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Uridylic Acid Synthesis in Ehrlich Ascites Carcinoma. Properties, Subcellular Distribution, and Nature of Enzyme Complexes of the Six Biosynthetic Enzymes†

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ABSTRACT: The six enzymes required for the biosynthesis of uridylic acid have not previously been characterized in a single cell line. To establish the subcellular localization, the general kinetic characteristics, and, more importantly, to be able to consider the possible rate-limiting step, we have studied the six enzymes in the mouse Ehrlich ascites carcinoma. All enzymes except dihydroorotase were studied in the biosynthetic direction. Five of the enzymes (carbamyl phosphate synthetase, aspartate transcarbamylase, dihydroorotase, orotidylate phosphoribosyltransferase, and orotidylate decarboxylase) are located predominantly in the soluble (100,000*g* for 60 min) supernatant fraction of the cell as two enzyme complexes, but a significant proportion of these activities can also be localized in the crude nuclear fraction. One complex contains the first three enzymes of the pathway, carbamyl phosphate synthetase, aspartate transcarbamylase, and dihydroorotase. This complex, isolated in sucrose gradients containing 30% dimethyl sulfoxide and 5% glycerol, has an apparent molecular weight of 800,000–850,000. The glutamine-dependent carbamyl phosphate synthetase can be dissociated from the complex by reducing the dimethyl sulfoxide concentration to 10% to yield a protein with an apparent molecular weight of 150,000–200,000 whose activity is very labile. When the carbamyl phosphate synthetase is dissociated from the complex, the aspartate transcarbamylase and dihydroorotase activities still cosediment in the sucrose gradient, but both enzyme activities now occur in two new protein peaks with apparent molecular weights of 400,000–450,000 and 650,000–700,000. These latter two peaks are present in a 0.25 M sucrose homogenate. The second enzyme complex contains the fifth and sixth enzymes of the pathway, orotidylate phosphoribosytransferase and orotidylate decarboxylase. This complex sediments in a sucrose gradient containing 30% dimethyl sulfoxide and 5% glycerol with an apparent molecular weight of 105,000–115,000; however, when only sucrose is present, the complex is unstable and a major portion of the activities sediments as a complex of 55,000–60,000 daltons. The fourth enzyme, dihydroorotate dehydrogenase, unlike the other five enzymes, is not found in the soluble supernatant, but sediments from the homogenate at 700*g*. The natural electron acceptor(s) for this enzyme has not been identified; a new assay is described for this enzyme. The dihydroorotate dehydrogenase will utilize dihydroorotate formed by the complex of the first three enzymes and the orotate so produced is converted to UMP by the orotidylate enzymes of the 100,000*g* supernatant. The apparent K_m values and pH optima were determined for four of these enzymes, since they have not been previously determined in extracts of these cells. Although the Ehrlich ascites cell carbamyl phosphate synthetase is subject to product inhibition (by UTP) and precursor activation (by 5′-phosphoribosyl 1′-pyrophosphate), as discovered for this enzyme in extracts of mouse spleen by M. Tatibana and his colleagues, it is not the enzyme with the slowest optimal rate; rather orotidylate phosphoribosyltransferase or perhaps dihydroorotase when it is measured in the biosynthetic direction seem(s) to be the slowest catalyst(s).

The six enzymes required for the biosynthesis of uridylic acid (Figure 1) have not previously been characterized simultaneously in a single cell line under apparently optimal conditions. It is, therefore, difficult from the literature to compare the relative rates of these enzymes for a given cell or to even begin to devise experiments to *properly* assess the factors that may regulate the rate of these enzymes for any given cell *in vivo*. This paper, therefore, represents a start on a study of the

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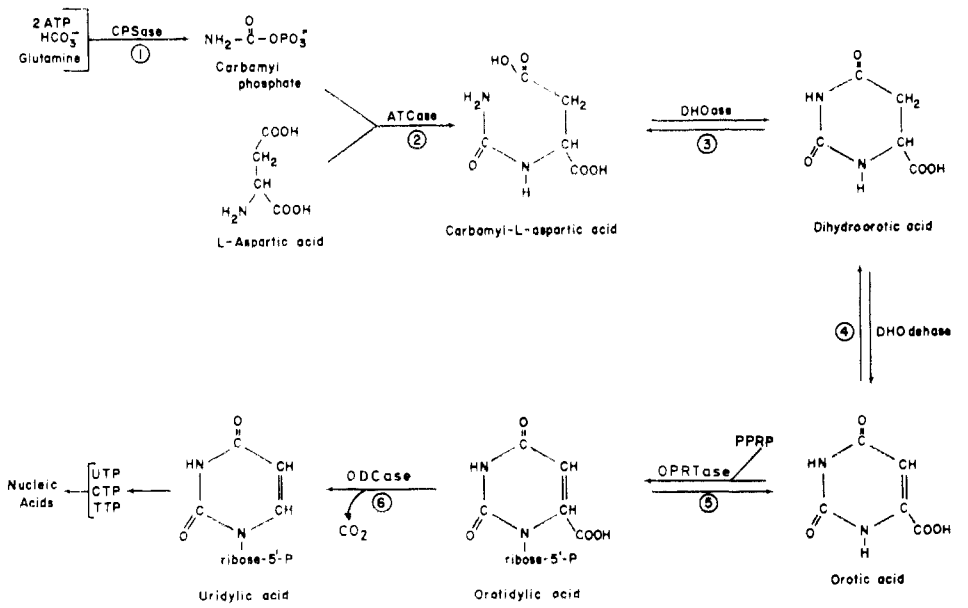

FIGURE 1: The pyrimidine biosynthetic pathway. The enzymes catalyzing the above reactions are as follows: CPSase, carbamyl phosphate synthetase, EC 2.7.2.5; ATCase, aspartate transcarbamylase, EC 2.1.3.2; DHOase, dihydroorotase, EC 3.5.2.3; DHOdehase, dihydroorotate dehydrogenase, EC 1.3.3.1; OPRTase, orotidylate phosphoribosyl transferase, EC 2.4.2.10; ODCase, orotidylate decarboxylase, EC 4.1.1.23.

regulation of all six of these enzymes in a single mammalian cell line, namely, the mouse Ehrlich ascites cell. Factors studied have been preliminary conditions for optimal rate and location of the enzymes during subcellular fractionation in two solvents.

Early in these studies, it became evident (Jones, 1971) that the first two enzymes of the pathway in Ehrlich ascites cells could be isolated as a complex as was independently observed in mouse spleen (Hoogenraad *et al.*, 1971) and as has been observed more recently in human lymphocytes (Ito and Uchino, 1973). However, preliminary experiments indicated that the ascites cell–enzyme complex probably also contained dihydroorotase (Shoaf and Jones, 1971); that finding is more fully verified here. The co-purification of orotidylate phosphoribosyltransferase and decarboxylase from calf thymus (Kasbekar, *et al.*, 1964) and bovine brain (Appel, 1968) had shown, although it was not emphasized by either laboratory, that these enzymes probably are normally associated in a complex. The data presented here indicated that this is the case for these enzymes in the Ehrlich ascites cell and for the first time the size of this complex, as well as its stability in sucrose homogenates, have been studied.

Materials and Methods

Radioactive Chemicals. The following compounds were purchased from New England Nuclear Corporation: [^{14}C]-carbamyl phosphate, 4.89 mCi/mmol; ^{14}C-labeled DL-*N'*-carbamyl-L-aspartate, 3.75 mCi/mmol; DL-[6-^{14}C]dihydroorotate, 3.03 mCi/mmol; [*carboxyl*-^{14}C]orotate, 10.2 mCi/mmol; [*carboxyl*-^{14}C]orotidylate, 21 mCi/mmol; uridine 5'-[2-^{14}C]monophosphate. Sodium [^{14}C]bicarbonate, 60 mCi/mmol, was purchased from Amersham/Searle Corporation. [^{14}C]Carbamyl phosphate from New England Nuclear was recrystallized by the method of Adair and Jones (1972), to remove radioactive impurities.

Preparation of Ehrlich Ascites Tumor Cell Extract. Ehrlich ascites tumor cells were obtained from mice (Swiss-Webster, male, 30 g, injected with 0.1 ml of ascitic fluid) 7–9 days after injection. The cells were separated from the ascitic fluid by a low-speed centrifugation for 2 min. The cells were then suspended to the original total volume in 0.9% room-temperature NaCl and pelleted in the same way three more times. For each milliliter of packed cells, 5 ml of room-temperature distilled water was added, the cells were resuspended and centrifuged immediately for 5 min, and a small volume of supernatant water was discarded. Water-swollen cells (4.0 ml) were then extracted with 2.0 ml of dimethyl sulfoxide–glycerol mixture (6:1) or after addition of 2 ml of 0.75 M sucrose. The mixture was cooled to 0° and homogenized in a Ten Broeck glass tissue grinder for 5 min. To remove whole cells and nuclei, the extract was centrifuged for 10 min at room temperature in the clinical centrifuge. The pelleted material was discarded while the supernatant solution was centrifuged at 4° for 30 min at 100,000*g*. The pellet was discarded. The 100,000*g* supernatant obtained after a 30-min centrifugation will be called the D–G supernatant.[1]

Protein Determination. Protein was assayed by a modification (Oyama and Eagle, 1956) of the method of Lowry *et al.* (1951).

General Conditions for Pyrimidine Enzyme Assays. When dimethyl sulfoxide–glycerol or sucrose extracts were assayed at various protein concentrations, the amount of the homogenizing solvent was kept constant at the level used for the highest protein concentration tested. In other experiments, when a constant volume of extract was used, no additional solvent was added.

Carbamyl Phosphate Synthetase Assay. The enzyme assay used a slightly modified procedure from that described by Hager and Jones (1967a). The routine assay system contained 150 mM Tris-Cl (pH 7.5), 150 mM KCl, 25 mM ATP, 25 mM $MgCl_2$, 10 mM ornithine, 0.1 mM glutamine, 3 mM $NaHCO_3$, 0.667 mM [^{14}C]bicarbonate, excess ornithine transcarbamylase

[1] Abbreviations used are: PRibPP, 5'-phosphoribosyl 1'-pyrophosphate; D–G supernatant, the 100,000*g* supernatant obtained from a homogenate prepared in 30% (v/v) dimethyl sulfoxide and 5% (v/v) glycerol which was centrifuged at this speed for 30 min.

