and allowed to stand overnight prior to addition of 18.0 ml of toluene/Triton X-100 scintillant for counting.

Assay of Dihydroorotate—To determine the maximal activity of dihydroorotate, the procedure of Christopherson et al. (1978) was used. Assay mixtures contained in a total volume of 25 μl: 50 mM KHepes, pH 7.4, 10% (v/v) glycerol, 2.0 mM dithiothreitol, 5.0 mM N-[14C]carbamyl-L-aspartate (110 Ci/mol) and the reaction was initiated by addition of 4 different dilutions of ME pyrl-3 to incubation mixtures. Samples of 5 μl were spotted onto PEI-C at 2, 4, and 6 min and the [14C]dihydroorotate synthesized was isolated by chromatography and quantitated as described by Christopherson et al. (1978).

For determination of the K₅₀ values of N-carbamyl Lac-L-aspartate and L-5,6-dihydroorotate, assay mixtures contained in a total volume of 25 μl: 50 mM KHepes, pH 7.4, 10% (v/v) glycerol, 2.0 mM dithiothreitol, appropriate concentrations of N-[14C]carbamyl-L-aspartate (520 Ci/mol), or L-[14C]dihydroorotate (220 Ci/mol) and the reaction was initiated by addition of 0.93 ng of hamster ME pyrl-3. Samples of 7 μl were spotted onto PEI-C chromatograms maintained at 4°C at 2, 4, 10.0, 20.0, 10.0, 20.0, 10.0, and 21 min and the substrate and product were isolated chromatographically and quantitated as described by Christopherson et al. (1978).

Assay of the Overall Reaction Catalyzed by ME pyrl-3—To determine the overall activity of ME pyrl-3, a procedure described in detail elsewhere was used. Briefly, assay mixtures contained in a total volume of 50 μl: 50 mM KHepes, pH 7.4, 10% (v/v) glycerol, 2.0 mM dithiothreitol, 10 mM ATP, 15 mM MgCl₂, 3.26 mM Na[14C]HCO₃, (52.4 Ci/mol), 4.0 mM L-glutamine, 0.2 mM PRPP, 10 mM phosphoenolpyruvate, 150 milliunits of pyruvate kinase, 5.0 mM L-aspartate and the assay was initiated by addition of 0.93 μg of hamster ME pyrl-3. When required, the final product of ME pyrl-3, L-5,6-dihydroorotate, was converted enzymatically toOMP by inclusion of the following "coupling enzyme mixture" in the total assay volume of 50 μl: 10% (v/v) pyruvate kinase, 100 milliunits of rabbit pyruvate kinase, 10 mM NAD+, 63 milliunits of dihydroorotate dehydrogenase, 10 mM acetaldehyde, 5.0 milliunits of equine alcohol dehydrogenase, 1.0 mM PRPP, 300 milliunits of yeast orotate phosphoribosyltransferase, 50 mM 6-azauridine 5'-monophosphate, and 20 milliunits of yeast inorganic pyrophosphatase. Samples of 5 μl were spotted into PEI-C chromatograms maintained at 4°C. Radiolabeled carbamyl phosphate, N-carbamyl-L-aspartate, 1,5-6-dihydroorotate, orotate, andOMP were separated by ascending chromatography at 4°C using 0.19 mM LiCl, 0.1% (v/v) formic acid as developing solvents. Vials of concentrated NaOH were included in the chromatography tank to trap residual [14C]. Detection of [14C]-labeled intermediates by autoradiography and their quantitation by scintillation counting have been described elsewhere.

**Comparison between Carbamyl Phosphate Synthesized by ME pyrl-3 and Exogenous Carbamyl Phosphate for the Aspartate Transcarbamylase Reaction**—To study possible substrate channeling of carbamyl phosphate by ME pyrl-3, endogenous carbamyl phosphate synthesized from HCO₃⁻ of a particular radiolabel (* *) was competed with exogenous carbamyl phosphate of a different radiolabel (? following to the according scheme:

\[
\begin{align*}
\text{L-glutamine} & \quad \text{L-glutamate} \\
\text{HCO}_3^- & \quad \text{CAP} \\
& \quad \text{L-aspartate} \\
\text{L-aspartate} & \quad \text{CA-asp} = \text{DHO}
\end{align*}
\]

The relative contributions of endogenous carbamyl phosphate and various concentrations of exogenous carbamyl phosphate to the synthesis of N-carbamyl-L-aspartate was determined by performing two parallel experiments concurrently. In one experiment, a series of incubation mixtures were set up containing all the components described above for assay of the overall reaction (except the coupling enzyme mixture) including 3.26 mM [14C]HCO₃⁻ (52.4 Ci/mol) and various concentrations of unlabeled carbamyl phosphate (0 to 90 mM). A second set of incubation mixtures of opposite radiolabeling contained unlabeled 3.26 mM HCO₃⁻ as precursor for endogenous carbamyl phosphate and [14C]carbamyl phosphate (10.4 Ci/mol) at the same concentrations used in the first set of incubation mixtures. Reactions were initiated by addition of 56 ng of hamster ME pyrl-3 to give a total volume of 50 μl and 5-μl samples were spotted onto PEI-C chromatograms maintained at 4°C at 2, 4, 6, and 8 min. Carbamyl phosphate, N-carbamyl-L-aspartate, and 1-dihydroorotate were separated by chromatography for 18 cm with 0.5 M LiCl, 0.1% (v/v) formic acid at 4°C. The higher concentration of LiCl enabled a clear separation of high concentrations of exogenous [14C]carbamyl phosphate from N-[14C]carbamyl-L-aspartate. Re values obtained were: carbamyl phosphate, 0.18; N-carbamyl-L-aspartate, 0.40; and 1,5,6-dihydroorotate, 0.65. The [14C]-labeled intermediates were detected by autoradiography and quantitated by scintillation counting. Relatively high concentrations of [14C]INCO derived from exogenous [14C]carbamyl phosphate sometimes contaminated the L-5,6-[14C]dihydroorotate spot. Because 1,5,6-[14C]dihydroorotate constituted only 4.4% of the total N-[14C]carbamyl-L-aspartate plus L-[14C]dihydroorotate synthesized (see Fig. 4A and Christopherson et al., 1979), it was not included in calculations for determination of the contributions of endogenous and exogenous carbamyl phosphate to the aspartate transcarbamylase reaction. The Na[14C]HCO₃ used in the first set of incubations was standardized as described above and solutions of unlabeled NaHCO₃ used in the second set were prepared on the same day from the solid. The unlabeled carbamyl phosphate

* K⁺ ion was present as a counterion of a number of assay components giving a total concentration of approximately 90 mM.
used in the first set of incubations and the \(^{14}\text{C}\)carbamyl phosphate used in the second set were standardized concurrently against \(^{14}\text{C}\)aspartate as described above. Because conditions for both sets of incubations were identical except for the radiolabeling, the contribution of endogenous carbamyl phosphate to the aspartate transcarbamylase reaction at various concentrations of exogenous carbamyl phosphate could be determined.

**Competition between N-Carbamyl-L-aspartate Synthesized by ME pyrl-3 and Exogenous N-Carbamyl-L-aspartate for the Dihydroorotate Reaction**—To determine if \(N\)-carbamyl-L-aspartate synthesized by ME pyrl-3 is utilized preferentially compared with exogenous \(N\)-carbamyl-L-aspartate, endogenous \(N\)-carbamyl-L-aspartate derived from \[^{14}\text{C}\text{HCO}_3^-\] was competed with exogenous \(N\)-carbamyl-L-[^{1}\text{H}\text{asp}].

![Diagram](image_url)

**RESULTS**

Many of the experiments described in this paper utilize HCO\(_3^-\) as a precursor for the synthesis of carbamyl phosphate and subsequent intermediates by ME pyrl-3. Fig. 1A shows that under the conditions of assay used for these experiments, \([^\text{14}\text{C}\text{HCO}_3^-]\) evolves as CO\(_2\) from open incubation tubes with a half-time of 39.5 min. Thus to obtain the \(K_m\) value of ME pyrl-3 for HCO\(_3^-\) where concentrations of HCO\(_3^-\) were sub-saturating, sampling times of 1, 2, and 3 min were used for determination of initial reaction velocities to minimize the loss of HCO\(_3^-\). Using this precaution and \([^\text{14}\text{C}\text{HCO}_3^-]\) standardized as described under "Experimental Procedures," a \(K_m\) value of hamster carbamyl phosphate synthetase for HCO\(_3^-\) of 610 \(\mu\text{M}\) was obtained in the presence of 10 mM ATP, 15 mM MgCl\(_2\), and 100 \(\mu\text{M}\) FRPP (Fig. 1b). The \(K_m\) value obtained for HCO\(_3^-\) was not changed significantly in the absence of PRPP or when 500 \(\mu\text{M}\) MgUTP was substituted for FRPP in assay mixtures containing 50 mM ATP, 55 mM MgCl\(_2\) to ensure saturation of carbamyl phosphate synthetase with this substrate. The \(K_m\) value of 610 \(\mu\text{M}\) obtained for HCO\(_3^-\) is considerably lower than values reported previously from mouse spleen of about 10 \(\mu\text{M}\) at pH 7.4 (Levine et al., 1971) and 9.5 \(\mu\text{M}\) at pH 7.2 (Tatibana and Shigesada, 1972a), but is comparable to a value of 1.2 \(\mu\text{M}\) obtained for carbamyl phosphate synthetase II from *E. coli* (Anderson and Meister, 1966).

