RICE UNIVERSITY

UREA CYCLE ENZYMES IN THE
LAND SNAIL, OTALA LACTEA

by

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A THESIS
SUBMITTED TO THE FACULTY
IN PARTIAL FULFILLMENT OF THE
REQUIREMENTS FOR THE DEGREE OF
MASTER OF ARTS

Houston, Texas
May, 1961
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ACKNOWLEDGEMENTS

I wish to express my sincere appreciation to Dr. J. W. Campbell whose advice and assistance made this study possible. My thanks also go to Dr. C. J. Beams for his continued aid and encouragement, to Mrs. Dorothy McRitchie for her technical assistance, and to the many other graduate students and staff members who have lent their support.
INTRODUCTION

The chemical synthesis of urea by Wöhler in 1828 was a landmark in physiological chemistry in that it was the first time that the synthesis of a biological product was performed without the intervention of a "vital force". Early investigations of the biological synthesis of urea were carried out in vivo. W. von Schröder (1830) showed that hepatectomy of dogs and most vertebrates (elasmobranchs excluded) resulted in a cessation of urea production. This served to localize the site of urea production to the liver of vertebrates. Subsequent experiments using perfused mammalian livers demonstrated that ammonium chloride was converted to urea by this organ. Although this conversion was early established, little was known of the mechanism of synthesis at this time. Two theories were widely held by the physiological chemists. In one, urea was formed from ammonium carbonate, in the other, from ammonium cyanate. Both theories were abandoned due to the failure of these compounds to stimulate urea production in vivo.

The first indication of the enzymic nature of the biosynthesis of urea was Kossel and Dakin's (1904) demonstration of the enzyme arginase, which acts on arginine to form urea and ornithine. A study of the distribution of this enzyme in the animal kingdom was carried out by Clementi (1914, 1922) and it was he who made the generalization, now known as Clementi's rule of arginase, that this enzyme occurs in the livers of ureotelic organisms and is not found in the livers of uricotelic organisms. Recently, however, Brown and Cohen (1960) have shown exceptions to Clementi's rule in demonstrating low levels of arginase activity in the liver of the Western alligator lizard (Gerrhonotus multicarinatus), the garter snake (Thamnophis sirtalis), and the domestic chicken.
The next landmark in biological chemistry in which urea participated was brought about by Krebs (1932). Krebs showed that slices of liver will form urea from ammonium salts, thus confirming the earlier work with perfused livers. He also studied the effects of amino acids upon this production of urea and found that most amino acids stimulated urea production. He concluded that this was due to the quantitative conversion of the ammonium formed by deamination of the amino acids to urea: one mole of amino acid resulting in approximately one-half mole of urea, taking into consideration that with the different amino acids, different rates of deamination obtained. However, when the amino acid ornithine was used, it was found that ten or more moles of urea were produced. This suggested that ornithine might be catalytically involved in the production of urea. Following this, the effect of arginine was also investigated and it was found to have a similar effect on the production of urea by tissue slices. Krebs then postulated a cyclic process involving ornithine and arginine for the production of urea from bicarbonate and ammonium.

At this time, the structure of a substance isolated from the watermelon, *Citrullus vulgaris*, became known and was found to be chemically intermediate between ornithine and arginine. Citrulline, as this substance was named, was tested and was found to be active catalytically in the biogenesis of urea in tissue slices. This then led to the formulation of the classical ornithine cycle by Krebs and Henseleit (1933). This cycle involves the interconversion of the three amino acids, arginine, citrulline, and ornithine, to form urea.

The complete synthesis of urea from ammonium would take place only in intact cells having an external source of energy in the form of glucose or lactic acid. This indicated that the energy metabolism of
the cells was involved and any further progress in the elucidation of
the urea cycle had to await a better understanding of the mechanisms of
biological energy-transfer.