(to produce 125 μmol of citrulline/min at pH 8.5, using the assay of Nakamura and Jones (1970)), and tissue extract in a total volume of 1 ml. The incubation was for 30 min at 37°.

Aspartate Transcarbamylase Assay. Aspartate transcarbamylase was assayed by a modification of the method of Bethell *et al.* (1968). The reaction vessel contained 100 mM Tris-Cl^- and 100 mM Bis-Cl^- (2-amino-2-methyl-1,3-propanediol) at pH 8.5, 4 mM aspartate (pH 8.5), [^{14}C]carbamyl phosphate (2 mM when full saturation of the enzyme was required), and enzyme in a total of 1 ml. The time of incubation was 10 min at 37°. The reaction was stopped by the addition of 0.5 ml of normal perchloric acid. The samples were heated at 100° for 6 min and then cooled and bubbled for 30 min with carbon dioxide to remove $^{14}CO_2$ formed from carbamyl phosphate. The volume was readjusted to compensate for evaporation. The entire sample was then placed in a scintillation vial containing 10 ml of scintillation fluid.

Dihydroorotase Assay. Dihydroorotase activity was measured by converting dihydroorotate to *N'*-carbamyl-L-aspartate, which was then determined spectrophotometrically by the assay of Prescott and Jones (1969). The reaction mixture contained 200 mM Tris-Cl^- (pH 9.0), 0.4 mM dihydroorotate, and tissue extract in a total volume of 1 ml. The incubation was for 45 min at 37°; the reaction was stopped by the addition of 0.2 ml of 4 M perchloric acid. A modification of the assay of Yates and Pardee (1957), which measures the amount of dihydroorotate present by its absorbance at 240 nm and by its rate of hydrolysis at pH 13, was used in preliminary experiments to confirm that the loss of this substrate equaled the amount of carbamyl phosphate formed. The enzyme activity was also measured by converting [^{14}C]dihydroorotate to ^{14}C-labeled *N'*-carbamyl-L-aspartate. The labeled aspartate was then cyclized to hydantoinacetic acid and separated from dihydroorotate on Dowex-1–formate columns as described (Hager and Jones, 1965).

Dihydroorotate Dehydrogenase Assay. Enzyme activity was measured by converting [^{14}C]dihydroorotate (3.03 mCi/mmol) to [^{14}C]orotate, and then isolating the [^{14}C]orotate formed by thin-layer chromatography. The reaction was carried out at 37° for 60 min. The reaction mixture contained 200 mM Tris-Cl^- (pH 7.4), 0.5 mM DL-[^{14}C]dihydroorotate (the concentration at which the enzyme becomes saturated with the DL substrate), and extract in a volume of 0.1 ml. The reaction was stopped by the addition of 2 μl of 70% perchloric acid and neutralization with KOH and K_2CO_3. After centrifugation to remove precipitated protein and potassium perchlorate, 10 μl of the sample was spotted on thin-layer DEAE-cellulose plates. The plates were 5 × 20 cm; the DEAE-cellulose layer was 0.35 mm thick. The plates were developed in a methanol–10% glacial acetic acid solvent (1:3, v/v) for approximately 2 hr. Plates were then dried and scanned with a Packard radiochromatogram scanner system (Model 7201). The R_F for orotate was usually 0.12, and the R_F for dihydroorotate was between 0.75 and 0.82. Spots were scraped from the plates into scintillation vials to which 1 ml of water and 10 ml of scintillation fluid were added. Because some dihydroorotate might be converted to *N'*-carbamyl-L-aspartate by dihydroorotase, and some orotate can be converted to orotidylate and UMP, it was necessary to know where these compounds migrated. In this system, *N'*-carbamyl-L-aspartate migrated with dihydroorotate, while UMP migrated with or slightly ahead of the orotate peak, and orotidylate migrated somewhat more slowly. Thus, the area representing R_F values between 0.05 and 0.35 was the orotate area, but it contained orotate, orotidylate, and UMP, *i.e.*, all the products formed from dihydroorotate. The dihydroorotate area (R_F values between 0.5 and 1.0) represented the substrate added or *N'*-carbamyl-L-aspartate.

Orotidylate Phosphoribosyltransferase Assay. This enzyme was measured by converting [*carboxyl*-^{14}C]orotate (10.2 mCi/mmol) and PRibPP[1] to orotidylate, which was then converted to UMP and $^{14}CO_2$ by endogenous orotidylic decarboxylase or by adding a purified yeast orotidylic decarboxylase from the Sigma Chemical Co. The latter enzyme contained less than 1% orotidylate phosphoribosyltransferase activity. The reaction was carried out at 37° for 30 min in serum cap sealed scintillation vials. The serum cap supported a plastic center well which contained 0.2 ml of a mixture of ethylene glycol and ethanolamine (2:1, v/v) used to absorb the $^{14}CO_2$. The reaction mixture contained 100 mM Tris-Cl^- (pH 7.5), 2 mM $MgCl_2$, 0.11 mM PRibPP, 30 μM [^{14}C]orotate, and tissue extract in a volume of 1 ml. The reaction was stopped by injecting 0.2 ml of ice-cold 4 M perchloric acid through the serum cap. Diffusion of $^{14}CO_2$ was complete after 60 min at room temperature; the caps were then removed and the entire center well was placed into another scintillation vial with 10 ml of dioxane-based scintillation fluid. The method is a modification of that of Fausto (1969).

Orotidylic Decarboxylase Assay. Enzyme activity was measured by the release of $^{14}CO_2$ from the carboxyl group of orotidylate (21 mCi/mmol). The reaction was carried out at 37° and 30 min in serum-cap sealed scintillation vials, as described for the orotidylate phosphoribosyltransferase assay. The reaction mixture contained a citric acid (100 mM)–Tris (100 mM) buffer, adjusted to pH 6.8, 0.1 mM [^{12}C]orotidylate and 1.9 μM [^{14}C]orotidylate, and tissue extract in a volume of 1 ml. The reaction was terminated and activity was determined as described in the orotidylate phosphoribosyltransferase assay.

Sucrose Gradients. Five per cent and 20% (w/v) sucrose (Schwarz/Mann Ultra-pure) solutions containing 0.05 M Tris were titrated to pH 7.5 with HCl; linear gradients (Martin and Ames, 1961) were prepared at room temperature, with an ISCO Model 570 gradient former. The gradients were kept at 5° for approximately 4 hr before use. The sample (usually 0.5 ml) was layered on the gradients and centrifuged for the indicated time and speed, at 5°. The centrifuge was a Beckman L2-65 preparative ultracentrifuge: with the SW 41 Ti rotor, the 12-ml gradients were developed at 40,000 rpm; with the SW 65L Ti rotor, the 5-ml gradients were developed at 64,000 rpm. Fractions were collected from the top of the gradient with an ISCO Model 183 density gradient fractionator. All marker enzymes sedimented to positions expected for their molecular weight. Recovery of enzyme activity from the gradients was usually measured and was always less than 100%. A number of factors make it difficult if not impossible to be concerned at this time about the lack of total recovery. These problems include the following. (1) A constant loss of carbamyl phosphate synthetase activity with time in dimethyl sulfoxide–glycerol solutions (Figure 10) as well as its instability in sucrose solutions present a problem; the instability of the activity of the orotidylate enzymes in sucrose is a similar problem (see Table V). (2) The fact that the protein–activity curves for three enzymes, *i.e.*, carbamyl phosphate synthetase (Figure 3) and the two orotidylate enzymes (Figure 7), are not linear means that unless one assays each formation from the sucrose gradient over a wide range of protein concentrations, one must generally recover less than 100% of these activities. It is a practical impossibility to carry out a large enough sucrose gradient to do this for those fractions where the

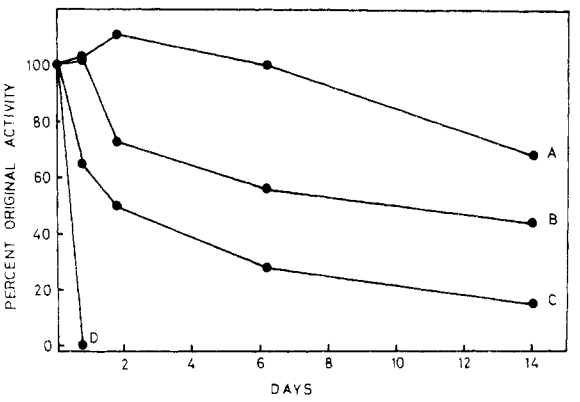

FIGURE 2: Stability of carbamyl phosphate synthetase to storage at −20°. The preparation used is the D–G supernatant fraction which contains most of the synthetase activity of the cell (see Table II). A is that fraction prepared so that the final concentration of dimethyl sulfoxide is 30% (v/v) and glycerol is 5% (v/v); B is 20% (v/v) dimethyl sulfoxide–5% (v/v) glycerol; C is 10% (v/v) dimethyl sulfoxide–5% (v/v) glycerol; D is enzyme in 0.05 M potassium phosphate buffer (pH 7.5) with 14 mM mercaptoethanol. Samples were stored for the interval indicated. The stored samples were used once and discarded. The initial activities were: A, 1553; B, 1974; C, 1871; D, 168 cpm per μmol of citrulline.

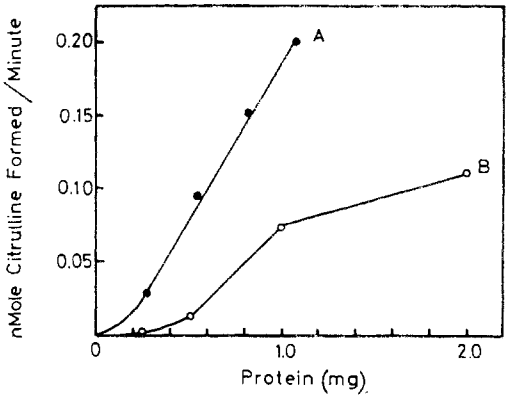

FIGURE 3: Carbamyl phosphate synthetase assay–protein concentration curve. An ATP regenerating system was required to obtain linearity over the large range of protein concentrations used, line A. The ATP-regenerating system contained 10 mM ATP, 10 mM phosphoenolpyruvate, and 10 μg of pyruvate kinase (Boehringer, 150 U/mg) to replace the ATP of the standard assay system listed under Materials and Methods. The lack of much enzyme activity at low protein concentrations was observed both in the presence (line A) and absence (line B) of the ATP-regenerating system.

enzyme is dilute. (3) It is at this time uncertain whether the specific activities of each of the various enzymes are identical in the various protein complexes recorded here, *i.e.*, whether the sum of the fractions should add up to 100%. Taking these considerations into account, one, at this moment, can only record what is observed; further work with purified complexes, purposely dissociated and recombined, will answer these questions.

