Carbamyl phosphate synthesis in a Land Snail, *Strophocheilus oblongus* *

(Received for publication, August 17, 1970)


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**SUMMARY**

A carbamyl phosphate synthetase has been detected and partially characterized in hepatopancreas tissue of the land snail *Strophocheilus oblongus*. The enzyme is localized in mitochondria, utilizes L-glutamine, and shows an absolute requirement for N-acetyl-L-glutamate. This unique combination of properties distinguishes the snail enzyme from either of the previously described animal carbamyl phosphate synthetases, carbamyl phosphate synthetase I and carbamyl phosphate synthetase II. The snail enzyme was measured as the fixation of 14C-bicarbonate into citrulline in the presence of excess L-ornithine and ornithine transcarbamylase. ATP and Mg$^{2+}$ are also required in the reaction; optimal activity is obtained when the Mg$^{2+}$:ATP ratio is 2. When this ratio is maintained, saturation with ATP occurs at approximately 5 mM. The $K_m$ of the enzyme for L-glutamine is estimated to be 2.5 mM and for N-acetylglutamate, 0.3 mM.

The major portion of carbamyl phosphate synthetase activity is localized in the mitochondrial fraction of the snail tissue and, since this is also the fraction in which ornithine transcarbamylase occurs, the enzyme is presumed to function in arginine biosynthesis. Aspartate transcarbamylase occurs in the cytosol of the tissue but the amount of carbamyl phosphate synthetase that can be measured in this fraction is negligible. The snail carbamyl phosphate synthetase may thus also function in pyrimidine biosynthesis.

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Carbamyl phosphate, the initial compound in both arginine and pyrimidine biosynthesis, is formed in vertebrate animals by two distinct enzymes, carbamyl phosphate synthetase I and carbamyl phosphate synthetase II. Carbamyl phosphate synthetase I (EC 2.7.2.5) utilizes NH$_3$, CO$_2$, and ATP for carbamyl phosphate synthesis and requires N-acetyl-L-glutamate as a cofactor. Carbamyl phosphate synthetase II utilizes L-glutamine, CO$_2$, and ATP, and does not require a cofactor. Enzyme I occurs in mitochondria and Enzyme II, in the cytosol or soluble cellular fraction. The mitochondrial localization of Enzyme I along with ornithine transcarbamylase (EC 2.1.3.2) indicates that its metabolic function is to provide carbamyl phosphate for arginine and urea biosynthesis, although carbamyl phosphate formed intramitochondrially may also be accessible to extramitochondrial enzyme systems (1). The extramitochondrial localization of Enzyme II along with aspartate transcarbamylase (EC 2.1.3.2) indicates that the glutamine enzyme functions in vivo to provide carbamyl phosphate for pyrimidine biosynthesis (2). Enzyme II, but not Enzyme I, occurs in tissues capable of pyrimidine, but not arginine synthesis de novo (3-6).

With the exception of a carbamyl phosphate synthetase I-like enzyme that has been reported in the soluble cellular fraction of earthworm gut tissue (7), the mechanism of carbamyl phosphate synthesis in invertebrate animals is unknown, although tracer studies in vivo with several species indicate that carbamyl phosphate synthesis does occur (8). In certain land snails, for example, 14C-bicarbonate is incorporated into the ureido-C of citrulline and the guanidino-C of arginine (9, 10) and into the pyrimidine nucleotides UMP and CMP (11). Attempts to demonstrate either a carbamyl phosphate synthetase I- or carbamyl phosphate synthetase II-like enzyme in these species have previously yielded negative or inconclusive results (8). The work reported here was therefore directed toward the detection and characterization of the enzyme or enzymes responsible for carbamyl phosphate synthesis in the land snail *Strophocheilus oblongus*.

**MATERIALS AND METHODS**

**Animals**—Specimens of *S. oblongus* were obtained from Porto Alegre, Brazil, through the courtesy of Dr. C. P. Jaeger of the Universidade do Rio Grande do Sul. They were kept under humid conditions and fed lettuce *ad libitum*.