The apparent Michaelis constants of \(S_{0.5}\) values of ME pyrl-3 for substrates are summarized in Table I. The \(K_m\) values for HCO\(_3^-\), carbamyl phosphate, \(N\)-carbamyl-L-aspartate, and L-

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\(^3\) Similar experiments using sealed plastic tubes (1.5 ml) indicated that CO\(_2\) was evolved at the same rate for 20 min until equilibrium was established between CO\(_2\) in the incubation mixture and in the air space above it. Further loss of CO\(_2\) then proceeded at a much slower rate.
5,6-dihydroorotate were determined under the standard conditions for assay of the overall activity described under "Experimental Procedures." Values for the other substrates were obtained for N-carbamyl-L-aspartate (92 μM) and L-dihydroorotate (3.1 μM) for dihydroorotase of mouse or hamster ME pyrl-3 (Table I). The Kₐ value of hamster aspartate transcarbamylase for carbamyl phosphate of 3.7 μM (Table I) is in good agreement with values reported by Shoa and Jones (1973) from mouse Ehrlich ascites tumor cells and by Hogenraad (1974) from mouse spleen. Mori et al. (1975) obtained a higher Sₒₐ for MgATP of carbamyl phosphate synthetase from mouse spleen in the absence of PRPP and UTP (5.2 mM, Table I) than their value of 1.7 mM from rat liver. However, this difference may not be significant because Sₒₐ values obtained in the presence of PRPP and UTP from both sources were comparable. Thus, the only major discrepancy between the apparent Kₐ or Sₒₐ values obtained for the three activities of ME pyrl-3 from different rodent sources is the Kₐ value for HCO₃⁻ of 610 μM obtained here with pure hamster ME pyrl-3 (Table I) compared with a value of approximately 9.5 mM obtained with partially purified ME pyrl-3 from mouse spleen (Levine et al., 1971; Tatibana and Shigesada, 1972a). This discrepancy may be attributed in part to evolution of HCO₃⁻ prior to and during assays (Fig. 1A) and/or the possible partial denaturation of the carbamyl phosphate synthetase from these wild type cells during purification.

Published activities of carbamyl phosphate synthetase, aspartate transcarbamylase and dihydroorotase have related to purification of ME pyrl-3 and assays have consequently been designed for convenience and maximal sensitivity (Mori and Tatibana, 1975; Coleman et al., 1977). To facilitate analysis of the overall reaction catalyzed by ME pyrl-3, the maximal enzymatic activities of hamster ME pyrl-3 were determined individually at pH 7.4 in 10% (v/v) glycerol, 2.0 mM dithiothreitol (Table II). Dihydroorotase was assayed in the biosynthetic direction using N(1-¹⁴C)carbamyl-L-aspartate as substrate (Christopherson et al., 1978) and with consideration of the unfavorable equilibrium ratio of N-carbamyl-L-aspartate to L-5,6-dihydroorotate of 16.6 to 1 existing at pH 7.4 (Christopherson and Jones, 1980). Plots of activity versus the concentration of pure ME pyrl-3 were linear for carbamyl phosphate synthetase and aspartate transcarbamylase after an initial lag at low protein concentration and the plot for dihydroorotase passed close to the origin. Extrapolated intercepts on the abscissa for protein concentration obtained from a least squares fit to the linear portions of the plots were: carbamyl phosphate synthetase, 2.02 μg/ml; aspartate transcarbamylase, 0.61 μg/ml; dihydroorotase, 0.094 μg/ml. The maximal specific activities presented in Table II are the slopes obtained from the linear portions of these plots. The maximal specific activity of aspartate transcarbamylase from a 100,000 × g supernatant of a crude homogenate of mouse Ehrlich ascites tumor cells prepared by the procedure of Shoa and Jones (1973) was 18.1 pmol/min/μg of protein. Assuming the turnover numbers of hamster and mouse aspartate transcarbamylase to be the same, ME pyrl-3 therefore constitutes 0.1% by weight of the total soluble cellular protein in these cells. To analyze the three reactions catalyzed by ME pyrl-3 under optimal conditions at pH 7.4, 10% (v/v) glycerol and 100 μM PRPP (Ishida et al., 1977) and 90 mM K⁺ ion (Levine et al., 1971) were included in assay mixtures to give maximal carbamyl phosphate synthetase activity. To further approximate conditions prevailing in vivo, a "coupling enzyme mixture," which inhibits carbamyl phosphate synthetase (Shoa and Jones, 1973), and to convert L-5,6-dihydroorotase to OMP, preventing accumulation of N-carbamyl-L-aspartate due to the unfavorable equilibrium. The overall reaction scheme is shown in Fig. 2. Because the equilibria between N-carbamyl-L-aspartate and L-5,6-dihydroorotate; L-5,6-dihydroorotate and orotate; and orotate and OMP all favor the first men-

### Table I

**Apparent Michaelis constants or Sₒ values of ME pyrl-3 for substrates**

| Enzymatic activity | Source | pH | Substrate | Kₐ or Sₒ (μM) | Reference |
|--------------------|--------|----|-----------|----------------|-----------|
| Carbamyl phosphate synthetase | Mouse | 7.2 | MgATP | 720 | |
| | Hamster | 7.4 | HCO₃⁻ | 5,200 | |
| | Mouse | 7.2 | L-Glutamine | 13 | |
| Aspartate transcarbamylase | Hamster | 7.4 | Carbamyl phosphate | 3.7 | |
| Dihydroorotase | Mouse | 7.5 | L-Aspartate | 700 | |
| | Mouse or hamster | 7.4 | N-Carbamyl-L-aspartate | 92 | |
| | Mouse or hamster | 7.4 | L-5,6-Dihydroorotate | 3.1 | |

* All substrates have Michaelis-Menten kinetics and may be characterized by a Kₐ value except for MgATP which exhibits positive homotropic cooperativity and L-aspartate which shows substrate inhibition above a concentration of approximately 100 μM (R. L. Christopherson and M. E. Jones, unpublished experiment). For these two cases, the substrate concentration which gives half-maximal activity (Sₒₐ) is presented.

* Sₒₐ in the presence of 50 μM PRPP.

* Sₒₐ in the presence of 2.0 mM UTP.

* Mori et al., 1975.

* See Fig. 1B (miniprint).

* Tatibana and Shigesada, 1972a.

* The Kₐ value for carbamyl phosphate obtained under the present conditions of assay (see Table III) agrees well with previous values (Shoa and Jones, 1973; Hogenraad, 1974).

* Christopherson and Jones, unpublished experiment.

* See Table III and Christopherson and Jones (1980).

* Christopherson and Jones (1980).

### Table II

**Maximal enzymatic activities of ME pyrl-3**

| Carbamyl phosphate synthetase | Aspartate transcarbamylase | Dihydroorotase |
|-------------------------------|---------------------------|---------------|
| Maximal specific activity (pmol/min/μg protein) | 329 | 18,600 | 2,910 |
| Activity relative to carbamyl phosphate synthetase | 1.0 | 57 | 8.8 |
tioned intermediate (Christopherson and Jones, 1979; Krakow and Vennesland, 1961; Traut and Jones, 1977) and orotate is a competitive inhibitor of dihydroorotase (Christopherson and Jones, 1980), it was necessary to hydrolyze the pyrophosphate produced in the synthesis of OMP (Fig. 2) to convert most of the L-5,6-dihydroorotate synthesized by ME pyr1-3 to OMP. Control incubation mixtures containing the coupling enzyme mixture prepared under “Experimental Procedures” contained low levels of aspartate transcarbamylase and carbamyl phosphate phosphatase activities which consumed [14C]carbamyl phosphate at a total rate of 0.019 μM/min when the concentration of carbamyl phosphate was 1 μM. This mixture also contained low levels of dihydroorotase activity which consumed 6 μM N-[14C]carbamyl-L-aspartate at a rate of 0.13 μM/min. Thus, the rates of consumption of carbamyl phosphate and N-carbamyl-L-aspartate at concentrations prevailing in assay mixtures by these contaminating activities were low compared with their rates of synthesis by ME pyr1-3 (5 μM/min, see Fig. 4).