Enzyme Marker Assays. Lactate dehydrogenase (rabbit muscle, mol wt 136,000) was assayed by measuring the rate of decrease in absorbancy at 340 nm as NADH is oxidized (Worthington Biochemical Corporation catalog). Catalase (beef liver, mol wt 247,000) was assayed by the disappearance of peroxide measured spectrophotometrically at 240 nm as described by Beers and Sizer (1952). Glutamine synthetase (*Escherichia coli*, mol wt 592,000) was assayed by a modification of the method described by Elliot (1955).

Experiments Measuring the Pathway Products Formed from Bicarbonate. The reaction mixtures contained 150 mM Tris-Cl (pH 7.5), 0.1 mM glutamine, 4 mM aspartate, 25 mM $MgCl_2$, 25 mM ATP, 0.265 mM phosphoribosyl pyrophosphate, 1.66 mM $NaH^{14}CO_3$ (sp act. 60 mCi/mmol), and enzyme as indicated in the legend of Figure 10, in a final volume of 0.4 ml. The reaction was stopped by the addition of 0.2 ml of 1 N perchloric acid; 1 μmol each of *N'*-carbamyl-L-aspartate, dihydroorotate, orotate, orotidylate, and UMP was added to each sample as carriers. The protein precipitate was removed by centrifugation and then the supernatant was heated at 100° for 60 min. Under these conditions, *N'*-carbamyl-L-aspartate cyclizes to hydantoinacetic acid which allows separation of the hydantoin from dihydroorotate, and all di- and trinucleotides are converted to mononucleotides. Samples of the supernatant were bubbled for 30 min with CO_2; the pH was carefully adjusted with constant mixing to 7–7.5 with KOH. Samples were placed on a 0.8 × 4.0 cm Dowex-1–formate column (100–200 mesh; 3.2 mequiv/g dry weight). The column was washed with 20 ml of water. *N'*-Carbamyl-L-aspartate (as the hydantoin), dihydroorotate, UMP, and orotate were eluted as described by Hager and Jones (1965) while orotidylate emerged with 1.25 M ammonium formate, pH 4.3, as illustrated in Figure 15. Two-milliliter fractions were collected and 1 ml of each fraction was counted by liquid scintillation. Compounds were identified by at least two of the following methods: comparing the elution patterns of unknowns with the elution patterns of standards, electrophoresis or thin layer chromatography in comparison with standards (Shafritz and Senior, 1967), or by the spectra for orotate, orotidylate, or UMP.

Electrophoresis. High voltage electrophoresis was used to help determine the identity of *N'*-carbamyl-L-aspartate, dihydroorotate, orotate, orotidylate, and UMP. Samples were applied to 80 × 3.75 cm Whatman No. 3MM chromatography paper strips. Seven strips were used on a Savant plate-type electrophoresis chamber. A pH 4 buffer contained 0.05 M sodium citrate with 1 mM EDTA which helped maintain sharper bands and reduced trailing. Electrophoresis using this buffer with 2500 V and ~20 mA initial current (final ~40 mA) for 3.5 hr at 0° resulted in the following migration distances: UMP, 28 cm; orotate, 33 cm; *N*^{1}-carbamyl-L-aspartate or dihydroorotate, 36 cm; orotidylate, 40 cm. *N'*-Carbamyl-L-aspartate and dihydroorotate were separated using a 0.1 M potassium phosphate buffer at pH 6.8. When the time used was 1.75 hr at 3500 V and 0°, the following migration distances were obtained: orotate, 41 cm; dihydroorotate or UMP, 53 cm; orotidylate, 64 cm; *N'*-carbamyl-L-aspartate, 70 cm. Trailing occurred with both systems if high salt concentrations were present in the sample.

Results

Carbamyl Phosphate Synthetase. The mammalian glutamine-dependent carbamyl phosphate synthetase is unstable in conventional aqueous buffers. It can be stabilized in 0.25 M sucrose containing Mg-ATP (Hager and Jones, 1967a) or by the use of 30% (v/v) dimethyl sulfoxide and 5% (v/v) glycerol (Tatibana and Ito, 1969). This latter solvent also gives marked stability to the Ehrlich ascites carbamyl phosphate synthetase on long term storage at −20°. A stability curve of the enzyme stored at −20° is shown in Figure 2 for extracts prepared in 5% (v/v) glycerol, plus 10, 20, and 30% (v/v) dimethyl sulfoxide, and of the enzyme in the absence of dimethyl sulfoxide and glycerol.

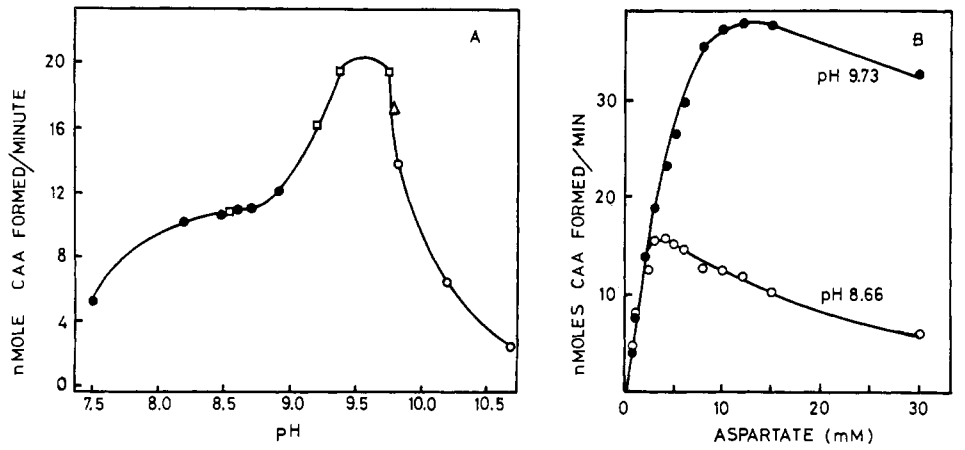

FIGURE 4: Aspartate transcarbamylase. (A) pH curve: the concentration of aspartate was 5 mM; the carbamyl phosphate concentration was increased to 6 mM to ensure that even after some hydrolysis at pH levels above 8.5 the enzyme would be saturated. The time of incubation was shortened to 5 min because of the increased instability of carbamyl phosphate at the higher pH values (Allen and Jones, 1964). Closed circles indicate the reaction in 200 mM Tris-Cl^-; squares, 200 mM Bis-Cl^-; triangles, 100 mM cyclohexylaminopropanesulfonic acid; and open circles, 200 mM butylamine. The enzyme was 0.1 ml of the D–G supernatant. (B) Aspartate concentration curves: at pH 8.66, the reaction was buffered with 200 mM Tris-Cl^-, and at pH 9.73 with 200 mM bis-Cl^-. Values in Tris-Cl^- or Bis-Cl^- at the same pH were identical (see part A). Carbamyl phosphate (2 mM) was saturating. The incubation time was 10 min.

The enzyme reaction is linear when the protein concentration is greater than 0.25 mg/ml and when phosphoenolpyruvate and pyruvate kinase are present (line A of Figure 3). At lower protein concentrations, little or no enzyme activity was observed. This lack of linearity has been noted previously (Hager and Jones, 1967a); to obtain linearity with protein concentrations above 0.25 mg/ml, it is necessary to use the ATP regenerating system (compare lines A and B of Figure 3).

As seen in Table I, the Ehrlich ascites carbamyl phosphate synthetase is inhibited by the end product, UTP, and activated by PRibPP, as is the mouse spleen synthetase (Tatibana and Shigesada, 1972).

Aspartate Transcarbamylase. The assay for aspartate transcarbamylase was linear with both time and protein concentration. The pH optimum (Figure 4A) for the reaction was between pH 9.4 and 9.75 using 6 mM carbamyl phosphate. Above this pH the rate drops rapidly. At pH values below the optimum, there is a broad plateau (pH 8–9), where the rate is half-maximal. The standard assay utilizes this plateau region, for it is also a region where the hydrolysis of carbamyl phosphate (Allen and Jones, 1964) is not base catalyzed.

Michaelis constants, calculated from Lineweaver–Burk plots, were determined for aspartate at pH 8.66 and 9.73; these K_m values were 2–3 and 6–8 mM, respectively. Substrate inhibition by aspartate is particularly prominent at pH 8.66, but is also observed at pH 9.73 (Figure 4B). The two curves are interesting, for they were obtained within the same experiment and they indicate that at subsaturating concentrations of aspartate, *i.e.*, below 2 mM aspartate, the rates are identical at pH 8.66 and 9.73. There would, therefore, be no pH optimum between pH 9.4 and 9.73 at low aspartate concentrations. However, above this concentration, the enzyme is strongly inhibited by aspartate at pH 8.66 while it is not at pH 9.73; therefore, one can observe the pH 9.4–9.7 maximum seen in Figure 4A, when 5 mM aspartate is utilized.

The apparent K_m for the other substrate, carbamyl phosphate, was determined at pH 8.66, and found to be 1000-fold lower than that for aspartate, namely, 3–4 μM when the aspartate concentration was 5 mM. The substrate–velocity curve is hyperbolic and the reverse plot is linear. In the course of testing whether aspartate transcarbamylase is inhibited by nucleotide mono-, di-, and triphosphates (we found no marked inhibition), an inhibition by phosphate (Figure 5) was observed at low (2.75 μM) carbamyl phosphate concentrations, which was independent of the cation used. The inhibition is competitive with carbamyl phosphate, for it was markedly reduced by raising the concentration of this substrate.

Dihydroorotase. This enzyme was routinely measured in the reverse direction (dihydroorotate to *N'*-carbamyl-L-aspartate). The enzyme has a pH optimum of 8.0–8.5; the curve obtained was like that previously reported by Bresnick and Hitchings (1961) for the enzyme extracted from Ehrlich ascites tumor cells. The dihydroorotate saturation curve was not reported by these authors; the apparent K_m value derived from a reciprocal plot of the hyperbolic substrate curve for L-dihydroorotate was 0.15–0.25 mM at pH 8.2.