While discrete enzymatic steps were implied in the original formul-
lation of Krebs and Henseleit (1933), they were first clearly established
by Srb and Horowitz (1944), working with mutants of Neospora crassa.
This study served to initiate investigations on the discrete enzymatic
steps involved in the cycle. Also, with a better understanding of the
energy-transfer reactions involving adenosine triphosphate, less complex
systems than whole organisms or tissue slices could be used for studying
these enzymatic steps. Cohen and Hayano (1946) demonstrated the formation
of urea from citrulline in a cell-free preparation. Following this,
Cohen and Hayano (1946, 1947) demonstrated the synthesis of arginine
from citrulline and of citrulline from ornithine in cell-free systems.
A complete understanding of the ornithine cycle has essentially involved
the elucidation of the discrete enzymatic steps leading from bicarbonate
and ammonium to citrulline and from citrulline to arginine.

A preparation was obtained by Cohen and Grisolia (1948) which would
carry out the conversion of ornithine to citrulline in the presence of
bicarbonate, ammonium, adenosine triphosphate (ATP), and glutamate. An
attempt was then made to find a compound which would replace the bicar-
bonate and ammonium. Of the compounds tested, only carbamyl-glutamate
would work (1951). However, this compound had a catalytic effect and
was not involved in the reaction. It was thus possible to separate the
original system into two enzyme activities. First, the formation of
compound "X" from bicarbonate, ammonium, and ATP and secondly, the
reaction of compound "X" with ornithine to form citrulline. Compound "X"
was an extremely labile compound with high energy which was identified when it was found that, in certain bacteria, citrulline was broken down to ornithine and carbamyl phosphate (Jones et al., 1955). It was thus established that this first enzymatic step in urea synthesis involved the formation of carbamyl phosphate. This compound then reacted enzymatically with ornithine to form citrulline.

The second stage of the ornithine cycle was also found to consist of more than one simple reaction. Borsook and Dubnoff (1941) showed that either aspartate or glutamate was required for arginine synthesis from citrulline by kidney slices. Ratner (1947) obtained cell-free preparations of liver which were capable of arginine synthesis upon the addition of ATP.

The arginine synthetase system, or overall conversion of citrulline and aspartate in the presence of ATP to yield arginine, fumarate, and adenosine diphosphate (ADP), has been demonstrated by Ratner et al. (1949) to proceed via two enzymatic steps. The first, the condensation of citrulline and aspartate to form argininosuccinate, requires ATP. The argininosuccinate is then enzymatically cleaved to yield arginine and fumarate.

At the present time, the following enzymatic mechanisms are considered to be involved in the biosynthesis of urea in the livers of mammals, amphibians, and certain species of reptiles.

1) ammonium / bicarbonate / ATP \rightarrow carbamyl phosphate / ADP / H₃PO₄
   acetyl glutamate, Mg^2+ ornithine
2) carbamyl phosphate / ornithine \rightarrow citrulline / H₃PO₄
   transcarbamylase
   condensing enzyme
3) citrulline / aspartate / ATP \rightarrow argininosuccinate / AMP / H₄P₂O₇
   Mg^2+
Carbamyl phosphate synthetase

Reaction 1 is very important in that it represents the primary fixation of both bicarbonate and ammonium. It is catalyzed by the enzyme carbamyl phosphate synthetase, which is located in the particulate fraction of the liver of ureotelic animals. In the mammal bicarbonate, ammonium, ATP (two moles per mole of citrulline formed) and an acyl derivative of glutamate are required for its activity.

A very few invertebrates have been assayed for carbamyl phosphate synthetase activity with predominately negative results. The enzyme was reported by Seaman (1954) to be present in *Tetrahymena* but subsequent attempts by Dewey (1957) to demonstrate the enzyme failed. The presence of this enzyme, or one similar, has been detected in *Neurospora crassa* (Brown and Cohen, 1960)

Observations on the substrate level generation of ATP in *Streptococcus faecalis* linked to the anaerobic conversion of citrulline to ornithine led to the discovery of carbamate kinase, an enzyme which catalyzes the reversible synthesis of carbamyl phosphate. This enzyme, though related to carbamyl phosphate synthetase, differs in that it utilizes only one mole of ATP per mole of citrulline formed and has no requirement for acetyl glutamate. The lower affinity for ammonium lends credence to the proposed mechanism in which there is a direct phosphorylation of the carbamic acid present in equilibrium with carbon dioxide and water.
Jones and Spector (1960) have proposed the following mechanism for the synthesis of carbamyl phosphate by the mammalian enzyme from data obtained in studies with labeled compounds.