**Chemicals**—L-Glutamine was obtained from Sigma and sodium 14C-bicarbonate from New England Nuclear. Sodium bicarbonate was added to the 14C-compound to adjust the specific radioactivity to 1 µCi per pmole. N-Acetyl amino acids and other substrates were also obtained from Sigma with the exception of N-acetyl-L-glutamine, N-acetyl-L-cysteine, and biotin which were obtained from Mann Research. The sources of the glutamine analogues were as follows: azaserine, S. W. Cancer Chemotherapy Study Group Headquarters, M. D. Anderson Hospital, Houston, Texas; 1-carbamyl-L-serine, Cyclo Chemicals, Los Angeles, California; N-acetyl-L-cysteine, Biochemical Research Laboratories, Cambridge, Massachusetts; and N-acetyl-L-glutamine, Sigma Chemical Co., St. Louis, Missouri.

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*This investigation was supported by United States Public Health Service Grants AI-55065 and 5-T1-GM-884 and National Science Foundation Grant GB-8172.
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§ To whom inquiries should be sent. Recipient of United States Public Health Service Career Development Award 5-K3-GM-6780.
Enzymes—Pyruvic kinase was obtained from Calbiochem. The ornithine transcarbamylase used as a supplementary enzyme was either purified from rat liver mitochondria through the second ammonium sulfate step as described by Caravaca and Grisolia (12) or was a preparation from Streptococcus D furnished by Dr. S. H. Bishop, Department of Biochemistry, Baylor College of Medicine, Houston, Texas. The specific enzyme activity of the rat enzyme was 20 units per mg of protein and of the bacterial enzyme, 1200 units per mg. A unit of enzyme activity corresponds to 1 μmole of product per min at 30°C. In experiments in which the effect of NH₄⁺ on enzyme activity was tested, endogenous NH₄⁺ in the supplementary enzyme preparations was removed by dialysis against 50 mM Tris-chloride, pH 7.4, or by chromatography on Sephadex G-25 equilibrated with this buffer.

Preparation of Enzyme Extract—Hepatopancreas tissue was homogenized in 9 volumes of 0.25 M sucrose by hand using a TenBroeck homogenizer kept cold in crushed ice. The homogenate was centrifuged at 600 × g for 10 min. This and subsequent centrifugations were at 0–4°C. The supernatant fluid was collected and centrifuged at 6,000 × g for 15 min. The sediment, which contained the mitochondria, was resuspended in 0.154 M KCl. This suspension was then centrifuged at 20,000 × g for 20 min. The composition of the mitochondrial fraction prepared in this manner is shown in Fig. 1. To solubilize the enzyme, the KCl-washed mitochondrial fraction was suspended in a solution containing 1% (w/v) cetyltrimethylammonium bromide, 20% (v/v) glycerol, and 1 mM dithiothreitol. The volume of this solution was such that 1 ml of the suspension contained the mitochondrial fraction from 1 g of tissue. The suspended mitochondria were subjected to two 30-sec pulses in a Bronwill Biosonic II sonic disrupter operating at maximum output intensity. During sonic disruption, the suspension was kept cold in an ice-water mixture. After sonic disruption, the solution was centrifuged at 48,000 × g for 20 min. The resulting supernatant fluid was used as the enzyme source. The solubilized enzyme retained 100% of its activity when kept at -15°C for 11 days; 40% of the original activity was lost during the same time period at 2–5°C. Both glycerol and dithiothreitol were necessary to retain enzyme activity (5, 14–16).