Dihydroorotate Dehydrogenase. Unlike the other five enzymes required for the biosynthesis of UMP, none of the dihydroorotate dehydrogenase activity is solubilized when the cells are extracted with either 30% (v/v) dimethyl sulfoxide and 5% (v/v) glycerol or with 0.25 M sucrose (Table II). Both

TABLE I: Effect of 5-Phosphoribosyl 1-Pyrophosphate or Uridine Triphosphate on Carbamyl Phosphate Synthetase Activity.[a]

ATP (mM)	UTP (mM)	PRibPP (mM)	% Act.
2	0	0	100
2	1	0	33
2	2	0	13
2	0	0.1	868
2	0	0.5	1257
2	0	1	1350
2	0	2	1340
2	1	0.5	1023
0	0	2	0

[a] The standard conditions for the synthetase assay were used, except that 5 mM $MgCl_2$ and 1 mM $NaHCO_3$ were used. The D–G supernatant (0.2 ml) was the enzyme source.

TABLE II: Subcellular Distribution of the Six Enzymes Required for *de novo* UMP Biosynthesis.[a]

	CPSase		ATCase		DHOase		DHOdehase		OPRTase		ODCase	
	Suc	D–G	Suc	D–G	Suc	D–G	Suc	D–G	Suc	D–G	Suc	D–G
Homogenate	100		100	100	100	100	100	100	100	100	100	100
700*g* pellet	22		26	19	32	30	86	90	0	4	33	7
9200*g* pellet	0		0	0	1	2	4	5	0	0	0	0
100,000*g* pellet	3		5	4	5	0	2	1	0	0	0	1
100,000*g* supernatant	70		72	76	60	67	2	0	37	90	66	92

[a] Ehrlich ascites tumor cells were extracted in sucrose or dimethyl sulfoxide–glycerol so that a final concentration of 0.25 M sucrose (Suc) or 30% (v/v) dimethyl sulfoxide and 5% (v/v) glycerol (D–G) was obtained. Fractions were obtained by differential centrifugation and pellets were washed once, repelleted, and then resuspended in a volume of solvent equal to its original volume. The 700*g* 10-min and 9200*g* 15-min pellets were centrifuged at 5° in a Sorvall centrifuge using an SS-34 rotor. The 100,000*g* 60-min pellet and supernatant were obtained by centrifugation of the 9200*g* supernatant at 5° in a Beckman L2-65B preparative ultracentrifuge using a No. 40 rotor. All values are percentages, the activity of the whole homogenate being assigned a value of 100%. Numerical values for the specific activity of each enzyme of the whole homogenate are found in Table V. Enzyme abbreviations are: carbamyl phosphate synthetase, CPSase; aspartate transcarbamylase, ATCase, dihydroorotase, DHOase; dihydroorotate dehydrogenase, DHOdehase; orotidylate phosphoribosyltransferase, OPRTase; and orotidylate decarboxylase, ODCase.

homogenates were fractionated by differential centrifugation into four fractions (see Table II) enriched in nuclei (usually containing some adhering cytoplasm and cell membranes, but only a few or no whole cells), mitochondria, microsomes, and soluble supernatant; the majority of the dihydroorotate dehydrogenase activity sedimented at 700*g* with the nuclei and cell membrane fraction from either 0.25 M sucrose or 30% (v/v) dimethyl sulfoxide and 5% (v/v) glycerol homogenate. A small amount (4–5%) of the activity appears in the mitochondrial fraction, but essentially no activity is found in the microsomal fraction or in the soluble supernatant.

The formation of orotate by the "nuclear" enzyme was linear with time and protein as long as no more than 75 μg of protein from the 700*g* resuspended pellets is used. Slightly above this concentration, the rate fell off rapidly under the conditions used here. Activity was completely stable for at least 2 weeks when the 700*g* pellet supernatant fraction was stored at 5° in either of the two solvents. The pH curve of the enzyme is shown in Figure 6.

Orotidylate Phosphoribosyltransferase. The conversion of orotate to orotidylate and then to UMP and carbon dioxide by either endogenous or added orotidylate decarboxylase was used as an assay for this enzyme. The assay is linear with time. The velocity was not linear with protein concentration until at least 40 μg of the D–G supernatant was added (Figure 7A). The apparent K_m values derived from the linear reciprocal plots of the hyperbolic substrate curves at pH 7.5 were 2 μM for orotate (PRibPP was 0.2 mM) and 0.125 mM for PRibPP (orotate was 0.015 mM). The enzyme had a pH optimum of 7.75 (Figure 7B). Orotidylate decarboxylase was present in excess over the orotidylate phosphoribosyltransferase at each pH value.

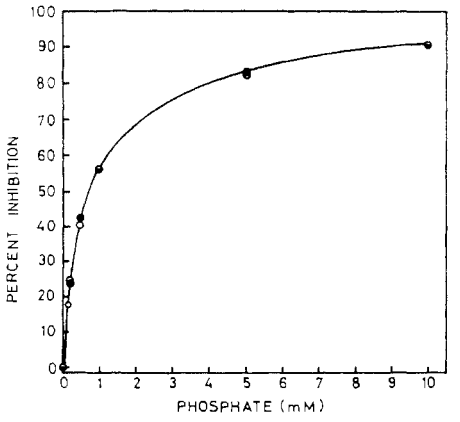

FIGURE 5: Aspartate transcarbamylase inhibition by phosphate. Aspartate was 2.5 mM and carbamyl phosphate was 2.75 μM. Closed circles are potassium phosphate; open circles, sodium phosphate. Both potassium and sodium phosphate were previously titrated to the pH of the reaction mixture (8.5). The amount of enzyme used was 0.5 μl of the D–G supernatant solution.

Orotidylate Decarboxylase. The assay which measured the $^{14}CO_2$ release as UMP is formed was linear with time. As with the orotidylate phosphoribosyltransferase, the reaction was not linear until a protein concentration of 40 μg of the D–G supernatant was obtained (Figure 7C). The apparent K_m for orotidylate derived from the linear reciprocal plot of the hyperbolic substrate curve was also low, 8–9 μM. When fully saturated with orotidylate (0.1 mM), the enzyme was inhibited by high concentrations (millimolar) of its product, UMP, and was more potently inhibited by 6-aza-UMP, 80% at 10 μM (Table III). The enzyme had a pH optimum of 6.8 (Figure 7D).

Subcellular Location of Enzyme Activities. As seen in Table II, the carbamyl phosphate synthetase, aspartate transcarb-

TABLE III: Inhibition of Orotidylate Decarboxylase by UMP and Aza-UMP.[a]

UMP (mM)	6-Aza-UMP (mM)	pmol of UMP/min	% Inhibition
		144	0
	0.0001	142	1
	0.001	103	28
	0.010	30	79
	0.100	2	99
0.1		148	0
1.0		137	5
5.0		109	24

[a] The orotidylate concentration was 0.1 mM. The enzyme used was 0.02 ml of the D–G supernatant.

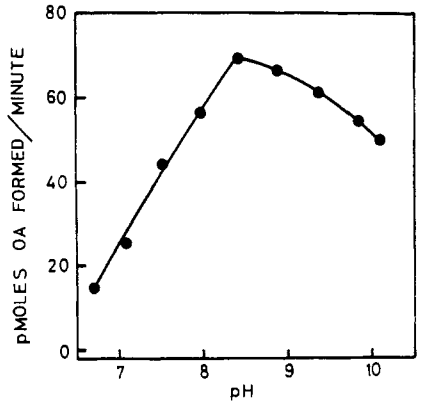

FIGURE 6: Dihydroorotate dehydrogenase assay; effect of pH. Standard assay conditions were used; 5 μl of the homogenate was used. The buffers were all 0.1 M Tris, adjusted with HCl to pH 6.5, 7.0, etc., to pH 10.5. The pH of each mixture of substrates and buffer plus the protein was measured, and this value is that reported on the abscissa.

amylase, and dihydroorotase activities were found predominantly in the soluble fraction of the cell (76, 72–76, and 60–67%, respectively), when either 0.25 M sucrose or 30% dimethyl sulfoxide–5% glycerol was used as the solvent for the homogenate. Approximately one-quarter of the synthetase and the transcarbamylase and one-third of the dihydroorotase activities were found in the crude nuclear fraction. All of the dihydroorotate dehydrogenase activity was particulate in both solvents.

In the dimethyl sulfoxide–glycerol solvent, 100% of the orotidylate phosphoribosyltransferase and decarboxylase activities of the homogenate were recovered in the soluble supernatant. When the orotidylate phosphoribosyltransferase

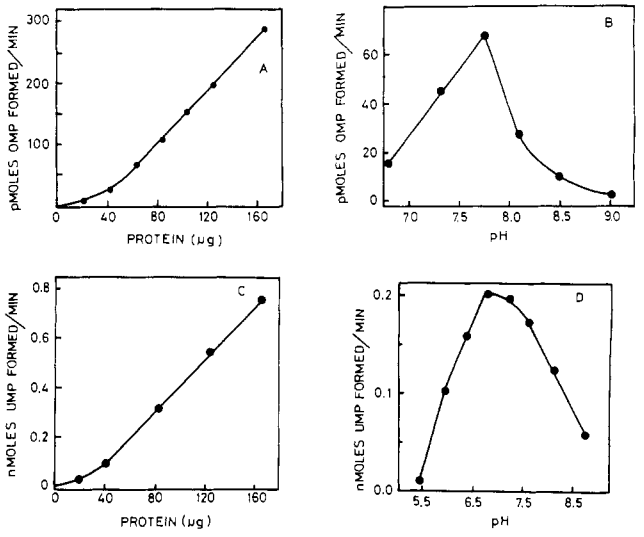

FIGURE 7: Effect of protein concentration and pH on orotidylate phosphoribosyltransferase and orotidylate decarboxylase. Orotidylate phosphoribosyltransferase activity: (A) protein concentration curve; purified yeast orotidylate decarboxylase (0.5 unit, Sigma Chemical Co.) was added to each sample; standard assay conditions were used; the protein added was the D–G supernatant; (B) pH curve; standard assay conditions were used, except that the orotate concentration was 7 μM and the PRibPP was 50 μM and Tris–citrate buffers were used. The enzyme used was 20 μl of the D–G supernatant. The pH listed is that observed when all components are present. Orotidylate decarboxylase activity: (C) standard assay conditions were used; (D) pH curve; standard assay conditons were used except that Tris–citrate buffers were used; the pH listed is that observed when all components are present.