1) \[ \text{ATP} + \text{HOCO}_2 \rightarrow \text{ADP} + \text{OOC-O-PO}^- \]

2) \[ \text{O-O-C-0-PO}^- \rightarrow \text{Acetylglutamate (AGA)} \rightarrow \text{AGA-CO}^- + \text{HOPO}^- \]

3) \[ \text{AGA-CO}^- + \text{ATP} \rightarrow \text{AGA-COPO}_3^- = \text{ADP} \]

4) \[ \text{AGA-COPO}_3^- \rightarrow \text{NH}_3 \rightarrow \text{NH}_2\text{COPO}_3^- = \text{AGA} \]

An active carboxyl derivative of acetyl glutamate has been postulated as the intermediate in two other studies but its existence still remains to be proven. This mechanism is consistent with the utilization of two moles of ATP per mole of carbamyl phosphate formed and the catalytic action of acetyl glutamate.

The optimum pH, as determined by Grisolia (1960), is between pH 7.4-7.8 for the mammalian system; between pH 8.5-8.8 for the bacterial system. The animal enzyme shows a very high affinity for ammonium \( (K_m 7.1 \times 10^{-1} \text{M}) \) in contrast to the bacterial enzyme.

Ornithine transcarbamylase

The second step in the cyclic formation of urea, the transcarbamylation of ornithine to form citrulline, is catalyzed by ornithine transcarbamylase. Characteristics of the mammalian enzyme, as determined from a purified
mitochondrial preparation of rat liver by Reichard (1957), are a relatively high degree of substrate specificity, no apparent cofactor requirements, and an equilibrium strongly in favor of citrulline synthesis. With the exception of the pH optimum, which was determined to be pH 7.4, Reichard's results are in agreement with those obtained by Burnett and Cohen (1957) working with a similar preparation of beef liver enzyme. Under the conditions of their assay, the pH optimum was 8.50-8.5.

The bacterial enzyme, citrulline phosphorylase, is essentially the same as the mammalian ornithine transcarbamylase but the equilibrium of the reaction it catalyzes is slightly in favor of citrulline degradation whereas the mammalian enzyme does not catalyze the reverse reaction to any great extent. All data available indicates that ornithine transcarbamylase also occurs in higher and lower plants. Until the present investigation, there were no reports of these enzymes in invertebrate animals.

Arginine Synthetase System

Condensing enzyme (Argininosuccinate synthetase)

The condensing enzyme, which mediates the first step of the conversion of citrulline to arginine, i.e., the condensation of citrulline and aspartate to form argininosuccinate, is found in the particulate fraction of the liver and requires Mg\(^{2+}\) and ATP. The amino group must be donated by aspartate. Because of the inhibitory effect of substrate levels of ATP, most assays contain an ATP-regenerating system, which consists of a lyophilized extract of rabbit muscle, 3-phosphoglyceric acid, and a catalytic amount of ATP. The pH optimum, determined by Ratner and assoc., (1953) is at pH 8.6 which is much higher than that of the overall system
(pH 7.5). As yet, this is the only reaction in the cycle which is not
detectibly reversible.

The mechanism of formation of argininosuccinate, a guanidine derivative,
is postulated by Ratner (1956) to be a condensation between aspartate and
the tautomeric isourea form of citrulline, analogous to the chemical
synthesis of guanidines from amines and S-methylisothiourea or O-methyl-
thioisourea. The enzymatic biosynthesis is as yet the only means of
obtaining argininosuccinate.