Fig. 3 shows a plot of the overall biosynthetic activity of ME pyr1-3 versus the concentration of pure protein, assayed in the absence and presence of the coupling enzyme mixture. The activity values obtained are very similar indicating that this mixture has no inhibitory effect upon ME pyr1-3 at the concentrations used. The slope of the linear portion of the plot gave a maximal specific activity for carbamyl phosphate synthetase (after correlation for saturation by 3.26 mM HCO₃⁻) of 329 pmol/min/μg of ME pyr1-3 (Table II), equivalent to a turnover number of 65.8 mol min⁻¹ (mol of subunit)⁻¹.

Without the coupling enzyme mixture, N-carbamyl-L-aspartate is the major product synthesized by ME pyr1-3 from HCO₃⁻ (Fig. 4A) accumulating to a concentration of 100 μM after 30 min. The concentration of carbamyl phosphate reaches a steady state level of 0.07 μM and L-5,6-dihydroorotate increases to a concentration of 4.5 μM after 30 min (Fig. 4A). The low levels of carbamyl phosphate observed confirm the finding of Coleman et al. (1977) that endogenous aspartate transcarbamylase activity is sufficient to convert virtually all carbamyl phosphate to N-carbamyl-L-aspartate when carbamyl phosphate synthetase is assayed. The ratio of L-5,6-dihydroorotate to N-carbamyl-L-aspartate (R) decreases to a steady state value of 0.046 which is lower than the value obtained at equilibrium at pH 7.4 of 0.060 (Christopherson and Jones, 1979).

Fig. 4B demonstrates that inclusion of the coupling enzyme mixture efficiently removes L-5,6-dihydroorotate by its conversion.

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**Fig. 2.** The synthesis of L-5,6-dihydroorotate (DHO) from ATP, HCO₃⁻, and L-glutamine by ME pyr1-3 and conversion of the DHO to OMP using the coupling enzyme mixture. Carbamyl phosphate (CAP) can decompose chemically to cyanate (NCO⁻), the direction favored by the equilibria between N-carbamyl-L-aspartate (Ca-asp) and DHO and between subsequent intermediates is indicated by long and short arrows. Components of the coupling enzyme mixture are described under “Experimental Procedures.”
version to OMP\(^2\) (Fig. 2). This prevents a significant back-reaction to N-carbamyl-L-aspartate which reaches a maximal concentration of 7.13 \(\mu\)M after 5 min and then decreases slowly. Carbamyl phosphate reaches a steady state concentration of 0.20 \(\mu\)M and the amount of L-5,6-dihydroorotate, the majority of which is converted to OMP\(^3\) (Fig. 2), was equivalent to 80 \(\mu\)M after 20 min. Carbamyl phosphate synthetase activity, calculated by summing the concentrations of all intermediates between carbamyl phosphate and OMP\(^3\) (Fig. 2) is constant and maximal during the first 5 min at a rate of 4.99 pM/min (Fig. 4B). Aspartate transcarbamylase activity, calculated in a similar manner is 4.96 pM/min during the first 5 min (Fig. 4B) and dihydroorotate is synthesized at a rate of 4.07 pM/min after an initial lag. This lag is better illustrated in Fig. 4C which shows that the net synthesis of N-carbamyl-L-aspartate (and therefore carbamyl phosphate also) proceeds with no detectable lag after addition of ME\(\text{pyr1-3})\), while extrapolation of the time course for net synthesis of L-5,6-dihydroorotate (most of which is converted to OMP, Fig. 2) to the abscissa indicates a transient time of 1.3 min.

The concentrations of carbamyl phosphate observed in Fig. 4A (0.72 \(\mu\)M) and Fig. 4B (0.20 \(\mu\)M) are of the same order of magnitude as the concentration of ME\(\text{pyr1-3})\) subunits or carbamyl phosphate synthetase-aspartate transcarbamylase active sites (0.093 \(\mu\)M, Fig. 3). If carbamyl phosphate were sequestered in some way to channel to the aspartate transcarbamylase site and were converted to OMP (Fig. 2)); \(\Delta\), L-5,6-dihydroorotate converted to OMP (Fig. 2)); \(\square\), L-5,6-dihydroorotate converted to OMP (Fig. 2). Assay mixtures contained 0.93 \(\mu\)g of ME\(\text{pyr1-3})\) in a total volume of 50 \(\mu\)l.

It was surprising to find that the absence of coupling enzymes affected the steady state level of carbamyl phosphate because this system (Fig. 2) was designed to investigate the steady state concentrations of N-carbamyl-L-aspartate when L-5,6-dihydroorotate is efficiently removed. The values for the steady state concentration of N-carbamyl-L-aspartate as a function of the concentration of ME\(\text{pyr1-3})\) (Fig. 5B) were obtained from the same experiment where carbamyl phosphate levels were measured in the presence of coupling enzymes (Fig. 5A). The curve obtained (Fig. 5B) is biphasic, having a linear upper portion which extrapolates to 5.0 \(\mu\)M at zero ME\(\text{pyr1-3})\) concentration. The steady state concentration of N-carbamyl-L-aspartate does not show a strong dependence upon ME\(\text{pyr1-3})\) concentration for the upper linear portion of the curve, increasing from 6.75 \(\mu\)M at 55 nM ME\(\text{pyr1-3})\) subunits to 12.1 \(\mu\)M at 220 nM ME\(\text{pyr1-3})\).

To quantitatively inter-relate the maximal velocities of the individual activities of ME\(\text{pyr1-3})\) (Table II) with the time course obtained for the overall reaction in the presence of coupling enzymes (Fig. 4B) and the steady state levels of carbamyl phosphate (Fig. 5A) and N-carbamyl-L-aspartate (Fig. 5B), the kinetic constants of aspartate transcarbamylase and dihydroorotase in the presence of the substrates for carbamyl phosphate synthetase were determined (Table III). The apparent K\(_{a}\) of aspartate transcarbamylase for carbamyl phosphate increases from 3.65 \(\mu\)M (see also Table I) to 48.7 \(\mu\)M in the presence of 10 \(\mu\)M ATP, 15 \(\mu\)M Mg\(\text{Cl2}\), 3.26 \(\mu\)M KH\(\text{CO3}\), and 4.0 \(\mu\)M L-glutamate, which was substituted for L-glutamine to avoid dilution of \(^{14}\)C-labeled carbamyl phos-
phate with endogenous unlabeled carbamyl phosphate. There is also a substantial increase in the $V_{\text{max}}$ (Table III). Values obtained in the absence of l-glutamate were similar. The remarkable increase in the apparent $K_a$ for carbamyl phosphate may be due to competitive inhibition with added anions or, more specifically, with ATP. Shoaf and Jones (1973) showed that 10 mM inorganic phosphate is an effective inhibitor, more specifically, with ATP. Shoaf and Jones (1973) showed that 10 mM inorganic phosphate is an effective inhibitor, more specifically, with ATP. Shoaf and Jones (1973) showed that 10 mM inorganic phosphate is an effective inhibitor, more specifically, with ATP. Shoaf and Jones (1973) showed that 10 mM inorganic phosphate is an effective inhibitor, more specifically, with ATP. Shoaf and Jones (1973) showed that 10 mM inorganic phosphate is an effective inhibitor, more specifically, with ATP.

The substrates for carbamyl phosphate synthetase also increase the apparent $K_a$ of dihydroorotase for N-carbamyl-l-aspartate from 92.1 μM to 263 μM when 100 μM PALA is included in the incubation mixture; the $V_{\text{max}}$ is almost unaffected by these additions (Table III). Values obtained in the absence of PALA were similar. The increase observed in the apparent $K_a$ for N-carbamyl-l-aspartate may be attributed to competitive inhibition by anions because addition of 150 mM KCl increases the $S_0$ for l-aspartate (Table I) approximately 9-fold.7

The overall reaction (Fig. 4B) can only be explained by preferential utilization (substrate channeling) of carbamyl phosphate and N-carbamyl-l-aspartate.