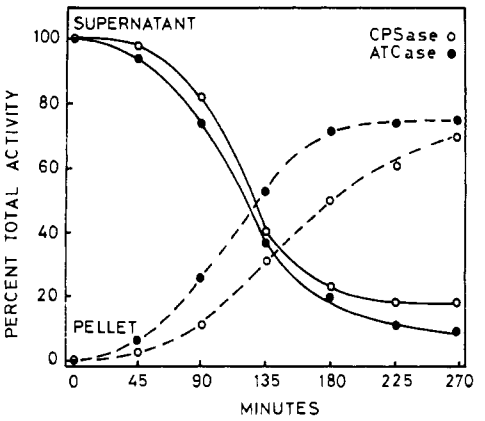

FIGURE 8: The copelleting of carbamyl phosphate synthetase and aspartate transcarbamylase. The extract was prepared as described under Materials and Methods. After centrifuging at 100,000g for 30 min at 5°, aliquots of the supernatant were further centrifuged at 100,000g in separate tubes which were removed from the centrifuge at 45-min intervals. Carbamyl phosphate synthetase and aspartate transcarbamylase activities were assayed in both the supernatant and the pellet which was resuspended in a volume of 30% (v/v) dimethyl sulfoxide–5% glycerol (v/v) to give the initial volume of the homogenate. Solid lines represent activitiy in the supernatant and dashed lines represent activity in resuspended pellet.

was fractionated in sucrose, the total recovery was always much less than 100%; the activity recovered (30–40%) was present in the 100,000g supernatant. Fractionation of orotidylate decarboxylase in 0.25 M sucrose always resulted in approximately two-thirds of the activity being recovered in the 100,000g supernatant. The remaining one-third was occasionally recovered in the "nuclear" fraction; this activity is, therefore, less stable than the supernatant activity.

The Carbamyl Phosphate Synthetase–Aspartate Transcarbamylase–Dihydroorotase Complex. Bottomley and Lovig (1967) have reported that rat liver aspartate transcarbamylase sediments from a 30-min, 105,000g soluble supernatant of a 0.25 M sucrose homogenate after an additional 4-hr centrifugation at 105,000g. Using Ehrlich ascites cell extracts, 80–90% of the aspartate transcarbamylase, as well as the carbamyl phosphate synthetase, can be pelleted in 4–5 hr of centrifugation at 100,000g (Figure 8). This allows one to concentrate the enzymes several-fold and also gives a fivefold purification of these activities. The fact that the two enzymes cosedimented suggested that the activities might be associated in a complex; it was subsequently found that the pellet also contained dihydroorotase (see Figure 9).

When resuspended, the carbamyl phosphate synthetase was less stable than it was in the original supernatant (100,000g for 30 min), even in 30% (v/v) dimethyl sulfoxide and 5% (v/v) glycerol. The stability of both the synthetase and the transcarbamylase when resuspended in 5% (v/v) glycerol with 10, 20, and 30% (v/v) dimethyl sulfoxide is shown in Figure 10. Aspartate transcarbamylase activity was stable under all conditions studied; however, the molecular weight of the aspartate transcarbamylase changed with the solvent used, as is discussed later. Carbamyl phosphate synthetase was unstable under all conditions studied, but its stability increased as the dimethyl sulfoxide concentrations were increased. In an attempt to see if the instability of the synthetase was related to its physical state, the rates of its sedimentation in sucrose gradients, as well as those of aspartate transcarbamylase and dihydroorotase, were examined. In a sucrose gradient containing 30% (v/v) dimethyl sulfoxide and 5% (v/v) glycerol, the activities of these three enzymes always co-

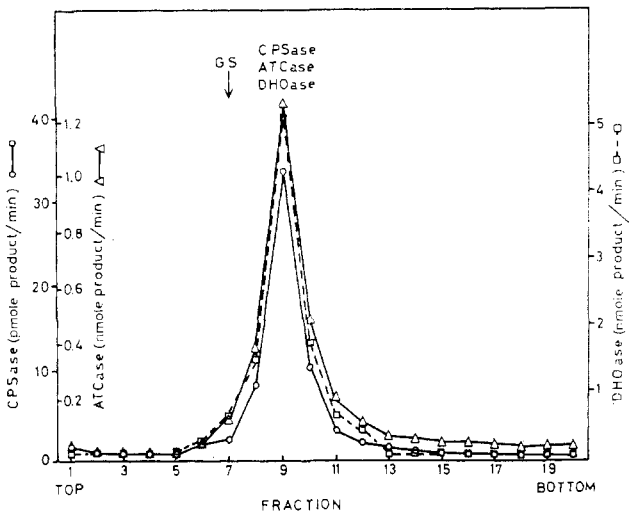

FIGURE 9: Cosedimentation of carbamyl phosphate synthetase, aspartate transcarbamylase, and dihydroorotase activities. The homogenate was prepared in 30% (v/v) dimethyl sulfoxide and 5% (v/v) glycerol and centrifuged for 30 min at 100,000*g* and 5°. The supernatant (24 ml) was centrifuged, as described in Figure 10, for 4.5 additional hours at 100,000*g* at 5°. The pellet obtained was resuspended in one-twelfth the original volume of the supernatant and 0.5 ml was layered on a sucrose gradient containing 5–20% (w/v) sucrose and 30% (v/v) dimethyl sulfoxide, 5% (v/v) glycerol, and 0.05 M Tris-Cl⁻, all at pH 7.5. The 12-ml gradient was centrifuged at 5° for 20 hr at 40,000 rpm using the SW 41 Ti rotor. Gs represents the peak for *E. coli* glutamine synthetase (0.36 mg) added as an internal marker. The apparent molecular weight of this complex was determined by measuring relative migration rates with respect to glutamine synthetase (and in other experiments, bovine liver catalase as well as glutamine synthetase). The enzymatic rates reported are those for a 0.2-ml sample from each 0.59-ml fraction. A sample of the protein solution applied to the gradient was held at 5° for 20 hr and assayed for the three enzymes at the same time as the fractions from the gradient. Using this sample to represent 100% of the activity that could be recovered, the following recoveries were usually observed: carbamyl phosphate synthetase, 31%; aspartate transcarbamylase, 45%; dihydroorotase, 47%; glutamine synthetase, 85%.

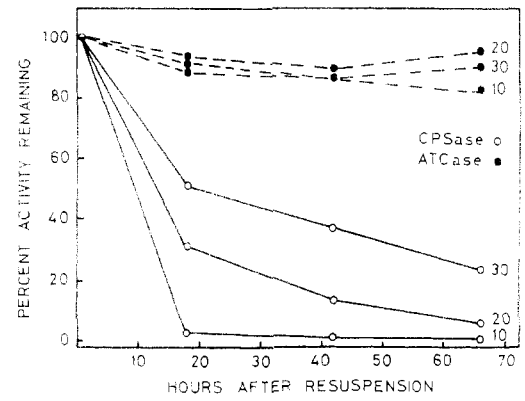

FIGURE 10: Effect of dimethyl sulfoxide on the stability of carbamyl phosphate synthetase and aspartate transcarbamylase after sedimentation of a pellet at 100,000*g* and resuspension. The extract was prepared in 30% (v/v) dimethyl sulfoxide and 5% (v/v) glycerol and centrifuged at 100,000*g* for 30 min at 5°. This supernatant was then centrifuged at 100,000*g* for 4 additional hours. The pellets from three tubes were resuspended in their original volume of either 10, 20, or 30% (v/v) dimethyl sulfoxide with 5% (v/v) glycerol present in all and held at −20° in individual tubes. The activities were measured at intervals following resuspension up to 66 hr by removing a tube and discarding the unused sample after assay. The per cent dimethyl sulfoxide is indicated on the right, at the end of each line. The 100% values for carbamyl phosphate synthetase in 30, 20, and 10% (v/v) dimethyl sulfoxide and 5% (v/v) glycerol are, respectively, 74.1, 79.7, and 63.5 pmol of citrulline formed per minute with 0.32 mg of protein; for aspartate transcarbamylase, 16.5, 18.8, and 18.9 nmol, respectively, of *N*′-carbamyl-L-aspartate formed per minute with 0.16 mg of protein. The synthetase was assayed by the usual procedure with the addition of an ATP regenerating system containing phosphoenolpyruvate and pyruvate kinase, to replace ATP (25 mM).

sediment in a single sharp peak, as shown in Figure 9. The apparent molecular weight of this peak was between 800,000 and 850,000, as determined from several experiments. However, when the 4-hr 100,000*g* pellet was resuspended in 10% (v/v) dimethyl sulfoxide and 5% (v/v) glycerol, the synthetase activity dissociated from the initial complex (Figure 11) and sedimented as if it had a molecular weight of 175,000. The activity was lower than that observed in the complex of the three enzymes and frequently was not measurable. Aspartate transcarbamylase and dihydroorotase activities remained associated, but now migrated as two peaks of lower molecular weight. The apparent molecular weights were 420,000 and 698,000.

Similar findings were made when the pellets were resuspended in a small volume of distilled water which was layered on a 5–20% (w/v) sucrose gradient containing Tris-Cl⁻ (pH 7.5); however, carbamyl phosphate synthetase was generally no longer measurable. A profile of such a gradient is seen in Figure 12; the molecular weights obtained for aspartate transcarbamylase and dihydroorotase were 430,000 and 680,000.

The relative height of the two aspartate transcarbamylase peaks seemed to vary slightly from preparation to preparation; we have not investigated the reason for this variation. It has been reported for rat, mouse, and chick tissues that the variation in the relative heights of two aspartate transcarbamylase peaks could be correlated with developmental levels and was also characteristic of the tissue extracted (Koskimies *et al.*, 1971).