Measurement of Enzyme Activity—Carbamyl phosphate synthesis was measured as the formation of ¹⁴C-citrulline in a coupled assay system containing l-ornithine and ornithine transcarbamylase. The reaction was performed in thick walled, 12-ml centrifuge tubes maintained at 30°C in a water bath. The reaction mixture for the basic assay system contained, in micromoles per 1 ml: ATP (sodium salt, pH 7), 5; MgSO₄, 10; l-glutamine, 10; sodium ¹⁴C-bicarbonate (1 μCi per μmole), 10; N-acetyl-L-glutamate (sodium salt, pH 7.5), 5; l-ornithine (l-ornithine hydrochloride adjusted to pH 7.4 with NaOH), 10; Tris-chloride,
pH 7.4, 50, sodium phosphoenolpyruvate, 2.5, pyruvate kinase, 6.6 units; ornithine transcarbamylase, 2 to 3 units; and 0.1 ml of Strophocheilus enzyme preparation. The reaction mixture components were mixed and kept at 0°C until used. After they had equilibrated at 50°C, the reaction was started by the addition of the mitochondrial extract. The reaction was stopped after 20 min by the addition of 0.25 ml of 30% (w/v) trichloracetic acid. After centrifuging to remove the protein precipitate, the supernatant fluid was gassed with CO₂ to remove unreacted 14C-acid. After centrifuging, 50 μl of the deproteinized solution were spotted on Whatman No. 3MM paper. The citrulline area was cut out and placed in a standard scintillation mixture (0.5% 2,5-diphenyloxazole and 0.05% 1,4-bis(2-(4-methyl-5-phenoxyazolyl))benzene in toluene) for counting. The efficiency of counting was determined by the channels-ratio method (17). Enzyme activity is expressed as disintegrations per min incorporated into citrulline; 2.2 × 10⁶ dpm therefore corresponds approximately to the formation of 1 nmole of carbamyl phosphate (Ehrlich's reagent). The area containing the 14C-citrulline was identified by paper and automated ion exchange chromatography and was measured colorimetrically (19, 20).

Glutamine synthetase activity was estimated in a reaction mixture containing, in 1 ml volume: 10 μmoles of ATP (sodium salt, pH 7); 10 μmoles of MgCl₂; 100 μmoles of Tris-chloride, pH 7.2; 25 μmoles of NH₄Cl; 2 μmoles of 3,4-¹⁴C-glucose acid (4.3 × 10⁶ dpm per pmole); and 0.1 ml of the same mitochondrial extract used for the assay of carbamyl phosphate synthetase activity. The reaction was stopped by adding 0.5 ml of 30% (w/v) trichloracetic acid. After removing the protein precipitate by centrifuging, 50 μl of the deproteinized solution were spotted on Whatman No. 3MM paper and developed with 75% (w/w) phenol in an ammoniacal atmosphere. The glutamine area on the chromatogram was located by reference to a glutamine standard and was cut out and counted as described above for citrulline. Incorporation of ¹⁴C into glutamine was linear with both time of incubation and enzyme concentration under the conditions used and was dependent upon the addition of both NH₄⁺ and ATP to the reaction mixture.

Protein Measurement—Protein was estimated by the method of Lowry et al. (21) using dry bovine serum albumin as the standard.

RESULTS

The general requirements for carbamyl phosphate synthetase activity in Strophocheilus hepatopancreas tissue are shown in Table I. Maximal activity is dependent upon the addition of L-glutamine, N-acetyl-L-glutamate, ATP, and L-ornithine as an accessory substrate for the conversion of carbamyl phosphate to citrulline. The extent of dependence upon these compounds varies somewhat from one enzyme preparation to the other. This is also true of the activity found when NH₄⁺ is used as the amino donor (see below).

The level of carbamyl phosphate synthetase activity in Strophocheilus hepatopancreas tissue ranges from around 150 to 500 nmoles of carbamyl phosphate formed per g per hour at 30°C. This is higher than most extrahepatic mammalian tissue but falls within the range reported for several tumor tissues (6).

L-Glutamine Requirement—Evidence that L-glutamine is the substrate for carbamyl phosphate synthesis includes the inhibition of the reaction by L-glutamine analogues (Table II). In concentration ratios with L-glutamine of 50:1, aaseresine and O-carbamyl-L-serine inhibit the Strophocheilus enzyme 20% and 80%, respectively. Somewhat similar results have been obtained using both of these inhibitors with the Ehrlich ascites carcinoma enzyme (9).

The effect of L-glutamine concentration on Strophocheilus carbamyl phosphate synthetase is shown in Fig. 2. Saturation oc-
Inhibition of carbamyl phosphate synthesis by glutamine analogues

The basic assay procedure was modified as follows: the L-glutamine concentration was decreased to 0.5 mM and the reaction was started by the addition of L-glutamine and the inhibitor. Incubation was for 20 min using 0.1 ml of the enzyme preparation.