Another experimental approach to the detection of substrate channeling is to compete the endogenous intermediate, which may be channeled, with exogenous intermediate added at increasing concentrations to assay mixtures. The final product of a multifunctional protein may then be derived from the endogenously synthesized intermediate or from the exogenous equivalent according to their effective concentrations at the active site of the subsequent enzymatic activity. Such experiments have been performed for the intermediates, carbamyl phosphate and N-carbamyl-l-aspartate, of ME pyr1-3 as described under “Experimental Procedures” (Schemes 1 and 2). The rate of synthesis of product (v) from endogenous intermediate (A) in the presence of various concentrations of exogenous A is given by the expression:

$$V = \frac{[V][A]D}{K_m + [A]}$$

where $V$ is the maximal activity and $K_m$ is the apparent Michaelis constant for A under the conditions of assay of the catalytic site utilizing A as a substrate (Table III), [A] is the average total concentration of endogenous plus exogenous A during the period of assay, and D is the fractional dilution of the pool of endogenous A with exogenous A assuming uniform mixing (no compartmentation of endogenous A). Values for [A] and D have been calculated from experimentally determined concentrations of endogenous and exogenous A prevailing during the period of the assay, as described under “Appendix.”

To investigate possible channeling of carbamyl phosphate between the carbamyl phosphate synthetase and aspartate transcarbamylase sites of ME pyr1-3, endogenous [14C]carbamyl phosphate synthesized from [14C]HCO₃⁻ was competed with unlabeled carbamyl phosphate for conversion to N-carbamyl-l-aspartate. A parallel experiment was carried out concurrently under identical conditions using unlabeled HCO₃⁻ as precursor for endogenous carbamyl phosphate and exogenous [14C]carbamyl phosphate as shown in Scheme 1. With a final concentration of ME pyr1-3 of 1.1 ng/μl, the rate of consumption of exogenous carbamyl phosphate was approximately constant for the 4-min assay period and the rate of appearance of endogenous carbamyl phosphate was constant over this period when the average concentration of exogenous carbamyl phosphate was greater than 124 μM (Fig. 6A). In the

7 T. Mateuura and M. E. Jones, unpublished experiment.

**Table III** Effects of the substrates for carbamyl phosphate synthetase upon the kinetic constants of aspartate transcarbamylase and dihydroorotase

| Condition                      | Apparent $K_a$ (μM) | $V_{\text{max}}$ (nM/min) |
|-------------------------------|---------------------|---------------------------|
| Aspartate transcarbamylase     | (CAP)               |                           |
| No addition                   | 3.65                | 240                       |
| 10 mM ATP + 15 mM MgCl₂ + 3.26 mM KHCO₃ + 4.0 mM l-glutamate | 48.7                | 380                       |
| Dihydroorotase                | (CA-sap)            |                           |
| No addition                   | 92.1                | 71.6                      |
| 10 mM ATP + 15 mM MgCl₂ + 3.26 mM KHCO₃ + 4.0 mM l-glutamate | 263                | 74.4                      |

**Conditions** Determined by the manufacturer were better than 0.99.

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**Graphs**

- Fig. 5a. The effect of the concentration of ME pyr1-3 upon the steady-state concentration of carbamyl phosphate in the absence (○) or (●) of the coupling enzyme mixture. Conditions of the experiment were the same as for Fig. 5 and are described in the text.

- Fig. 5b. The effect of the concentration of ME pyr1-3 upon the steady-state concentration of N-carbamyl-L-aspartate in the presence of the coupling enzyme mixture. Data were obtained in the same experiments as Fig. 5a.

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**Footnote**: T. Mateuura and M. E. Jones, unpublished experiment.
absence of exogenous carbamyl phosphate, the endogenous carbamyl phosphate rapidly reached a steady state concentration within the 4-min assay period (see Fig. 4B). Fig. 6A shows that increasing concentrations of exogenous carbamyl phosphate rapidly dilute the endogenous carbamyl phosphate at the aspartate transcarbamylase site resulting in a very marked reduction in the rate of synthesis of N-carbamyl-L-aspartate derived from HCO₃⁻. There is also a corresponding rapid increase in the concentration of endogenous carbamyl phosphate present in the assay medium but this increase is not equal to the decreased concentration of N-carbamyl-L-aspartate, indicating that exogenous carbamyl phosphate has inhibited carbamyl phosphate synthetase activity (upper curve, Fig. 6A). This inhibition of carbamyl phosphate synthetase by exogenous carbamyl phosphate means that the decreased rate of synthesis of endogenous N-carbamyl-L-aspartate (Fig. 6A) cannot be analyzed by using Equation 3. However, the data clearly show that carbamyl phosphate is not tightly channeled by ME pyr1-3 although maintenance of some synthesis of N-carbamyl-L-aspartate at a high concentration of exogenous carbamyl phosphate of 2.6 mM (Fig. 6A) suggests partial channelling of carbamyl phosphate. This result is at variance with the data of Lue and Kaplan (1970) who found total channelling of carbamyl phosphate to a concentration of exogenous carbamyl phosphate of 1 mM by the bifunctional carbamyl phosphate synthetase aspartate transcarbamylase from yeast. Their data could be reconciled with our findings if a major portion of the exogenous carbamyl phosphate was rapidly consumed within the assay period by exogenous aspartate transcarbamylase activity. However, the bifunctional yeast protein may have different channelling properties to the trifunctional mammalian protein.

Experiments were performed to investigate possible channeling of N-carbamyl-L-aspartate between the aspartate transcarbamylase and dihydroorotate sites of ME pyr1-3. Endogenous N-[¹⁴C]carbamyl-L-aspartate synthesized from [¹⁴C]HCO₃⁻ was competed with various concentrations of exogenous N-carbamyl-L-[¹⁴C]aspartate for conversion to L-5,6-dihydroorotate (Fig. 6B) according to Scheme 2. The L-5,6-dihydroorotate was converted to OMP using the coupling enzyme mixture (Fig. 2) and concentrations of [H]labeled orotate and OMP were calculated using a specific radioactivity one-third the value of the N-carbamyl-L-[¹⁴C]aspartate used (58.0 Ci/mol). This corrected for the loss of 2 atoms of H as [¹⁴C]ethanol and [¹⁴C]water when L-5,6-[¹⁴C]dihydroorotate was converted to [¹⁴C]orotate (Fig. 2). Using a final concentration of ME pyr1-3 of 3.7 ng/μl, the rate of appearance of endogenous N-[¹⁴C]carbamyl-L-aspartate and the rate of consumption of exogenous N-carbamyl-L-[¹⁴C]aspartate were approximately constant during the 3-min assay period. If higher concentrations of ME pyr1-3 or longer sampling times were used, endogenous N-[¹⁴C]carbamyl-L-aspartate approached a steady state concentration (see Fig. 4B) and exogenous N-carbamyl-L-[¹⁴C]aspartate was greatly depleted, preventing analysis by the relatively simple procedure defined by Equation 3. The total rate of synthesis of L-[¹⁴C]dihydroorotate from endogenous N-[¹⁴C]carbamyl-L-aspartate is only moderately decreased by concentrations of exogenous N-carbamyl-L-[¹⁴C]aspartate is only moderately decreased by concentrations of exogenous N-carbamyl-L-[¹⁴C]aspartate (plus small quantities of carbamyl phosphate) are less precise than those for total L-[¹⁴C]dihydroorotate because of the presence of relatively high amounts of N-carbamyl-L-[¹⁴C]aspartate in these samples isolated for double label counting as described under “Experimental Procedures.”

Theoretical curves for the expected decrease in the total rate of synthesis of L-[¹⁴C]dihydroorotate and the consequent increased accumulation of N-[¹⁴C]carbamyl-L-aspartate were generated using Equation 3 with values for [A] and D calculated from the measured concentration of [¹⁴C] and [H]-labeled N-carbamyl-L-aspartate (see “Appendix”) and values for K₈ of 100, 200, 300, and 400 μM (Fig. 6B). These values were used because the apparent K₈ for N-carbamyl-L-aspartate increases from 92.1 μM to 305 μM in the presence of substrates for carbamyl phosphate synthetase (Table III). The theoretical curves for K₈ = 300 μM, which would best approximate the predicted operation of the system if endogenous and exogenous N-carbamyl-L-aspartate mixed freely prior to binding at the dihydroorotate site, do not coincide with the experimental values obtained (Fig. 6B). Considerably more total L-5,6-dihydroorotate is actually synthesized from endogenous N-carbamyl-L-aspartate by the system if endogenous and exogenous N-carbamyl-L-aspartate mixed freely prior to binding at the dehydroorotate site, do not coincide with the experimental values obtained (Fig. 6B). Considerably more total L-5,6-dihydroorotate is actually synthesized from endogenous N-carbamyl-L-aspartate by the system it was therefore of interest to investigate the effect of an inhibitor upon the overall reaction catalyzed by ME pyr1-3. The time course for the synthesis of pyrimidine intermediates from [¹⁴C]HCO₃⁻ in the presence of 0.80 μM PALA (Fig. 7A), is very different from that obtained in the absence of PALA (Fig. 4B). The rate of synthesis of total N-carbamyl-L-aspartate is initially low due to inhibition of aspartate transcarbamylase, but increases as carbamyl phosphate accumulates in the incubation medium.