Orotidylate Phosphoribosyltransferase-Orotidylate Decarboxylase Complex. The last two enzymes required for UMP biosynthesis are orotidylate phosphoribosyltransferase and orotidylate decarboxylase. Nearly all of the activities of these two enzymes are released into the soluble supernatant of homogenates in either 0.25 M sucrose or 25 mM Tris (pH 7.5) or 30% (v/v) dimethyl sulfoxide and 5% (v/v) glycerol. The enzymes are very stable in 30% (v/v) dimethyl sulfoxide and 5% (v/v) glycerol when stored at −20° (these extracts do not freeze at this temperature). When a sample of the 100,000*g* 30-min supernatant is placed on a 5–20% (w/v) sucrose gradient containing 30% (v/v) dimethyl sulfoxide, 5% (v/v) glycerol, and 0.05 M Tris-Cl⁻ at pH 7.5, the two activities cosediment in a single peak having an apparent mol wt of 105,000–115,000 (Figure 13).

When the Ehrlich ascites cells are extracted in 25 mM Tris-Cl⁻ at pH 7.5, and placed on a gradient with 5–20% (w/v) sucrose and 0.05 M Tris-Cl⁻, with a final pH of 7.5, most of the two activities cosediment, but the apparent mol wt, 55,000–60,000, is about half of that observed in dimethyl sulfoxide and glycerol (Figure 14). The orotidylate decarboxylase migrated as a nearly symmetrical peak but the orotidylate phosphoribosyltransferase always had a shoulder toward a higher molecular weight.

Intermediate Products of the Pyrimidine Biosynthetic Pathway. As shown in Figure 15C, D, or E, the two enzyme complexes of the soluble supernatant can be coupled by the addition of the particulate dihydroorotate dehydrogenase, to produce [^{14}C]UMP from [^{14}C]bicarbonate, ATP, Mg^{2+},

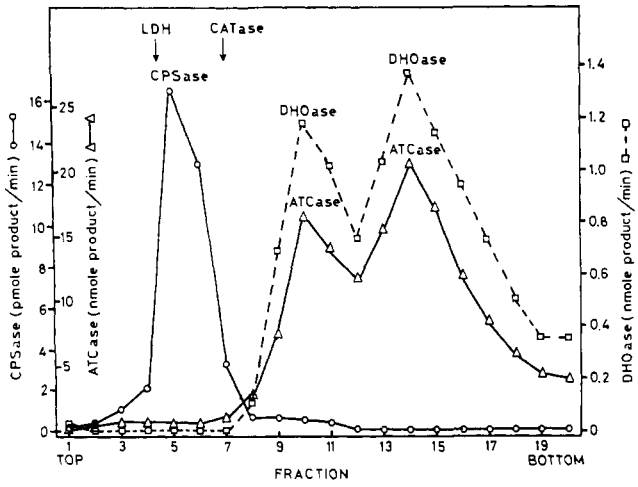

FIGURE 11: Dissociation of carbamyl phosphate synthetase from aspartate transcarbamylase and dihydroorotase. The enzymes were prepared and pelleted as indicated in Figure 9. The material pelleted at 100,000*g* after 4.5 hr was resuspended in 10% (v/v) dimethyl sulfoxide and 5% (v/v) glycerol; 0.5 ml was layered on a 12-ml sucrose gradient containing 5–20% (w/v) sucrose, 10% (v/v) dimethyl sulfoxide, 5% (v/v) glycerol, and 0.05 M Tris-Cl⁻, with a final pH of 7.5. The gradient was centrifuged at 5° for 15 hr at 40,000 rpm using the SW 41 Ti rotor. LDH and CATase indicate the peaks of activity of rabbit muscle lactate dehydrogenase and of bovine liver catalase, respectively, both of which were added as internal molecular weight markers. The enzymatic rates reported are those for a 0.2-ml sample from each 0.59-ml fraction. Recoveries of enzyme activities were not measured in this experiment.

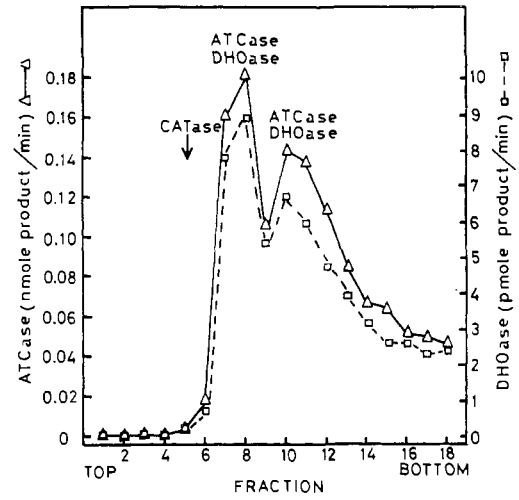

FIGURE 12: Sedimentation of aspartate transcarbamylase and dihydroorotase activities in a sucrose gradient. The enzyme was prepared and pelleted as indicated in Figure 11. The pellet was resuspended in distilled water and 0.5 ml was layered on a 5-ml sucrose gradient containing 5–20% (w/v) sucrose in 0.05 M Tris-Cl⁻ with a final pH of 7.5. The gradient was centrifuged at 5° for 2 hr at 64,000 rpm using the SW 65 Ti rotor. CATase indicates the peak of activity of bovine liver catalase, added as an internal molecular weight marker. The rates reported are those for a 0.2-ml sample from each 0.29-ml fraction. Recoveries of aspartate transcarbamylase were 46 and 30% in two separate experiments.

glutamine, aspartate, and PRibPP. If dihydroorotate dehydrogenase is not added to the 100,000*g* supernatant, synthesis of UMP from bicarbonate does not occur, and *N'*-carbamyl-L-aspartate and dihydroorotate accumulate (Figure 10A). Because of the instability of carbamyl phosphate in aqueous solutions, no attempt was made to detect the possible accumulation of this compound. The 700–100,000*g* pellet contains a portion of the dihydroorotate dehydrogenase, but does not contain significant amounts of the other five enzymes so no UMP is formed, and only a small amount of *N'*-carbamyl-L-aspartate and dihydroorotate accumulate (Figure 15B). When the dehydrogenase is added to the 100,000*g* supernatant, which contains the other five enzymes, synthesis of UMP occurred (Figure 15C), and by adding increasing amounts of the dehydrogenase, an increase in UMP formation and a decrease in the accumulation of carbamyl aspartate and dihydroorotate were observed (Figure 15D,E). This should not be interpreted to mean that the dehydrogenase is rate limiting in the cell since no electron acceptor was added, the samples were not aerated, and the 700–100,000*g* pellet contains only a fraction of the dehydrogenase in the cell. The total number of counts per minute which are measured as *N'*-carbamyl-L-aspartate, dihydroorotate, orotate, orotidylate, and UMP in Figure 15 are listed in Table IV; the sum of the peaks is comparable for all vessels containing the complete system for the synthesis of UMP.

Discussion

The data in this paper show that five of the enzymes required for UMP biosynthesis exist in 30% dimethyl sulfoxide–5% glycerol homogenates as two enzyme complexes. Since this is a nonphysiological solvent, one can question whether the complexes so observed occur as such in the cell or whether they are produced by this solvent.

Although solvents used for cell fractionation are selected for the fact that they are isotonic, they cannot be considered truly phyisological; this includes 0.25 M sucrose, the most common medium used for cell fractionation. This paper shows that the enzymes required for UMP biosynthesis distributed themselves in the same cell fractions whether the solvent used for homogenization was 0.25 M sucrose or 30% dimethyl sulfoxide–5% glycerol. The latter solvent proved most convenient in stabilizing the enzyme complex which contains the first three enzymes of the UMP biosynthetic pathway. The stabilization of carbamyl phosphate synthetase (Tatibana and Ito, 1969) by this solvent may be due to maintenance of the enzyme complex. Since these studies were initiated (Jones, 1971), two other laboratories have also observed that in 30% dimethyl sulfoxide–5% glycerol a protein complex containing carbamyl phosphate synthetase and aspartate transcarbamylase can be isolated and partially purified from mouse spleen

TABLE IV: Accumulation of Radioactivity in Intermediates of the Pyrimidine Biosynthetic Pathway.[a]

Figure	CAA-H, DHO	OA	OMP	UMP	Total
15A	143,000	900	1100	1,600	146,600
15C	114,000	4000	2300	18,700	139,000
15D	76,700	6200	3300	31,900	117,400
15E	49,400	8900	5500	54,400	118,200

[a] The figures here are the numerical values from A, C, D, and E in Figure 15. The numbers are total counts per minute incorporated into the given intermediates between bicarbonate and UMP, as described in Figure 15 and under Materials and Methods. Abbreviations used are: CAA-H, hydantoin acetic acid; DHO, dihydroorotate; OA, orotate; OMP, orotidylate.

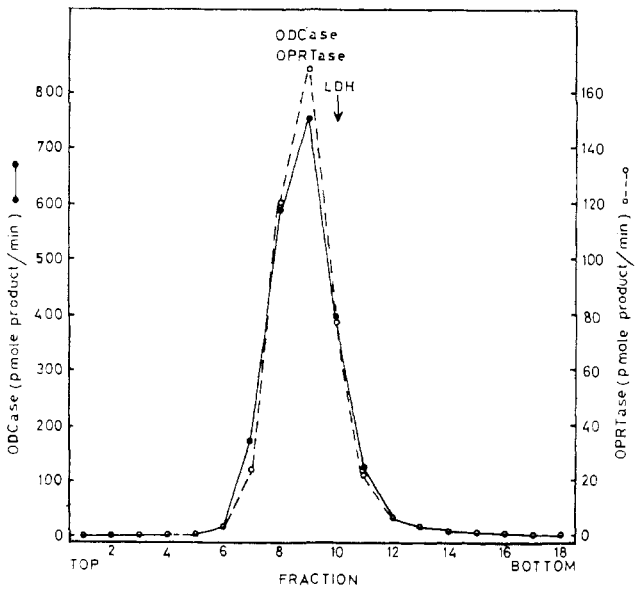

FIGURE 13: Cosedimentation of orotidylate phosphoribosyltransferase and orotidylate decarboxylase. The homogenate was prepared in 30% (v/v) dimethyl sulfoxide and 5% (v/v) glycerol and centrifuged at 100,000*g* for 30 min at 5°. One-half milliliter of the supernatant (containing 7.5 mg of protein/ml) was layered on a 5-ml sucrose gradient containing 5–20% (w/v) sucrose, 30% (v/v) dimethyl sulfoxide, 5% (v/v) glycerol, and 0.05 M Tris-Cl, with a final pH of 7.5. The gradient was centrifuged at 5° for 30 hr at 64,000 rpm using the SW 64 Ti rotor. LDH indicates the peak of rabbit muscle lactate dehydrogenase activity which was added as an internal molecular weight marker. The rates reported are those for a 0.1-ml sample from each 0.28-ml fraction; however, 0.1 and 0.05 ml of each fraction were used for the decarboxylase and the phosphoribosyltransferase activities, respectively. The amounts of the two activities recovered under these conditions were 40 and 80% for the phosphoribosyltransferase and the decarboxylase, respectively.