Experiment 1

None..............................
Minus N-acetyl-L-glutamate......
Plus azaserine....................
Plus azaserine....................
Plus azaserine....................
Plus azaserine....................
Plus O-carbamyl-L-serine....... 0.5
Plus O-carbamyl-L-serine....... 1.0
Plus O-carbamyl-L-serine....... 10.0
Plus O-carbamyl-L-serine....... 25.0

Experiment 2

None..............................
Minus N-acetyl-L-glutamate......
Plus azotomycin...................
Plus azotomycin...................
Plus azotomycin...................
Plus azotomycin...................

FIG. 2. Effect of L-glutamine concentration on the carbamyl phosphate synthetase reaction. The basic assay system in which the L-glutamine concentration was varied as indicated was used. Incubation at each substrate concentration was for 20 min with 0.1 enzyme preparation.

curs around 5 mM glutamine. A Lineweaver-Burk plot of the data indicates substrate inhibition at higher concentration. The K_m for L-glutamine, estimated by extrapolation from such a plot (---, Fig. 2), is around 2.5 mM, which is considerably higher than that of carbamyl phosphate synthetase II. The reported K_m values of carbamyl phosphate synthetase II for L-glutamine are around 0.01 mM (3, 6, 22). The higher K_m of the snail enzyme would not, however, render it nonfunctional in vivo since L-glutamine is a major free amino acid in snail tissues, occurring in concentrations of from 1 to 2 μmoles per g (23).

Because the snails are herbivorous and therefore ingest considerable L-asparagine in their diets, this compound was also tested as a substrate for the snail enzyme. This amide would not, however, substitute for L-glutamine as an amino donor: less than 2% of the glutamine activity was obtained with 25 mM asparagine.

Ammonia also does not appear to be a substrate for Strophocheilus carbamyl phosphate synthetase, although the nature of the reaction responsible for the small amount of 14C-bicarbonate fixation in the presence of NH_4^+ (Table I) is not known. The enzyme preparation does have glutamine synthetase activity but the measured level of this enzyme (less than 0.2 nmol per 0.1 ml per 20 min) is not sufficient to account for the amount of fixation observed in the presence of NH_4^+, assuming there were endogenous glutamate present in the preparation. The residual activity with NH_4^+ is also not generally dependent upon added acetylglutamate. When NH_4^+-NH_3 is used as substrate for the snail enzyme at 5 times the saturating glutamate concentration in the pH range 6.5 to 9.0 there is no optimum shown between pH 7 and 8 (Fig. 3). This is in contrast to the results obtained with carbamyl phosphate synthetase II which does utilize NH_4^+ as a substrate (3, 6). The pH optimum for the snail enzyme with glutamine as the substrate is around 7.8 as shown in Fig. 3.

N-Acyl Amino Acid Requirement—As shown in Table III, of the derivatives tested, N-acetyl-L-glutamate is the most effective cofactor for the snail enzyme. Carbamylglutamate, N-carboxyglutamine, and acetylaspartate are also utilized, but to a lesser extent than acetylglutamate. With acetylglutamate, the enzyme shows typical saturation kinetics when the concentration of this cofactor is varied between 0.125 and 10 mM (Fig. 4). The K_m of the enzyme for acetylglutamate, estimated from these data, is 2.5 \times 10^{-4} M.

The requirement for acetylglutamate is also shown by the enzyme present in "intact" mitochondria. The requirement of carbamyl phosphate synthetase I for acetylglutamate can be satisfied in freshly prepared mammalian mitochondria by an oxidizable substrate (24), presumably because of endogenous synthesis of this cofactor. The physiological integrity of the Strophocheilus mitochondria was not determined but, as shown in