The Overall Synthesis of 1-5,6-Dihydroorotate by ME pyr1-3

PALA is a transition state analog and inhibitor of aspartate transcarbamylase (Swyryd et al., 1974) with a K of 3 x 10⁻¹⁰ M (Kempe et al., 1976) and it was therefore of interest to investigate the effect of this potent inhibitor upon the overall reaction catalyzed by ME pyr1-3. The time course for the synthesis of pyrimidine intermediates from [¹⁴C]HCO₃⁻ in the presence of 0.80 μM PALA (Fig. 7A), is very different from that obtained in the absence of PALA (Fig. 4B). The rate of synthesis of total N-carbamyl-L-aspartate is initially low due to inhibition of aspartate transcarbamylase, but increases as carbamyl phosphate accumulates in the incubation medium.

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After carbamyl phosphate has reached an apparent steady state concentration of 18.9 µM, the total rate of synthesis of N-carbamyl-L-aspartate approaches the total biosynthetic activity of ME pyrl-3, as indicated by the dashed line (Fig. 7A). The concentration of N-carbamyl-L-aspartate of 4.6 µM after 20 min is lower than that of Fig. 4B due to its initially reduced rate of synthesis and NCO⁻, the degradation product of carbamyl phosphate at pH 7.4 (Allen and Jones, 1964), has accumulated to a concentration of 3.0 µM. The data of Fig. 7A indicate that inhibition of the overall biosynthetic activity of ME pyrl-3 by PALA can be overcome by accumulated carbamyl phosphate. To further investigate this effect, experiments similar to that of Fig. 7A with different concentrations of PALA were performed using a 40-min incubation time and a higher concentration of [¹⁴C]HCO₃⁻ of 6.5 mM (to compensate for the additional loss of ¹⁴CO₂, Fig. 1A). Under these conditions, the total rate of synthesis of N-carbamyl-L-aspartate again approached the total biosynthetic activity of ME pyrl-3 as the concentration of carbamyl phosphate reached a steady state. The results of this experiment performed at a concentration of ME pyrl-3 subunits of 0.093 µM (Fig. 7B) indicate that the accumulated steady state concentration of carbamyl phosphate required to overcome the block by PALA increases in direct proportion with the concentration of PALA after an initial lag. Blockade of ME pyrl-3 by concentrations of PALA higher than 2.4 µM (Fig. 7B) could have been tested using incubation times longer than 40 min, but significant evolution of ¹⁴CO₂ and possible inactivation of ME pyrl-3 were considered to be limiting factors.

The logical extension of the results of Fig. 7B is to ask whether carbamyl phosphate could accumulate in intact growing cells in response to similar intracellular concentrations of PALA, giving those cells resistance to the inhibitory effects of PALA. In this laboratory, the regulation of de novo pyrimidine nucleotide biosynthesis in vivo is under study in mouse Ehrlich ascites tumor cells. It was therefore of interest to determine whether these cells could maintain high intracellular concentrations of carbamyl phosphate. Fig. 8A is a control experiment showing the chemical decomposition of carbamyl phosphate to NCO⁻ at 37°C, pH 7.4 in 10% (v/v) glycerol using our recently developed assay procedure. The line of best fit passing through the experimental points for carbamyl phosphate concentration is a theoretical curve for first order decomposition with a half-time of 37.6 min, a value close to that obtained by Allen and Jones (1964) of 42.0 min at 37°C, pH 7.16. NCO⁻ increases in a corresponding manner from an intercept at zero time of 22 µM, indicating that the preparation of carbamyl phosphate used for this particular experiment was not pure. The total concentration of carbamyl phosphate plus NCO⁻ decreases slightly over the prolonged period of the incubation indicating some evolution of ¹⁴CO₂. When a crude dialyzed extract of mouse Ehrlich ascites tumor cells was added to a final concentration of 0.74 µg of protein/µl, the rate of decomposition of carbamyl phosphate was more rapid (Fig. 8B). NCO⁻ did not accumulate to the high levels of Fig. 8A and the total ¹⁴C in the assay mixture decreased with time indicating considerable evolution of ¹⁴CO₂. The data of Fig. 8B indicate that these cells contain a phosphatase activity capable of hydrolyzing carbamyl phosphate to carbamate (NH₂COO⁻) plus phosphate. NH₂COO⁻ decomposes rapidly to NH₃ plus CO₂ whereas NCO⁻ is relatively stable having a half-time for decomposition of 345 min at 37°C, pH 6.0 (Allen and Jones, 1964). This difference in stability of NCO⁻ and NH₂COO⁻ explains why the total ¹⁴C decreases rapidly in the presence of cell extract (Fig. 8B) due to evolution of ¹⁴CO₂ while the total ¹⁴C is relatively stable in the control (Fig. 8A).

In a previous publication (Christopherson and Jones, 1980), we showed that L-cysteine effectively inhibits mouse dihydroorotase in a time-dependent manner and that dihydroorotase was protected against this inactivation by the presence of 5.0 mM concentrations of either N-carbamyl-L-aspartate or L-5,6-dihydroorotate. Fig. 9 shows that dihydroorotase of pure hamster ME pyrl-3 is inactivated by 69 mM L-cysteine when ME pyrl-3 is synthesizing L-5,6-dihydroorotate from HCO₃⁻.
The time course for synthesis of pyrimidine intermediates proceeded in a manner similar to that of Fig. 4B until 5 min when L-cysteine was added after which the total rate of synthesis of L-5,6-dihydroorotate, as indicated by the slope of the time course, decreased to almost zero at 25 min. After 5 min, the concentration of N-carbamyl-L-aspartate increased in a corresponding manner and the overall rate of synthesis of pyrimidine intermediates was unaffected. It is concluded that although N-carbamyl-L-aspartate is partially channeled to the dihydroorotase site (Fig. 6B), the local concentration of this intermediate is not sufficient to protect dihydroorotase against inactivation by L-cysteine.

**DISCUSSION**

The three enzymatic activities of ME pyr1-3 are contained in a single oligomeric molecule (Coleman et al., 1977) and the data of Fig. 6, A and B indicate partial channeling of both the intermediates, carbamyl phosphate and N-carbamyl-L-aspartate. It is therefore of interest to quantitatively inter-relate the kinetic constants for the individual activities of ME pyr1-3 obtained under the conditions of the overall assay (Tables II and III) with the time course for appearance of pyrimidine intermediates during this assay (Fig. 4B). The predicted rates of the aspartate transcarbamylase and dihydroorotase reactions can be calculated by substituting the steady state concentrations of carbamyl phosphate (0.20 $\mu$M, Fig. 4B) and N-carbamyl-L-aspartate (7.13 $\mu$M, Fig. 4B) into appropriate forms of the Michaelis-Menten equation incorporating the maximal specific activities (Table II), the ratio of the $V_{\text{max}}$ values in the presence and absence of the substrate for carbamyl phosphate synthetase (Table III), and the apparent $K_m$ values for carbamyl phosphate and N-carbamyl-L-aspartate in the presence of these substrates (Table III). For aspartate transcarbamylase, the rate predicted is 2.29 $\mu$M/min compared with a rate of 4.96 $\mu$M/min actually observed (Fig. 4B). The 2.2-fold higher rate observed compared with that predicted can be explained if the local concentration of carbamyl phosphate at the aspartate transcarbamylase site is 0.44 $\mu$M rather than the average value of 0.20 $\mu$M, observed in the assay mixture (Fig. 4B). Similarly for dihydroorotase, the rate predicted from a steady state concentration of N-carbamyl-L-aspartate of 7.13 $\mu$M is 1.48 $\mu$M/min compared with that observed of 4.07 $\mu$M/min (Fig. 4B). The 2.8-fold higher rate actually observed could be explained if the local concentration of N-carbamyl-L-aspartate is 20.6 $\mu$M while the average concentration in the assay mixture is 7.13 $\mu$M (Fig. 4B).