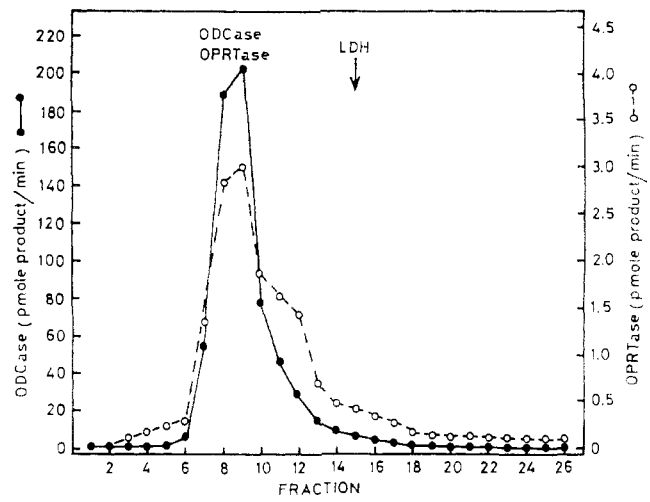

FIGURE 14: Cosedimentation of orotidylate phosphoribosyltransferase and orotidylate decarboxylase in the absence of dimethyl sulfoxide. The homogenate was prepared by adding to the water-swollen cells an equal volume of 50 mM Tris-Cl⁻ at pH 7.5 and homogenizing. One-half milliliter of the 100,000*g*, 30-min supernatant (containing 7 mg of protein/ml) was layered on a 5-ml sucrose gradient containing 5–20% (w/v) sucrose and 0.05 M Tris-Cl⁻ with a final pH of 7.5. The gradient was centrifuged at 5° for 24-hr at 64,000 rpm using the SW 65 Ti rotor. LDH indicates the peak of rabbit muscle lactate dehydrogenase activity which was added as an internal molecular weight marker. Tube 13 is the position at which a protein of mol wt 110,000 would sediment. The rates reported are those for a 0.1-ml sample from each 0.49-ml fraction. In the sample stored at 5° for 24 hr to estimate enzyme recovery from the gradient, the phosphoribosyltransferase could form 124 pmol of orotidylate/min while the decarboxylase could form 3250 pmol of UMP/min. Recovery of phosphoribosyltransferase in this experiment was 32%; however, the average of three experiments all giving a very similar but *not* identical distribution of the two enzymes was 47% (range 62–32%). Recovery of decarboxylase in this experiment was 54%; however, the average value for the three experiments mentioned above was 47% (range 54–40%).

(Hoogenraad *et al.*, 1971) and human lymphocytes (Ito and Uchino, 1973). Neither of these groups tested to see whether dihydroorotase activity was also associated with the first two enzymes of the pathway.

The manner in which dimethyl sulfoxide stabilizes these complexes is not known, and little is known of the specific effects of dimethyl sulfoxide on enzyme conformation. There are, however, an increasing number of reports describing its ability to affect protein conformation. These include the ability to (1) protect cold-labile enzymes such as phosphorylase *b* (Graves *et al.*, 1965), (2) affect substrate binding for allosteric proteins such as glutamic dehydrogenase (Rammler, 1967) and yeast phosphoenolpyruvate kinase (Ruwart and Suelter, 1971), and (3) change the conformation of glutamic dehydrogenase (Henderson *et al.*, 1969) such that the inactive monomers of this enzyme are stabilized.

Enzymes of Complex I, Carbamyl Phosphate Synthetase–Aspartate Transcarbamylase–Dihydroorotase. Data from several laboratories have indicated that the specific activities of pairs of these three enzymes change in a parallel manner in tissue homogenates in response to various physiological events. During fetal development and after birth of the rat, the glutamine-dependent carbamyl phosphate synthetase II and aspartate transcarbamylase decrease in a nearly parallel manner (Hager and Jones, 1967b). More recently it has been shown that lymphocytes stimulated by phytohemagglutinin show parallel changes in these two enzyme activities (Ito and Uchino, 1973), and it had previously been shown that the increase in carbamyl phosphate synthetase under these conditions was due to *de novo* synthetsis (Ito and Uchino, 1971). In a similar fashion, there is a positive correlation between aspartate transcarbamylase and dihydroorotase activities (Galofre and Kretchmer, 1970; Sweeney *et al.*, 1971). All of these data are compatible with the existence of a complex of the three enzymes functioning *in vivo;* however, further studies are necessary since the changes in aspartate transcarbamylase and dihydroorotase level are not always truly parallel (Bresnick *et al.*, 1968), and it is possible that the enzymes need not always be associated with one another, *i.e.*, perhaps one (or more) of these enzymes can exist both in and out of the complex within the cell (see Jones, 1971 and 1972).

(A) CARBAMYL PHOSPHATE SYNTHETASE. The results of Hager and Jones (1967a,b) established the optimal assay conditions for Ehrlich ascites cell carbamyl phosphate synthetase. Tatibana and his collaborators have demonstrated that this enzyme extracted or purified from mouse spleen is subject to feedback inhibition by UTP (Tatibana and Ito, 1969) and precursor activation by PRibPP. Both UTP (Levine *et al.*, 1971) and PRibPP (Tatibana and Shigesada, 1972) modify the sigmoidal binding of ATP to the enzyme (Hager and Jones, 1967a,b; Yip and Knox, 1970; Levine *et al.*, 1971; Tatibana and Shigesada, 1972). We have confirmed that this dual regulation is also observed with the enzyme of the Ehrlich ascites cell.

The maximum activation of the synthetase by PRibPP reported here (a 13.5-fold increase) is considerably greater than

the 2.6-fold activation observed with the mouse spleen enzyme (Tatibana and Shigesada, 1972). The amount of activation obtained with the Ehrlich ascites D–G supernatant varied from preparation to preparation, but was reproducible for any given preparation. It is possible that this variation reflects differences in the endogenous levels of PRibPP. The activation was observed, whether the carbamyl phosphate produced was trapped as citrulline in the presence of added ornithine transcarbamylase and ornithine, or as carbamyl aspartate by endogenous aspartate transcarbamylase and added aspartate.

(B) ASPARTATE TRANSCARBAMYLASE. The apparent K_m values for aspartate (5–9 mM) of the mammalian aspartate transcarbamylase are in good agreement (this paper; Bresnick and Mossé, 1966; Inagaki and Tatibana, 1970; Hoogenraad *et al.*, 1971). The inhibition by aspartate has only been observed before by Inagaki and Tatibana (1970) with the mouse spleen enzymes. Since they obtain a bell-shaped pH curve for these enzymes, they only studied the aspartate concentration curve at the pH optimum, 9.2. Because the Ehrlich ascites enzyme did not have a bell-shaped pH curve, we investigated the aspartate concentration curve both at pH 8.5 (center of the shoulder) and 9.2.[2] At both pH values a clear inhibition was seen at high levels of aspartate, but the inhibition is more marked at pH 8.5. The reported apparent K_m values for carbamyl phosphate, however, have varied since a value of about 30 μM (Bresnick and Mosse, 1966) was obtained for the partially purified rat liver enzyme, and 2 μM was obtained for the partially purified enzyme from mouse spleen (Hoogenraad *et al.*, 1971). The value obtained here for the Ehrlich ascites enzyme is 3–4 μM and is thus in the same range as that reported for the spleen enzyme.

Dihydroorotate Dehydrogenase. The apparent K_m value for L-dihydroorotate is 0.1–0.2 mM; the pH optimum observed here is similar to that observed previously (Smith and Baker, 1959; Bresnick and Hitchings, 1961).

Enzymes of Complex II, Orotidylate Phosphoribosyltransferase–Orotidylate Decarboxylase. The enzyme complex containing the two orotidylate enzymes is more firmly established; in fact, some complex has apparently been obtained in a homogeneous state and with rather low yields from calf thymus (Kasbekar *et al.*, 1964) and bovine brain (Appel, 1968). Once again, a considerable number of papers indicate that the two enzyme activities parallel one another under a number of conditions (Smith *et al.*, 1961; Pinsky and Krooth, 1967$_{a,b}$; Fox *et al.*, 1971; Pausch *et al.*, 1971). Two proposals have suggested alternate but not exclusive reasons why one generally observes parallel changes in these activities. Fox *et al.* (1971) observe that azauridine nucleotides stabilized the two enzymes against destruction in aging erythrocytes, while Pinsky and Krooth (1967a,b) have conducted studies that suggest that this agent may induce the synthesis of these two enzymes in cultured fibroblasts. It would be interesting to study the effect of changes in metabolite or analog additions to cultured cells to observe what intermediates accumulate *in vivo* in these cells or with extracts as in Figure 15.