### Table II

**Inhibition of carbamyl phosphate synthesis by glutamine analogues**

<table>
<thead>
<tr>
<th>Assay system modification</th>
<th>Final inhibitor concentration</th>
<th>L-Glutamine to inhibitor ratio</th>
<th>Enzyme activity</th>
<th>Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mM</td>
<td>dpm incorporated into citrulline</td>
<td>%</td>
<td></td>
</tr>
<tr>
<td>Experiment 1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>None</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Minus N-acetyl-L-glutamate</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Plus azaserine</td>
<td>0.5</td>
<td>1:1</td>
<td>14,700</td>
<td>0</td>
</tr>
<tr>
<td>Plus azaserine</td>
<td>1.0</td>
<td>1:2</td>
<td>15,300</td>
<td>0</td>
</tr>
<tr>
<td>Plus azaserine</td>
<td>10.0</td>
<td>1:90</td>
<td>13,900</td>
<td>10.1</td>
</tr>
<tr>
<td>Plus azaserine</td>
<td>25.0</td>
<td>1:60</td>
<td>8,300</td>
<td>43.7</td>
</tr>
<tr>
<td>Plus O-carbamyl-L-serine</td>
<td>0.5</td>
<td>1:1</td>
<td>15,200</td>
<td>0</td>
</tr>
<tr>
<td>Plus O-carbamyl-L-serine</td>
<td>1.0</td>
<td>1:2</td>
<td>13,800</td>
<td>0.2</td>
</tr>
<tr>
<td>Plus O-carbamyl-L-serine</td>
<td>10.0</td>
<td>1:20</td>
<td>11,000</td>
<td>25.5</td>
</tr>
<tr>
<td>Plus O-carbamyl-L-serine</td>
<td>25.0</td>
<td>1:50</td>
<td>4,670</td>
<td>68.3</td>
</tr>
<tr>
<td>Experiment 2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>None</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Minus N-acetyl-L-glutamate</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Plus azotomycin</td>
<td>0.5</td>
<td>1:1</td>
<td>35,900</td>
<td>0</td>
</tr>
<tr>
<td>Plus azotomycin</td>
<td>1.0</td>
<td>1:2</td>
<td>35,800</td>
<td>0</td>
</tr>
<tr>
<td>Plus azotomycin</td>
<td>10.0</td>
<td>1:20</td>
<td>35,400</td>
<td>0</td>
</tr>
<tr>
<td>Plus azotomycin</td>
<td>25.0</td>
<td>1:50</td>
<td>27,620</td>
<td>21.8</td>
</tr>
</tbody>
</table>

![Fig. 3](http://www.jbc.org/)

**Fig. 3.** Effect of pH on carbamyl phosphate synthesis from L-glutamine (1 μM) and NH_4Cl (0.025 μM). The basic assay system was used except that L-glutamine was omitted when NH_4Cl was added. Incubation was for 20 min with 0.1 ml of the enzyme preparation.
### Table III

<table>
<thead>
<tr>
<th>N-Acylamino acid</th>
<th>Enzyme activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>420</td>
</tr>
<tr>
<td>N-Acetyl-L-glutamate</td>
<td>44,800</td>
</tr>
<tr>
<td>N-Acetyl-L-cysteine</td>
<td>750</td>
</tr>
<tr>
<td>N-Acetyl-L-aspartate</td>
<td>780</td>
</tr>
<tr>
<td>N-Acetyl-L-alanine</td>
<td>690</td>
</tr>
<tr>
<td>N-Carbamyl-L-aspartate</td>
<td>4,740</td>
</tr>
<tr>
<td>N-Acetyl-L-ornithine</td>
<td>4,740</td>
</tr>
<tr>
<td>N-Acetyl-L-glutamine</td>
<td>7,140</td>
</tr>
<tr>
<td>N-Acetyl-L-phenylalanine</td>
<td>570</td>
</tr>
<tr>
<td>N-Acetyl-L-tryptophlan</td>
<td>630</td>
</tr>
<tr>
<td>N-Carbamyl-L-alanine</td>
<td>780</td>
</tr>
<tr>
<td>N-Carbamyl-L-ornithine</td>
<td>750</td>
</tr>
<tr>
<td>N-Carbamyl-L-phenylalanine</td>
<td>690</td>
</tr>
<tr>
<td>N-Carbamyl-L-tryptophlan</td>
<td>630</td>
</tr>
<tr>
<td>N-Carbamyl-L-alanine</td>
<td>780</td>
</tr>
<tr>
<td>N-Carbamylglycine</td>
<td>570</td>
</tr>
<tr>
<td>N-Carbamylglycine</td>
<td>540</td>
</tr>
</tbody>
</table>

**Effect of N-acyl amino acids on carbamyl phosphate synthetase**

The basic assay system in which the following N-acyl derivatives were added at 5 mM concentration was used. Solutions of these compounds were adjusted to pH 7.4 with NaOH. Incubation was for 20 min with 0.1 ml of enzyme preparation.