Argininosuccinate has been studied by Ratner (1953) and found to
readily undergo nonenzymatic conversion to the metabolically inert cyclic
anhydride when subjected to heat or dilute acid. Although the cyclic
anhydride cannot be differentiated from the metabolic form of arginino-
succinate by chemical methods, it is not acceptable as a substrate for
the cleavage enzyme. It can be reconverted by exposure to dilute alkali.

The enzyme has been partially purified from mammalian liver, kidney,
and brain (Ratner, 1953a, 1960) and is present in amphibian liver (Brown,
1959). It is found in yeast and other microorganisms and its occurrence
must be presumed wherever the urea cycle is present unless evidence to
the contrary is presented (Brown and Cohen, 1960).

Cleavage enzyme (Arginine synthetase)

The cleavage enzyme catalyzes the freely reversible nonhydrolytic
cleavage of the C-N bond. It has a pH optimum of 7.5, the activity
declining rapidly on either side of neutrality. No cofactor requirement
has been detected (Ratner, 1953c). The forward reaction is endergonic
(Brown and Cohen, 1960), but the reaction goes forward as long as arginase
is present in the system to remove the arginine produced.
Arginase

The highly exergonic hydrolytic cleavage of arginine to form ornithine and urea is catalyzed by arginase, an enzyme present in the soluble fraction of the liver cell. This cleavage is not dependent on oxidative processes and was thus recognized long before the role of arginase in the urea cycle was understood. The enzyme is specific for an amino acid containing a free guanidine and free carboxyl group, which explains the highly basic pH optimum of 9.8 for the manganese activated enzyme, for at this pH value arginine is predominantly present in the active form (Greenberg, 1956). The enzyme requires manganese which is usually bound tightly to the enzyme. Activation studies show that the mammalian enzyme is activated primarily by Mn$^{2+}$, Co$^{2+}$ with Cd$^{2+}$ and V$^{2+}$ reported to show effects in some instances.

Hunter and Dauphinee (1925) concluded that "arginase is an enzyme almost, if not entirely, peculiar to the vertebrates." Due to what was known of the distribution of arginine and creatine at this time, this view was held until Baldwin and Needham (1934, 1935) carried out an extensive survey of the invertebrates and demonstrated arginase in several molluscan species and the crab *Carcinus means*.

It has been suggested by Baldwin (1953) that invertebrates lack a urea cycle since only arginase, of the enzymes known to be involved in the biosynthesis of urea in vertebrates, has ever been irrevocably demonstrated in an invertebrate animal. Seaman (1954) reported the complete ornithine cycle in *Tetrahymena pyriformis*. However, subsequent attempts by Dewey et al. (1957) to confirm this were not successful.

The correlation between high levels of arginase activity and the relatively large percentage of total nitrogen excreted as urea found
in some members of the class Gastropoda (Pelaunay, 1927) was the basis for experimental studies by Baldwin and Needham (1934) in which they tried to determine the origin and mode of formation of the urea excreted by the land snail, Helix pomatia. Using a modification of the Kreb's and Henseleit (1932) method, Baldwin and Needham studied the formation of urea in tissue slices of the hepatopancreas and could not detect the presence of urea after incubation with Ringer's saline solution buffered with bicarbonate to which ammonium chloride had been added. Experiments were also done in vivo with starved animals and the data showed that only arginine, of the amino acids injected, resulted in a marked increase over the basal level of urea excretion. These findings led to the conclusion that the action of tissue arginase on ingested arginine is sufficient to account for the amount of urea excreted by the snail.

Cohen and Lewis (1949, 1950) demonstrated the production of urea by the earthworm, Lumbricus terrestris, and the presence of arginase in several tissues of this species. They showed that the arginase activity was related to the urea production by the intact worms, being much higher in fasting than in feeding individuals. It was concluded that the urea produced by the earthworms may arise by the urea cycle or some variant thereof. Needham (1960) noted that the difference in arginase activity between the worms Lumbricus and Eisenia shows a relationship to the difference in urea output by living worms of the two species under the same dietary regime. This would seem to offer further support to the theory of a Krebs-Henseleit type of mechanism for urea production in earthworms.