A general treatment of the relationship between kinetic constants and the steady state concentrations of intermediates in coupled enzyme assays has been reviewed by Rudolph et al. (1979). Such an analysis is also applicable to multifunctional proteins catalyzing successive reactions although the steady state concentrations of intermediates predicted at the active sites may be higher than the average concentration prevailing in the bulk solvent due to the physical association of these sites. The following kinetic properties of ME pyr1-3 under the conditions of assay of Fig. 4B are consistent with the assumptions necessary for this type of analysis. The concentrations of MgATP and L-glutamine are saturating for carbamyl phosphate synthetase (Table I) and the concentration of HCO$_3^-$ is 5.3-fold higher than the $K_m$ value of 610 $\mu$M (Table I). Thus, because only a small fraction of these substrates are utilized (Fig. 4B), the carbamyl phosphate synthetase reaction can be considered an irreversible, zero order step where carbamyl phosphate is continuously removed by aspartate transcarbamylase. The zero order rate constant for this reaction is simply the initial rate calculated from the maximal specific activity for carbamyl phosphate synthetase (329 pmol/min/$\mu$g of protein, Table II) multiplied by the concentration of ME pyr1-3 used for Fig. 4B (18.5 $\times$ 10$^{-3}$ $\mu$g/µl) and a factor to correct for the saturation of the enzyme with 3.26 mM HCO$_3^-$:

$$k_1 = 329 \times 18.5 \times 10^{-3} \times \frac{3.26}{0.619 + 3.26}$$

$$= 5.12 \mu\text{M/min}$$

This value is in excellent agreement with the actual rate of carbamyl phosphate synthetase reaction observed (Fig. 4B) of 4.99 $\mu$M/min.

The aspartate transcarbamylase reaction is irreversible, saturated for L-aspartate and first order with respect to the concentration of carbamyl phosphate since the apparent $K_m$ under these conditions (48.7 $\mu$M, Table III) is 244-fold higher than the steady state concentration of carbamyl phosphate observed (0.20 $\mu$M, Fig. 4B). The first order rate constant for this reaction is the ratio $V_{\text{max}}/K_m$ for aspartate transcarbamylase calculated from the maximal specific activity (18,600 pmol/min/$\mu$g of protein, Table II), the concentration of ME pyr1-3 (18.5 $\times$ 10$^{-3}$ $\mu$g/µl), the ratio of maximal activity in the presence and absence of carbamyl phosphate synthetase substrates (390:240, Table III) and the apparent $K_m$ for carbamyl phosphate under these conditions (48.7 $\mu$M, Table III):

$$k_2 = \frac{18,600 \times 18.5 \times 10^{-3}}{48.7} \times \frac{390}{240}$$

$$= 11.5 \text{ min}^{-1}$$

The dihydroorotase reaction is assumed to be irreversible because the coupling enzyme mixture removes L-5,6-dihydroorotate as it is synthesized and first order with respect to N-carbamyl-L-aspartate because the apparent $K_m$ for N-carbamyl-L-aspartate under these conditions (263 $\mu$M, Table III) is 37-fold higher than the steady state concentration of N-carbamyl-L-aspartate of 7.13 $\mu$M (Fig. 4B). The first order rate

![Fig. 9. Effect of L-cysteine upon the synthesis of pyrimidine intermediates by ME pyr1-3 in the presence of the coupling enzyme mixture. L-Cysteine was added to the assay mixture after 5 min (see arrows) to a final concentration of 69 $\mu$M. Carbamyl phosphate; N-carbamyl-L-aspartate; L-5,6-dihydroorotate converted to OMP (Fig. 2); carbamyl phosphate + N-carbamyl-L-aspartate + OMP; N-carbamyl-L-aspartate + OMP. Conditions of assay were the same as for Fig. 4.](image)
constant (V_{max}/K_m) of dihydroorotase under the conditions of Fig. 4B can be calculated from the maximal specific activity (2910 pmol/min/μg of protein, Table II), the concentration of ME pyr1-3 (18.5 × 10^{-3} μg of protein/μl), the ratio of maximal activities in the presence and absence of carbamyl phosphate synthetase substrates (74.4:71.6, Table III) and the apparent K_m for N-carbamyl-L-aspartate under these conditions (263 μM, Table III):

$$k_2 = \frac{2910 \times 18.5 \times 10^{-3}}{263} \times \left( \frac{74.4}{71.6} \right) = 0.213 \text{ min}^{-1}$$

From the values of the rate constants k_1, k_3, and k_2 as they apply to the experiment of Fig. 4B, steady state concentrations of carbamyl phosphate and N-carbamyl-L-aspartate can be predicted using the following equations:

$$[\text{CAP}]_p = \frac{k_2}{k_1 \cdot k_3}$$  \hspace{1cm} (4)
$$[\text{CA-asp}]_p = \frac{k_1}{k_1 \cdot k_3}$$  \hspace{1cm} (5)

where [CAP]_p and [CA-asp]_p are the steady state concentrations of carbamyl phosphate and N-carbamyl-L-aspartate, respectively. Substituting the values for k_1, k_2, and k_3 obtained above into Equations 4 and 5, values for [CAP]_p of 0.45 μM and for [CA-asp]_p of 24.1 μM are obtained. These values compared very well with the local concentrations of carbamyl phosphate of 0.44 μM and of N-carbamyl-L-aspartate of 20.6 μM calculated above from the Michaelis-Menten equation required to maintain the observed rates (Fig. 4B) of aspartate transcarbamylase and dihydroorotase, respectively. Thus, calculations using the kinetic constants presented in Tables II and III and the data of Fig. 4B for synthesis of L-5,6-dihydroorotase from HC03\(^-\) by ME pyr1-3 demonstrate by two independent methods that the local concentrations of carbamyl phosphate and N-carbamyl-L-aspartate at the active sites of aspartate transcarbamylase and dihydroorotase, respectively, are approximately 2.2-fold and 3.1-fold the average concentrations prevailing in the assay medium. An alternative kinetic explanation for rates of the aspartate transcarbamylase and dihydroorotase reactions higher than those predicted from the steady state concentration of intermediates (Fig. 4B) is that when ME pyr1-3 is catalyzing the overall synthesis of L-5,6-dihydroorotase from HC03\(^-\), there is activation of these two activities. This explanation seems unlikely since kinetic constants for aspartate transcarbamylase were obtained in the presence of MgATP, HC03\(^-\), and L-glutamine to occupy the first active site without synthesis of unlabeled carbamyl phosphate, and N-carbamyl-L-aspartate synthesized was partially cyclized to L-5,6-dihydroorotase by ME pyr1-3.

High local concentrations of carbamyl phosphate and N-carbamyl-L-aspartate at the active sites of aspartate transcarbamylase and dihydroorotase could be due to the close proximity of active sites catalyzing successive reactions or to physical containment of the intermediates by partially buried active sites. The latter possibility is particularly applicable to carbamyl phosphate whose concentration of 0.2 μM is in the same order of magnitude as the concentration of carbamyl phosphate synthetase sites (0.093 μM, Fig. 4B). However, the steady state concentration of carbamyl phosphate (measured in the presence of the coupling enzyme mixture (Fig. 5A) and N-carbamyl-L-aspartate (at concentrations of ME pyr1-3 above 10 ng/μl (Fig. 5B)) did not increase in proportion to the concentration of ME pyr1-3. This is consistent with the proposal that these intermediates are not sequestered or contained during transit between the first and second sites and second and third sites, respectively. There is a strong dependence of the steady state concentration of N-carbamyl-L-aspartate upon ME pyr1-3 at lower concentrations which can be attributed to a decrease in the ratio of the rate constants k_1/k_2 (see above). As discussed under "Results," carbamyl phosphate synthetase (Fig. 3) and, to a lesser extent, aspartate transcarbamylase, are sensitive to dilution below concentrations of ME pyr1-3 of 10 ng/μl (Fig. 3), while dihydroorotase activity remains stable under these conditions. Thus, at low concentrations of ME pyr1-3, k_1 decreases after allowance is made for the protein concentration while k_2 remains constant resulting in a decrease in [CA-asp]_p (Equation 5, Fig. 5B).

The very small increase observed for [CAP]_p in the presence of coupling enzymes, the marked increase in [CA-asp]_p in their absence (Fig. 5A), and the moderate increase in [CA-asp]_p above ME pyr1-3 concentrations of 10 ng/μl (Fig. 5B) can be attributed to various degrees of product inhibition by accumulated N-carbamyl-L-aspartate and L-5,6-dihydroorotase, respectively.

The two independent kinetic arguments presented above for high local concentrations of carbamyl phosphate and N-carbamyl-L-aspartate at the active sites of aspartate transcarbamylase and dihydroorotase, respectively, and evidence that these two intermediates are not physically contained (Fig. 5A and B) indicates that the partial channeling observed (Fig. 6, A and B) can be attributed to close proximity of the active sites on bifunctional ME pyr1-3. This channeling probably occurs for purely kinetic reasons. The three active sites are located on a single particle in solution and therefore exist within concentration gradients of the two intermediates and are able to operate at effectively higher concentrations than those existing in the bulk medium.