Table IV, neither glutamate nor pyruvate would replace the requirement of these mitochondria for N-acetylglutamate for carbamyl phosphate synthesis.

**Nucleotide Triphosphate Requirement**—The ATP requirement of *Strophocheilus* carbamyl phosphate synthetase cannot be replaced by either GTP, ITP, CTP, or UTP in 5 mM concentration.

**Divalent Cation Requirement**—Magnesium ion is the most effective of the divalent cations tested with the snail enzyme (Table V). Cobaltous ion gives approximately 50% of the activity obtained with Mg2+, whereas Mn2+ will substitute for Mg2+ to only a limited extent.

**Kinetics of ATP and Mg2+ Binding**—As shown in Fig. 5A, maximal activity of the snail enzyme is obtained when the Mg2+:ATP ratio is 2. With a ratio of 1, approximately one-half of the activity is obtained, and below 1, the activity is negligible. Although Mg2+ in excess of that required for a Mg2+:ATP ratio of 2 appears to increase the binding of ATP (Fig. 5B), it is inhibitory. When the concentration of ATP is varied under conditions where the Mg2+:ATP ratio is kept at 2, a sigmoid curve is obtained (Fig. 5B). The sigmoid relationship can be shifted to a more characteristic hyperbolic one by the addition of 5 mM excess Mg2+. These results are somewhat analogous to those reported with mammalian carbamyl phosphate synthetase I (25) and carbamyl phosphate synthetase II (4, 6) except that, with these enzymes, a sigmoid relationship is obtained when the Mg2+:ATP ratio is 1. With this ratio, the addition of 5 mM excess Mg2+ to mammalian Enzyme I converts the sigmoid curve to a more typical hyperbolic saturation curve (25). The Km of the snail enzyme for ATP, estimated from the data shown in Fig. 5B, is around 2.5 mM. The addition of 5 mM excess Mg2+ lowers this to around 1 mM (4 mM ATP also has this effect).

**Cellular Localization of Strophocheilus Carbamyl Phosphate Synthetase, Ornithine Transcarbamylase, and Aspartate Transcarbamylase**—The cellular localization of *Strophocheilus* hepatopancreas carbamyl phosphate synthetase is shown in Table VI. The specific enzyme activity, based on 14C-bicarbonate incorporation per mg of protein, is highest in the mitochondrial fraction.

![Graph](image-url)
TABLE VI

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Assay modifications in enzyme activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Homogenate (15.0)</td>
<td>None, Minus N-acylglutamate, Minus glutaminase, and N-acylglutamate, plus NH₄⁺</td>
</tr>
<tr>
<td>600 × g</td>
<td>970</td>
</tr>
<tr>
<td>Residue (28.5)</td>
<td>235</td>
</tr>
<tr>
<td>Supernatant fluid (11.3)</td>
<td>1480</td>
</tr>
<tr>
<td>6,600 × g</td>
<td>8030</td>
</tr>
<tr>
<td>Residue (11.3)</td>
<td>155</td>
</tr>
<tr>
<td>Supernatant fluid (9.0)</td>
<td>120</td>
</tr>
<tr>
<td>110,000 × g</td>
<td>209</td>
</tr>
</tbody>
</table>

a The protein content of each fraction as milligrams per ml is given in parenthesis.
TABLE VII

Effect of purine and pyrimidine nucleotides on carbamyl phosphate synthesis

The basic assay system was modified by reducing the ATP concentration to 2.5 mm. The nucleotides were added in 10 mm concentration. Incubation was for 20 min with 0.1 ml of enzyme preparation.

<table>
<thead>
<tr>
<th>Nucleotide added</th>
<th>Enzyme activity</th>
<th>dpm incorporated into citrulline</th>
<th>% of total</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td></td>
<td>31,800</td>
<td>100</td>
</tr>
<tr>
<td>UTP</td>
<td></td>
<td>40,800</td>
<td>117</td>
</tr>
<tr>
<td>ADP</td>
<td></td>
<td>31,100</td>
<td>90</td>
</tr>
<tr>
<td>CTP</td>
<td></td>
<td>26,500</td>
<td>76</td>
</tr>
<tr>
<td>AMP</td>
<td></td>
<td>11,200</td>
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<td>TTP</td>
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<td>3,040</td>
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</tr>
<tr>
<td>GTP</td>
<td></td>
<td>350</td>
<td>1</td>
</tr>
</tbody>
</table>

the distribution of the two transcarbamylases is much the same as in mammalian tissue (28, 29).