It was the purpose of this investigation to detect and partially characterize those enzymes of the urea cycle which are present in the
land snail, *Otala lactea*, and to determine to what extent they differed from or resembled the vertebrate and bacterial enzymes.
MATERIALS AND METHODS

The land snails, *Otala lactea*, were collected locally (Houston, Texas) throughout the year. This species is found very commonly attached to or feeding upon the lily, *Lilium* sp. and the oleander, *Nerium oleander* L. The snails were maintained in the laboratory in a state of "hibernation" until used. In the absence of adequate water, these snails attach themselves to their containers and remain inactive until water again becomes available. Needham and Baldwin (1934) found that the excretion of urea by *Helix* reaches a basal level approximately one-tenth the activity of feeding individuals after five days of starvation. An apparent decrease in the level of ornithine transcarbamylase activity was noted in the present study but no marked differences were found in the levels of arginase activity in "estivating" and feeding individuals. Three inactive snails gave an average arginase activity of 165 micromoles arginine converted per mg. protein per hour whereas three feeding individuals gave an average activity of 202 micromoles converted per mg. protein per hour when assayed. Nevertheless, all enzyme experiments were carried out on individuals which had been allowed to feed upon lettuce for eight or more hours. Active feeding can be brought about by placing the inactive snails in the presence of adequate moisture and fresh lettuce. Ordinarily, they will feed within a few minutes after being exposed to these conditions. Only the right hepatopancreas, due to its accessibility, was used in this study. The average wet, blotted weight of this portion of the hepatopancreas was found to be approximately 200 mg. (range 100-350 mg.).

The basic method of extraction of the urea cycle enzymes from this tissue was a slight modification of the method proposed by Brown and Cohen (1959) and was carried out as follows. The freshly excised tissue
was gently blotted on filter paper, weighed to the nearest .1 mg on a torsion balance, and placed in a Ten-Brock homogenizer at room temperature. To this was then added 4.5 volumes 0.1% (v/v) hexyldecyltrimethyl ammonium bromide (CTB) at room temperature. A few excursions of the homogenizer were made and the homogenizer was then transferred to an ice bucket.

Homogenization was carried out by hand and usually consisted of around 20 excursions of the plunger. The homogenate (referred to subsequently as "crude extract") was then transferred to chilled polyethylene centrifuge tubes and centrifuged at 4500 times gravity for 15 minutes at 0°C. After collecting the supernatant fluid (S₁), which was then kept at 0°C, the residue (R₁) was rehomogenized at 0°C with 4.5 volumes of CTB as before and recentrifuged. The supernatant fluid (S₂) from this centrifugation was collected and in certain cases was combined with S₁ and the residue (R₁) discarded. According to Brown and Cohen (1959), the major part of the activity for the overall arginine synthetase system is found in the S₁ fraction whereby the S₂ fraction contains the majority of the activities for the carbamyl phosphate synthetase, ornithine transcarbamylase, and arginase. For the assay of the latter three enzyme systems the combined S₁ and S₂ fractions were used. R₂ is reported to contain very little if any of these enzyme activities. Other modifications of this procedure are described with the specific assay systems.

Acetone powders of the hepatopancreas were prepared as follows. Ten grams of the freshly excised tissue were homogenized for 1 minute with 7 volumes redistilled acetone (added to tissue at -20°C) in an Omni-Mixer kept at 0°C. The homogenate was then filtered by suction until a fairly dry cake was formed. This semi-dry cake was rehomogenized as before and refiltered, the resulting cake being broken up at room temperature
and air dried for approximately 30 minutes. The powder which was formed was stored at -20° until assayed. Since low or negligible activities for
the condensing or cleavage enzyme were obtained from this preparation it was not routinely used.

In certain experiments, water homogenates of the tissue were checked for activity. They were prepared by homogenizing the tissue at 0°C in 9 volumes glass distilled water. The homogenate was then centrifuged at 25,000 rpm at 0°C for 15 minutes and the clarified supernatant fluid used as the enzyme source.