No significant transient (lag) time could be detected for aspartate transcarbamylase activity (Fig. 4C) and therefore the transient time for synthesis of L-5,6-dihydroorotase from HC03\(^-\) of 1.3 min (Fig. 4C) can be attributed to the transient for dihydroorotase. Because the maximal rates of the second and third activities are well in excess of the operating rate of the first activity (Table II) and the respective steady state concentrations of carbamyl phosphate and N-carbamyl-L-aspartate are well below their K_m values (Fig. 4B, Table III), equations formulated by Easterby (1973) and further discussed by Welch (1977) can be used to calculate the predicted transient times.

$$\tau_i = \frac{1}{k_i}$$  \hspace{1cm} (6)

where \(\tau\) is the transient time for the \(i\)th enzyme of a sequence of reactions and \(k_i\) is the first order rate constant for that reaction.

Substituting the value for k_2 obtained above for aspartate transcarbamylase into Equation 6, a maximal transient time of 5.2 s is obtained, a value below the limits of detection of our experiment (Fig. 4C). Similar substitution of k_3 into Equation 6 gives a transient time of 4.7 min for dihydroorotase. The total transient time for the synthesis of L-5,6-dihydroorotase from HC03\(^-\) is simply the sum of the individual transient times for aspartate transcarbamylase and dihydroorotase (Easterby, 1973). Since the value of \(\tau\) for aspartate transcarbamylase predicted (5.2 s) and obtained experimentally (Fig. 4C) is low, the total transient time for ME pyr1-3 of 1.3 min can be compared directly with that calculated above for dihydroorotase of 4.7 min. The discrepancy between these two values can be attributed to substrate channeling of
N-carbamyl-L-aspartate. Welch and Gaertner (1975) found a considerable reduction in the transient time from that predicted for the aromatic complex of Neurospora crassa which they interpreted in terms of the containment of intermediates within or on the surface of the aggregate. To account for the approximately 3.1-fold higher local concentration of N-carbamyl-L-aspartate at the active site of dihydroorotase (see above), the first order rate constant \( k_1 \) should be multiplied by this factor giving a value for \( \tau_2 \) of approximately 1.5 min (Equation 6). The transient time for dihydroorotase may be predicted from another equation formulated by Easterby (1973):

\[
\tau_2 = \frac{[\text{CA-asp}]}{k_1}
\]  

(7)

using values for \([\text{CA-asp}]\), the steady state concentration of N-carbamyl-L-aspartate, of 7.13 \( \mu \text{M} \) (Fig. 4B) and 5.13 \( \mu \text{M}/\text{min} \) for \( k_1 \). Equation 7 gives a value for \( \tau_2 \) of 1.4 min, in close agreement with that observed experimentally of 1.3 min (Fig. 4C). The transient time of 1.3 min (Fig. 4C) is the time required for the local concentration of N-carbamyl-L-aspartate to reach approximately 20 \( \mu \text{M} \) at the third active site while the average concentration in the assay medium reaches 7.13 \( \mu \text{M} \) (Fig. 4B). Thus, the channeling of N-carbamyl-L-aspartate predicted by two kinetic procedures and demonstrated by a competition experiment (Fig. 6B) is also supported by a reduction in the predicted transient time from 4.7 min to 1.3 min (Fig. 4C).

Our observation that steady state concentrations of carbamyl phosphate accumulated in the presence of PALA can overcome the blockade of this potent inhibitor (Fig. 7, A and B) has kinetic interest and implications for possible cancer chemotherapy. For simple competitive inhibition, the data of Fig. 7B could be analyzed using the equation:

\[
\frac{[\text{CAP}]_1}{[\text{CAP}]_m} = 1 + \frac{[\text{PALA}]}{K_{\text{PALA}}}
\]  

(8)

where \([\text{CAP}]_1\) and \([\text{CAP}]_m\) are the local steady state concentrations of carbamyl phosphate at the aspartate transcarbamylase site in the presence and absence of PALA, respectively, and \( K_{\text{PALA}} \) is the inhibition constant for PALA. However, Equation 8 describes a straight line and Fig. 7B exhibits curvature at low PALA concentrations. Since PALA is a reversible tight binding inhibitor and the concentration of aspartate transcarbamylase sites of 0.093 \( \mu \text{M} \) is of a similar magnitude to the concentrations of PALA where the effect is observed (0 to 0.4 \( \mu \text{M} \), Fig. 7B), the curvature of Fig. 7B may be attributed to significant depletion of free PALA concentrations by formation of enzyme-inhibitor complex. To fully characterize this effect and derive an equation with predictive value, a detailed analysis similar to those described by Williams and Morrison (1979) should be performed. Carbamyl phosphate is unstable, having a half-time for decomposition under these conditions of 37.5 min or a first order rate constant for decomposition of 0.0184 min\(^{-1}\) (Fig. 8A). The rate of decomposition of carbamyl phosphate accumulated in response to PALA inhibition will equal its rate of synthesis, (5.14 \( \mu \text{M}/\text{min} \), Fig. 4B) when a concentration of 278 \( \mu \text{M} \) is reached. In practice, this concentration of carbamyl phosphate would not be attained because leakage through the PALA block would lower its net rate of synthesis.

The physiological significance of these findings, therefore, depends upon the cellular concentration of ME pyr-1-3 and its capacity for carbamyl phosphate synthesis. Fig. 7B suggests that cells containing high levels of ME pyr-1-3 that are able to maintain high intracellular concentrations of carbamyl phosphate would be resistant to quite high cellular concentrations of PALA. Jayaram et al. (1979) have found intracellular concentrations of PALA to vary between 1 and 100 \( \mu \text{M} \) in tumor cells. By extrapolation of the linear portion of Fig. 7B to 100 \( \mu \text{M} \) PALA, a very approximate steady state concentration of 3.0 \( \mu \text{M} \) carbamyl phosphate is obtained which would require a synthetic rate of 55 \( \mu \text{M}/\text{min} \) to balance its rate of decomposition (see above, Fig. 8A), plus the rate necessary for de novo biosynthesis of pyrimidine nucleotides. The blockade by PALA would result in depletion of cellular UTP and likely elevation of the PRPP concentration, resulting in maximal activation of carbamyl phosphate synthetase (Mori et al., 1975) similar to that obtained in our in vitro assays in the presence of 100 \( \mu \text{M} \) PRPP. A concentration of 3.0 \( \mu \text{M} \) carbamyl phosphate would approximately halve the carbamyl phosphate synthetase activity (Fig. 6A), presumably due to product inhibition. Using the specific activity of carbamyl phosphate synthetase of 329 pmol/min/\( \mu \text{g} \) of ME pyr-1-3 (Table III) and considering the factors mentioned above, a cellular concentration of ME pyr-1-3 subunits of approximately 1.7 \( \mu \text{M} \) would be required to maintain a rate of carbamyl phosphate synthesis of 56 \( \mu \text{M}/\text{min} \).

The intracellular levels of ME pyr-1-3 in tumor cells have been correlated with their susceptibility to PALA. Tumors sensitive to PALA have low levels of ME pyr-1-3 (Johnson et al., 1978) while stable mutants resistant to PALA contain up to 100 times the wild type levels of ME pyr-1-3 (Kempe et al., 1976). As mentioned by Kempe et al. (1976) and demonstrated in Fig. 7B, high levels of carbamyl phosphate synthesized by these cells in the presence of inhibitory concentrations of PALA would decrease the effectiveness of PALA by competition. The question as to whether cells resistant to high concentrations of PALA can sustain intracellular carbamyl phosphate concentrations of the order of 3 \( \mu \text{M} \) remains unanswered. Such intracellular concentrations are not inconceivable since Escherichia coli K12, which has low levels of OMP decarboxylase (Womack and O’Donovan, 1978), maintains a cellular concentration of carbamyl phosphate of approximately 0.84 \( \mu \text{M} \) during growth in minimal medium (Christopherson and Finch, 1978). In addition, Cohen et al. (1980) found carbamyl phosphate concentrations in the matrix of isolated respiring rat liver mitochondria of 3 \( \mu \text{M} \) which increased to 15 \( \mu \text{M} \) in the absence of l-ornithine. They also found that high concentrations of carbamyl phosphate coincided with inhibition of carbamyl synthetase I activity, an observation that we have made here with carbamyl phosphate synthetase II of ME pyr-1-3 (Fig. 6A).

Fig. 8B demonstrates that mouse Ehrlich ascites tumor cells contain a phosphatase activity capable of degrading carbamyl phosphate to phosphate plus carbamate. Several nonspecific phosphatases have been characterized which are capable of hydrolyzing carbamyl phosphate (Diederich et al., 1971; Herzfeld and Knox, 1972; Lueck et al., 1972), but the cellular location and kinetic properties of these enzymes under physiological conditions will determine whether accumulated carbamyl phosphate is hydrolyzed enzymatically in intact cells. Indeed, cells able to hydrolyze accumulated carbamyl phosphate may be sensitive to PALA while cells lacking such phosphatases may have more resistance. Work in progress in this laboratory will determine whether accumulation of cellular carbamyl phosphate is a significant mechanism for PALA resistance.