Inhibition by Nucleotides—Since, if the *Stropheiucleis* carbamyl phosphate synthetase was functioning in pyrimidine biosynthesis, it might be subject to feedback inhibition by pyrimidine nucleotides (30), the effect of these compounds on its activity was tested. As shown in Table VIII, neither UTP nor CTP have a pronounced effect on the enzyme, although UTP gives a slight inhibition when these nucleotides are present in 10 mm concentration. UTP may possibly be an allosteric activator of the enzyme. In experiments identical to that described in Fig. 5B, the addition of 4 mm UTP in place of 5 mm excess Mg2+ had the same effect as excess Mg2+ in converting the sigmoid activity-concentration curve to a hyperbolic one, decreasing the apparent K_m for ATP from 2.5 to 1 mm.

In contrast to the effect of the pyrimidine nucleotides, the purine nucleotides AMP, ITP, and especially GTP markedly inhibit the enzyme. Since ADP acts as an end product inhibitor of carbamyl phosphate synthetase I (25, 31), the inhibition of the snail enzyme by AMP was of some interest. However, attempts to obtain an enzyme preparation free of interfering enzyme activities with which the stoichiometry of ATP hydrolysis during the reaction could be measured have been unsuccessful. Since carbamyl phosphate is not known to be involved in purine biosynthesis, no physiological significance can be attributed to the inhibitions given by GTP and ITP and they may, in fact, result from Mg2+ binding. The effect of both ITP and GTP is concentration dependent; in an experiment identical to that described in Table VIII, low concentrations of both ITP and GTP gave progressive stimulation up to around 3 mm. Increasing the nucleotide concentrations above 3 mm resulted in progressive inhibition of the enzyme. This concentration dependence may explain the various results obtained when these nucleotides have been tested on other carbamyl phosphate synthetases (5, 32).

Miscellaneous Compounds—Phosphate, which stimulates the *Ehrlich ascites cell* enzyme (3), is without effect on the snail carbamyl phosphate synthetase in 10 mm concentration. Sodium fluoride in 5 mm concentration inhibits the enzyme around 40%. Fluoride (3 mm KF) completely inhibits the pea seedling enzyme (33), although it stabilizes *Agricult carbamyl phosphate synthase II* (22). High concentrations of L-arginine (10 mm) inhibit the snail enzyme around 27%. This inhibition by arginine is, however, unlikely to be of physiological significance because of the generally low concentrations of free arginine in the tissues of land snails (23). Avidin and biotin, alone and in various combinations, have no effect on the snail enzyme.

**DISCUSSION**

Previous attempts to demonstrate either a carbamyl phosphate synthetase I- or carbamyl phosphate synthetase II-like enzyme in land snails were unsuccessful, although studies with intact organisms and tissues indicated that carbamyl phosphate was formed in vivo (8). The unique properties of the carbamyl phosphate synthetase in *Stropheiucleis* hepatopancreas tissue may explain the negative results obtained using assay systems designed for either of the vertebrate enzymes. Although the snail enzyme shares certain properties with carbamyl phosphate synthetase I and carbamyl phosphate synthetase II, its unique combination of properties distinguishes it from either of these enzymes. The snail enzyme shares the properties of requiring N-acetyl-L-glutamine and being a mitochondrial enzyme with vertebrate Enzyme I, the enzyme that provides carbamyl phosphate for arginine and urea biosynthesis. However, unlike Enzyme I, the snail enzyme utilizes L-glutamine as a substrate and apparently does not utilize ammonia. Its utilization of glutamine is a property shared with carbamyl phosphate synthetase II from microorganisms (22, 30, 34, 35), avian liver (4), and several mammalian tissues and tumor cells (4–6). In vertebrates, the latter enzyme is localized in the cytosol and provides carbamyl phosphate for pyrimidine biosynthesis.