Although the orotidylate enzyme activities co-purify (Kasbekar *et al.*, 1964; Appel, 1968) and although both activities are stabilized *in vitro* and *in vivo* in parallel by nucleotides (Appel, 1968; Fox *et al.*, 1971), these enzymes can be separated by electrophoresis (Kasbekar *et al.*, 1964); however, this treatment leads to a severe or complete loss of the phosphoribosyltransferase activity (Kasbekar *et al.*, 1964; Appel, 1968). This result suggests, but does not prove, that the complex either stabilizes or is essential for maximal orotidylate phosphoribosyltransferase activity. A similar conclusion is supported by the data of Table II where studies on the cellular location of these enzymes showed that the dimethyl sulfoxide–glycerol solvent stabilizes 67% of the activity of phosphoribosyltransferase, possibly because it stabilizes the 110,000-dalton complex. However, when 0.25 M sucrose is the solvent, only 37% of the phosphoribosyltransferase was recovered and presumably was present mainly as the 55,000-dalton complex. Wild and Belser (see Wild, 1972) have partially purified the two orotidylate enzymes from *Serratia marcescens* as a complex. When the enzymes are separated, they are both less active, but when the separated enzymes are recombined, their original activity is regained. Thus, the complex of these enzymes for *Serratia* appears to be required to confer the

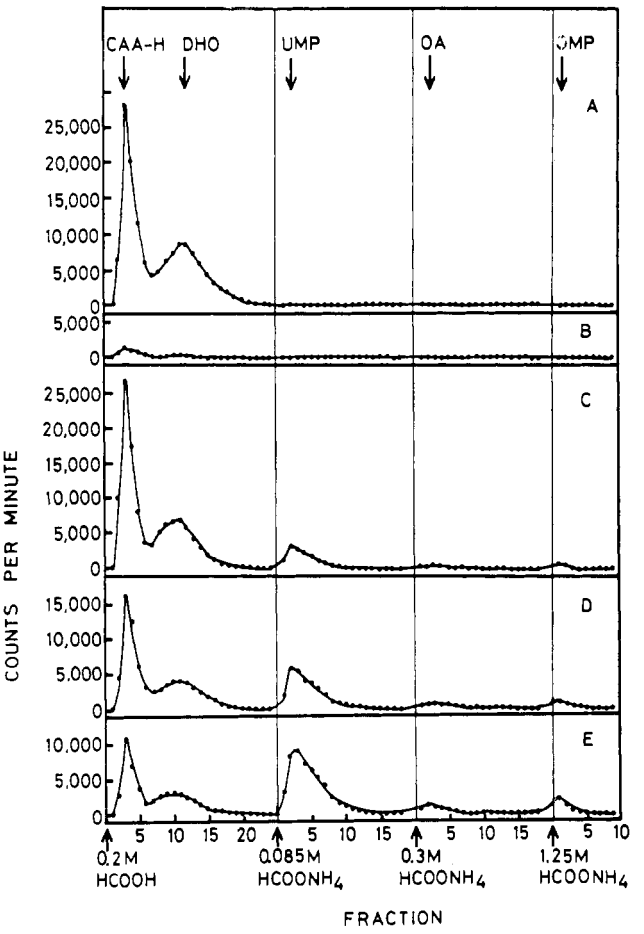

FIGURE 15: Formation of intermediates of the UMP biosynthetic pathway with the 100,000*g* supernatant, in the presence or absence of dihydroorotate dehydrogenase. Part A shows that *N'*-carbamyl-L-aspartate and dihydroorotate are formed from the 100,000*g* supernatant (prepared from cells extracted to give a final concentration of 30% (v/v) dimethyl sulfoxide and 5% (v/v) glycerol) from $NaH^{14}CO_3$, ATP, Mg^{2+}, glutamine, aspartate, and 5-phosphoribosyl 1-pyrophosphate (see Materials and Methods for concentrations). The reaction mixture contained 1.1 mg of protein. B shows the effect of dihydroorotate dehydrogenase activity (0.2 mg of protein) remaining in the 700–100,000*g* supernatant, a fraction which contains almost none of the other five enzymes. C shows the effect of addition of dehydrogenase (0.2 mg of protein) as in B to the amount of supernatant used in A (1.1 mg of protein). D shows the effect of two times (0.4 mg of protein) and E' of four times (0.8 mg of protein) the amount of dehydrogenase used in B with the same amount of supernatant used in A (1.1 mg of protein).

[2] Hoogenraad *et al.* (1971) do not obtain a bell-shaped curve for aspartate transcarbamylase purified from mouse spleen. Under the conditions used, the enzyme has pH optima at 9.4 and 10.2.

TABLE V: Rates of Reaction of the Six Enzymes Required for *de Novo* UMP Biosynthesis.[a]

Enzyme Assayed	pH of Assay	Homogenates Prepared in	
		Sucrose	D–G
A. In biosynthetic direction			
OPRTase	7.5	0.09	0.23
DHOdehase	7.4	0.80	0.52
ODCase	6.8	1.13	1.54
CPSase	7.5		2.02
ATCase	8.5	18.0	24.3
B. In degradative direction			
DHOase	8.5	0.22	0.13

[a] All values are in nanomoles/minute per milligram of protein. The values are for the homogenates described in Table II. Standard assay conditions were used, except that the CPSase was assayed in the presence of 0.1 mM PRibPP. Enzyme abbreviations are given in Table II.

proper conformation for maximal activity on each enzyme of the complex.

Nonlinear protein concentration *vs.* activity curves were seen for these two enzymes as well as for the carbamyl phosphate synthetase. All three enzymes show very little activity until the protein concentration reaches a given level, and then the curve can increase in a linear fashion. The same result has been observed for the orotidylate decarboxylase of rat liver (Fausto, 1969) and for the "activity" of the two orotidylate enzymes of the intact human erythrocyte (Smith *et al.*, 1961). In the studies on the erythrocyte enzyme, the addition of stabilizing agents such as glutathione, cysteine, or albumin did not increase the activity observed when the amount of erythrocytes added was low, nor did the addition of boiled extract change the erythrocyte concentration curve. This initial nonlinearity may merely indicate enzyme denaturation at low protein concentration or it is equally possible that it might reflect the breakdown of the enzyme complex if such a complex is essential for full enzyme activity. It may be significant that the same protein concentration, 40 μg, should be the point after which both orotidylate enzyme protein concentration curves become linear (Figure 7A and C).

The use of two solvents for fractionation of the six pyrimidine enzymes seemed desirable for two reasons: (1) 30% dimethyl sulfoxide and 5% glycerol were required to obtain and routinely maintain an adequate level of synthetase activity and this solvent has been used in the purification of this enzyme (Tatibana and Ito, 1969); (2) 30% dimethyl sulfoxide and 5% glycerol stabilized both the three-enzyme complex containing carbamyl phosphate synthetase, aspartate transcarbamylase, and dihydroorotase, and a two-enzyme complex of orotidylate phosphoribosyltransferase and orotidylate decarboxylase of the soluble supernatant so that it seemed desirable to compare the dimethyl sulfoxide–glycerol solvent with a solvent, 0.25 M sucrose, normally used for the separation of subcellular fractions. As seen in Tables II and V, the reaction rates and subcellular location of the enzymes are similar with both solvents.

From the data in Table V, one can consider which enzyme might be rate limiting for this pathway. It has been suggested in previous discussions on the *in vivo* regulation of this pathway that the synthetase was rate limiting (Hager and Jones, 1967a,b; Tatibana and Ito, 1969; Ito and Uchino, 1971). Of the enzymes tested in the biosynthetic direction, *i.e.*, all but dihydroorotase, and at pH values between 6.8 and 7.5, the enzyme with the lowest activity, under the conditions used here, was the orotidylate phosphoribosyltransferase, and not the glutamine-dependent carbamyl phosphate synthetase. Since the synthetase and transferase activities are both sensitive to and were assayed in the presence of 0.1 mM PRibPP, it is important to consider what effect this metabolite has on the rates obtained in Table V. When the PRibPP concentration is 0.1 mM, the synthetase is activated nearly ninefold (Table I), while the transferase activity is approximately half-maximal. If the PRibPP concentration were lowered, the transferase activity would eventually become zero, while the synthetase might always have some activity. If the PRibPP concentration were increased to 1 mM, the synthetase would be activated 13.5-fold, whereas the transferase rate would, at most, be doubled. Therefore, it appears possible that at concentrations of PRibPP up to 2 mM, the transferase might be the rate-limiting enzyme for UMP biosynthesis in the Ehrlich ascites cell. The *in vivo* levels of PRibPP have been measured in the Ehrlich ascites cell by Henderson and Khoo (1965) and found to be between 0.28 and 0.66 mM. The situation *in vivo*, however, is much more complex, for the ratio of ATP and UTP as well as the concentration of PRibPP are important.

Although aspartate transcarbamylase was assayed at the rather nonphysiological pH of 8.5, one can estimate from the pH–activity curve (Figure 4) that the rate of this enzyme at pH 7.5 would only be halved, *i.e.*, changed from 20 to 10 nmol/min per μg, so that it would not be rate limiting unless aspartate concentrations were low. Since dihydroorotase activity was not determined in the biosynthetic direction, we cannot state now whether or not it might be a rate-limiting step.

The dihydroorotate dehydrogenase is probably not rate limiting and the activity measured here may be minimal since no electron acceptor was added, nor were the assay vessels aerated (the latter appeared to have no effect). Preliminary studies (T. Matsuura and M. E. Jones, unpublished results) indicate that the activity can be increased several-fold by electron acceptors. Further studies will be necessary to establish the nature of the natural electron acceptor. The exact subcellular location of the enzyme in Ehrlich ascites extract (this paper) and human leucocytes (Smith and Baker, 1959) remains to be established. Using the method of Fukuda *et al.* (1970) to separate the nuclei from other membranes in the crude nuclear pellet, we have had conflicting results as to the location of the enzyme. In three experiments (Shoaf, 1972), the majority of the activity was sedimented with the nuclei and the majority of the DNA from 2.4 M sucrose containing 1 mM $MgCl_2$ at 80,000*g*, but in additional experiments, it remained with the vesicles and nuclei that did not sediment (W. T. Shoaf, T. Matsuura, and M. E. Jones, unpublished results). The crude nuclear pellet contains very few intact cells, but the majority of the nuclei generally have some adhering cytoplasm which is difficult to remove.

We are intrigued by the possibility that in the cell the two complexes observed here may be physically associated with the dihydroorotase dehydrogenase to form a larger complex which includes all of the enzymes of UMP biosynthesis. This suggestion is only supported by two indirect observations at the moment. The first observation is the fact that when intact Ehrlich ascites cells form UMP from bicarbonate, no inter-

mediate, *i.e.*, *N'*-carbamyl-L-aspartate, dihydroorotate, orotate, or orotidylate, accumulates (Hager and Jones, 1965), unless an inhibitor is added, even though the synthetase may not be the "rate-limiting" enzyme of this pathway (Table V). Obviously if the dihydroorotate dehydrogenase is in the interior of either the mitochondria (Miller *et al.*, 1968) or the nuclei such a master complex is improbable.

We hope, in the future, to see whether the two complexes observed here have special significance either for (1) the stability of the component enzymes, (2) the ability to "channel" (Davis, 1964; Lue and Kaplan, 1970) substrates more efficiently to product, or (3) maintaining each enzyme in a particularly active or regulated conformation.

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