Citrulline and urea were determined colorimetrically by modifications of the Archibald method (1945). Citrulline was determined as follows. To the sample (containing 0.01 to 0.6 μmoles citrulline) in 1.0 ml. volume were added 5.0 ml. of the acid reagent (modified from Ratner, (1955) by the addition of 0.2 ml. 1% CuSO₄·5H₂O to 1000 ml. reagent). 0.25 ml. of 3% (v/v) 2,3-butanedione-2-oxime was then added and the sample thoroughly mixed. The test tubes were capped with glass marbles and heated in a boiling water bath for 30 minutes. The reaction is carried out in low actinic test tubes and protected from light during heating and cooling due to its photosensitivity. The optical densities were determined with a Coleman Junior Spectrophotometer at 490 millimicrons. Linearity is not obtained at low levels (0.01-0.05) and standards must be run at all levels to determine the shape of the curve.

Urea was determined by its reaction with 1-phenyl-1,2-propanedione-2-oxime. To the sample (containing .05 to 0.4 μmoles of urea) in 5.00 ml. volume were added 2.0 ml. of acid reagent (prepared by mixing 750 ml. 85% o-phosphoric acid, 250 ml. concentrated sulfuric acid, 1.0 ml. 1% alcoholic 4% 1-phenyl-1,2-propanedione-2-oxime was added to each tube,
they were then mixed, and heated in a boiling water bath for one hour. After cooling, optical densities were determined at 540 millimicrons. This reaction is also light-sensitive and the same precautions must be taken as with citrulline. Linearity of color intensity as a function of urea concentration is obtained in the range of 0.0 to 0.4 μmoles.

Arginine was determined using the colorimetric method of Wu (1959). Protein was estimated in the enzyme extracts by the method of Lowry et al. (1951). The standard curve was prepared using crystalline bovine serum albumin (Armour).

All substrates and co-factors with the exception of argininosuccinate, were obtained from the Sigma Chemical Company, St. Louis, Missouri. The barium salt of argininosuccinic acid was obtained from the California Corporation for Biochemical Research, Los Angeles, California. Other reagents were commercial preparations of highest obtainable purity. Purified bovine liver arginase and Jack-bean urease (type II) were obtained from the Sigma Chemical Company. Ornithine transcarbamylase was purified from rat liver by the method of Caravaca and Grisolia (1960). A lyophilized extract of rabbit muscle (containing pyruvic kinase as an ATP-generating system) was prepared according to the method of Racker (1947). The arginase used had an average activity of 80 μmoles urea produced per mg protein per hour at 20°C and pH 9.5. The activity of the purified ornithine transcarbamylase was approximately 400 μmoles citrulline formed per mg protein per hour at 38°C and pH 7.4. The activity of the muscle extract measured as the conversion of diphosphopyridine nucleotide was 13.1 μmoles converted per mg extract per minute at pH 7.3 with sodium pyruvate as the substrate and 0.325 μmoles converted per mg per minute with 3-phosphoglyceric acid as the substrate.
Enzyme Assays

Carbamyl phosphate synthetase

Two assay systems were used for this enzyme. The first employed was that described by Brown and Cohen (1959) and consists of the following: 50 μmoles NH₄HCO₃, 5 μmoles ATP, 5 μmoles L-ornithine, pH 8.0, 5 μmoles N-acetyl-L-glutamate, 10 μmoles MgSO₄, 0.46 mg purified rat liver ornithine transcarbamylase and 0 to 600 μl Otala combined S₁ and S₂ fractions in a final volume of 1.0 ml. The reaction was started by the addition of the snail enzyme, incubation being carried out in 12 ml conical centrifuge tubes for 15 minutes at 25°C. The reaction was stopped by the addition of 5.0 ml 0.5 M HClO₄. The precipitated protein was removed from the solutions by centrifugation and citrulline determinations were carried out on 1.0 ml aliquots of the supernatant solution. No activity was obtained for Otala using this assay system. The S₁ / S₂ fraction of rat liver gave 277 μmoles per gram per hour using their assay method. Omission of N-acetyl-L-glutamate or ATP from the rat assay mixture resulted in loss of activity.