The properties the snail enzyme shares with carbamyl phosphate synthetase I and II are qualitative and not necessarily quantitative ones. The K_m of the snail enzyme for L-glutamine is, for example, different by three orders of magnitude from that of carbamyl phosphate synthetase II, being 2.5 mm versus 0.01 mm for the latter (3, 6, 22). Unlike Enzyme II, the snail carbamyl phosphate synthetase does not utilize ammonia in addition to glutamine, although it is possible that the affinity for ammonia was lost during subcellularization of the enzyme. The affinity for glutamine of the enzyme from *Escherichia coli* is much more labile
animal iirease rather than accumulat,ing for excretion as in ureo-
argininc even though it is subsequently hydrolyzed by a unique
phatc synthetasc I (a;), but much less than that required by
about the same as that required by mammalian carbamyl l,hos-
The metabolism of some land snails is unique because they are the
vide c~arbamyl phosphate for arginine and urea biosynthesis.
quantitative differences. Maximal activity of the snail enzyme
is obtained when the ratio of Mg2+ to ATP is 2. With this ratio,
the snail enzyme is localized along with ornit,hine transcarbamyl-
zyme activity is inhibited (25).
excess R4g2+ to both Enzyme I and the snail enzyme in the pres-
and Enzyme II when the XIg2+:9TI' ratio is 1. The addition of
the snail enzyme similar to that obtained with both Enzyme I
concentration under conditions where the >Ig2+:ATP ratio is
normal substrate of the enzyme. Its inhibition by glutamine
ammonia, the lower I<, for glutamine led to the conclusion that
than that for ammonia and may be lost during purification of the
though Enzyme II utilizes both glutamine and ammonia, the lower Km
Activity for glutamine in terms of what is the
significance of its high li, for ghltamine in terms of what is the
snails are indirect evidence that glutamine is the physiological substrate of the enzyme. The Km
of the snail enzyme for acetylglutamate is also higher than that of
vertebrate carbamyl phosphate synthetase I, being 0.3 mM
versus 0.03 to 0.06 mM for the latter (25, 31). The anomalous
behavior of the snail enzyme toward ATP and Mg2+ is similar
to that shown by Enzymes I and II although, again, there are
quantitative differences. Maximal activity of the snail enzyme
is obtained when the ratio of Mg2+ to ATP is 2. With this ratio,
the enzyme appears to be saturated around 5 mM ATP, which is
about the same as that required by mammalian carbamyl phosphate synthetase I (25), but much less than that required by
carbamyl phosphate synthetase II (4, 6). Varying the ATP
concentration under conditions where the Mg2+:ATP ratio is
kept at 2 results in a sigmoid activity-concentration curve with
the snail enzyme similar to that obtained with both Enzyme I
and Enzyme II when the Mg2+:ATP ratio is 1. The addition of
excess Mg2+ to both Enzyme I and the snail enzyme in the presence
of their respective Mg2+:ATP ratios gives a more typical
hyperbolic activity-concentration relationship even though
enzyme activity is inhibited (25).
The mitochondrial localization of Strophocelisus carbamyl
phosphate synthetase also serves to distinguish it from Enzyme II.
In vertebrates, Enzyme II is localized along with aspartate transcarbamylase in the soluble cellular fraction or cytosol and is
therefore felt to function in pyrimidine biosynthesis (2). Since
the snail enzyme is localized along with ornithine transcarbamylase in the mitochondrial fraction, it presumably functions to
provide carbamyl phosphate for arginine and urea biosynthesis.
The metabolism of some land snails is unique because they are the
only invertebrate animals known that have retained the ability to
synthesize arginine (8). In these snails, urea is formed from argininc even though it is subsequently hydrolyzed by a unique
animal urease rather than accumulating for excretion as in ureatelic species (37, 38). 14C-Bicarbonate is incorporated into
nucleic acid pyrimidine nucleotides by the snails (11) and their possession of aspartate transcarbamylase indicates that their synthesis
of pyrimidines is the same as in other species. The mitochondrial
carbamyl phosphate synthetase described here could thus also function in pyrimidine biosynthesis since intramito-
condochondrial carbamyl phosphate has been shown to be available to
extramitochondrial enzyme systems in vitro (1) and the glutamine
enzymes from at least two bacteria are known to have dual func-
REFERENCES
Carbamyl Phosphate Synthesis in a Land Snail, *Strophocheilus oblongus*
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