The time-dependent and ultimately total inhibition of dihydroorotase activity by L-cysteine when ME pyr-1-3 is synthesizing L-5,6-dihydroorotate from \( \text{HCO}_3^- \) (Fig. 9) indicates that substrate protection against this inactivation (Christopherson and Jones, 1980) by endogenous N-carbamyl-L-aspartate partially channeled to the dihydroorotase site is not
sufficient to prevent this inhibition. Although t-cysteine is not of interest as an inhibitor of pyrimidine biosynthesis in intact cells, the inhibition observed (Fig. 9) and previous detailed studies with inhibitors (Christopherson and Jones, 1980) does allow design of potential inhibitors of high potency. As postulated previously, the hydrolysis by dihydroorotase of the "peptide-like" bond of L-5,6-dihydroorotate may resemble the catalytic mechanism of carboxypeptidase A (Christopherson and Jones, 1980) where a zinc atom interacts with the carbonyl group of the peptide bond to be cleaved. Onetti et al. (1979) have developed potent inhibitors of carboxypeptidases A and B which combine thio groups capable of coordinating specifically with the zinc atoms and functional groups able to interact with other areas of the active sites of these enzymes. A similar inhibitor has been developed by Nishino and Powers (1979) for thermonolysis. This approach could be used for designing potent inhibitors of dihydroorotase combining the structure of L-5,6-dihydroorotate with an —SH or —S group in position 4 of the pyrimidine ring to interact with the putative zinc of this enzyme. Such an inhibitor could fit the active site and coordinate to the zinc atom giving rapid inactivation of dihydroorotase rather than the time-dependent inactivation shown in Fig. 9.

Data presented in this paper that ME pyr1-3 partially channels the two intermediates carbamyl phosphate and N-carbamyl-L-aspartate due to close proximity of the active sites of this oligomeric trifunctional protein. This channeling can be accounted for by simple substrate kinetics if the local concentration of carbamyl phosphate at the active site of aspartate transcarbamylase is 2.2-fold higher, and the concentration of N-carbamyl-L-aspartate at the dihydroorotate site is 3.1-fold higher, than their average concentrations in the assay medium. The low steady state concentrations of carbamyl phosphate and N-carbamyl-L-aspartate prevailing during synthesis of L-5,6-dihydroorotate from HCO₃⁻ by ME pyr1-3 (Fig. 4B) are primarily due to the favorable ratios of the rate constants k₁/k₂ and k₅/k₆ (Tables II and III) as discussed above. Maintenance of low concentrations of these two intermediates would have the general advantages of conservation of the solvent capacity of the cytoplasm and minimization of chemical side reactions (Atkinson, 1977) and the specific advantages of preventing futile cycling of carbamyl phosphate and possible toxic side effects of N-carbamyl-L-aspartate (Christopherson et al., 1980). Coordinate expression of the first three enzymes of de novo pyrimidine biosynthesis as a single polypeptide chain enables the favorable ratios of rate constants of the three activities to be maintained under all conditions of cellular growth, thus minimizing the accumulation of these intermediates. The major selective advantage to higher animals of having the trifunctional ME pyr1-3 instead of three distinct and separable enzymes would seem to be that these three activities are expressed coordinately, substrate channeling occurs but appears to be of secondary importance and simply a consequence of having three catalytic activities on a single particle in solution.

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The Overall Synthesis of L-5,6-Dihydroorotate by ME pyr-1-3

Appendix: Derivation of Theoretical Relationships for Monitoring Studies

Experiments to directly investigate possible channeling of carbamyl phosphate and N-carbamoyl-L-aspartate by ME pyr-1-3 have been done by comparing the endogenous intermediates with increasing concentrations of exogenous intermediate. The final products of ME pyr-1-3 have been analyzed for incorporation of 2 different radioisotopes derived either from the endogenous or exogenous intermediate (see Schemes 1 and 2). The activity of carbamyl phosphate synthetase decreased in the presence of exogenous carbamoyl phosphate (Fig. 6a) but was unaffected by exogenous N-carbamoyl-L-aspartate (Fig. 6b). The following simplified analysis was used to test for channeling of N-carbamoyl-L-aspartate but is not applicable to channeling of carbamyl phosphate. The concentration of N-carbamoyl-L-aspartate in assay mixtures is below its apparent Km value (Fig. 4b, Table 1) and therefore allowance must be made for the changing saturation of substrate at the dihydroorotate 1-3 as well as for dilution of endogenous with exogenous intermediate.

The average total concentration of the intermediate A, [A], is the sum of the average concentrations of endogenous A, [A]e, and exogenous A, [A]x, during an assay period of 1 min:

\[ [A] = 0.5 \left( [A]_e^{1/2} + [A]_x^{1/2} \right) \]

where the subscripts "e" and "x" denote the initial and final concentrations, respectively, of A.

The fractional dilution, D, of endogenous A with exogenous A is the average concentration of endogenous A, divided by the average total concentration of A:

\[ D = \frac{\left( [A]_e^{1/2} + [A]_x^{1/2} \right)}{\left( [A]_e^{1/2} + [A]_x^{1/2} \right)} \]

The degree of dilution of endogenous A with exogenous A is detected as the relative amounts of the 2 radioisotopes in the product(s) of the reaction.

In the absence of added exogenous A, the average rate of synthesis of product, \( \nu \), during an assay period of 1 min can be expressed in terms of the initial, [A]e, and final [A]f, concentrations of endogenous A and the apparent Michaelis constant for A under the conditions of assay, \( K_{mA} \), using a simplified form of Equation 8 for the average concentration of A and the Michaelis-Menten equation:

\[ \nu = \frac{[A]}{K_{mA}} \frac{[A]_e}{[A]_e^{1/2} + [A]_x^{1/2}} \]

Rearranging for \( K_{mA} \), the maximal activity:

\[ \nu \cdot \left( \frac{[A]_e}{[A]_e^{1/2} + [A]_x^{1/2}} \right) \]

\[ = \frac{0.5 \left( [A]_e^{1/2} + [A]_x^{1/2} \right)}{\left( [A]_e^{1/2} + [A]_x^{1/2} \right)} \]

The average rate of synthesis of product (\( v \)) from endogenous A in the presence of exogenous A may be expressed as:

\[ v = \frac{\nu [A]_x}{K_{mA} + [A]} \]

This is Equation 3 near "Results." Substituting expressions for [A], D, and V from Equations 9, 10, and 11 into Equation 3:

\[ v = \frac{\left( K_{mA} + 0.5 \left( [A]_e^{1/2} + [A]_x^{1/2} \right) \right) \nu \left( [A]_e^{1/2} + [A]_x^{1/2} \right)}{0.5 \left( [A]_e^{1/2} + [A]_x^{1/2} \right) \left( [A]_e^{1/2} + [A]_x^{1/2} \right)} \]

\[ X \left( [A]_e^{1/2} + [A]_x^{1/2} \right) \left( [A]_e^{1/2} + [A]_x^{1/2} \right) \]

Simplifying:

\[ v = \frac{\left( [A]_e^{1/2} + [A]_x^{1/2} \right) \left( [A]_e^{1/2} + [A]_x^{1/2} \right)}{K_{mA} + 0.5 \left( [A]_e^{1/2} + [A]_x^{1/2} \right)} \]

If the rate of appearance of endogenous A is approximately constant for 1 min from the addition of ME pyr-1-3, then \([A]_e = 0\). To simplify the analysis, \( \nu \) and \( v \) can be expressed as the net concentrations of product synthesized after 1 min of reaction time.

Theoretical values for \( v \) in the presence of various concentrations of exogenous A (N-carbamoyl-l-aspartate) were calculated by substituting experimentally obtained values for \( \nu \), \( [A]_e^{1/2} \), \( [A]_x^{1/2} \), \( [A]_e^{1/2} \), \( [A]_x^{1/2} \), and \( [A]_x^{1/2} \) into Equation 12. Values for \( K_{mA} \) ranging from low values obtained for study of the individual enzymes to higher values obtained in the presence of other assay components (Table 3) were used to generate theoretical curves for \( v \) as a function of the average concentration of exogenous A, \( 0.5 \left( [A]_e^{1/2} + [A]_x^{1/2} \right) \). Comparison of these curves with the values for \( v \) obtained experimentally indicated the degree of substrate channeling of endogenous A (Fig. 6b).