The second system used to assay for carbamyl phosphate synthetase in Otala was as follows: 100 μmoles tris (hydroxy-methyl) aminomethane (Tris) buffer at pH 7.4, 8.0, or 8.5; 50 μmoles sodium bicarbonate, 50 μmoles ammonium chloride or 20 μmoles L-glutamine, 5 μmoles N-acetyl-L-glutamate, 20 μmoles MgSO₄, 4 μmoles ATP, 20 μmoles l-ornithine, pH 8.0, 8 μmoles reduced glutathione, 0.46 mg rat liver ornithine transcarbamylase plus 200 μl S₁ / S₂ fraction from Otala in a final volume of 1.0 ml. Incubation was for 30 minutes at 25°C. The reaction was stopped by the addition of 1 ml 15% trichloroacetic acid and citrulline determinations were carried out on 1.0 ml aliquots of the supernatant fluid after
centrifugation. No activity could be demonstrated for Otala with this system.

Ornithine Transcarbamylase

Two systems were used to assay for ornithine transcarbamylase. The first, modified from Brown and Cohen (1959), consists of 20 μmoles L-ornithine pH 8.0, 20 μmoles carbamylphosphate (CAP), 90 μmoles sodium glycylglycinate, pH 8.0 and 100 μl of Otala combined S₁ / S₂ fractions prepared by the method of Brown and Cohen (1959) in a total volume of 2 ml. The reaction was started by the addition of carbamyl phosphate and the tubes were incubated for 15 minutes at 25°C. 5 ml of 0.5 M HClO₄ were added to stop the reaction; the protein precipitate which formed was removed by centrifugation. A 1 ml aliquot of the supernatant solution was used for the citrulline determination. Omission of ornithine or carbamyl phosphate resulted in essentially no activity.

The components of the second assay system were 20 μmoles L-ornithine, pH 8.0, 20 μmoles carbamyl phosphate, 100 μmoles sodium glycylglycinate, pH 8.2 or pH 8.4 and 100 μmoles of Otala combined S₁ / S₂ fractions in a total volume of 1 ml. The reaction was started by the addition of carbamyl phosphate and stopped after 15 minutes incubation at 25°C with 1.0 ml 20% HClO₄. A citrulline determination was carried out on a 1 ml aliquot of the supernatant solution after centrifugation. Omission of carbamyl phosphate or ornithine resulted in no activity.

In addition to the usual zero time control, both systems require a control consisting of the complete assay system with heat killed enzyme to correct for the nonenzymatic transcarbamyllation and the effect of protein concentration which is relatively high in this buffer system.
Arginine Synthetase System

Two systems were also used for the assay of the overall arginine synthetase system. The first, based on the procedure of Brown and Cohen (1959), contained 5 μmoles citrulline pH 7.0, 5 μmoles aspartate, pH 7.0, 50 μmoles potassium phosphate buffer pH 7.0, 10 μmoles ATP, pH 7.0, 2.5 mg. bovine arginase, 0.30 mg urease and 100-500 μl of Otala S$_1$ fraction in a final volume of 1 ml. The reaction was initiated by the addition of the Otala enzyme preparation and after incubation at 25°C for 60 minutes was stopped with 5 ml 0.5 M HClO$_4$. After centrifugation to remove the precipitated protein which interferes with the color reactions, a 100 μl aliquot of the supernatant solution was used for the determination of citrulline.

As Ratner et al. (1952) have shown that addition of ATP at substrate level amounts proves inhibitory to some enzymes systems, a second assay preparation (modified from Ratner, 1955) which contained an ATP-generating system was used. The incubation mixture contained 10 μmoles citrulline pH 7.0, 10 μmoles aspartate pH 7.0, 50 μmoles potassium phosphate buffer pH 7.0, 7 μmoles MgSO$_4$, 4 μmoles ATP, ATP-generating system (1 mg lyophilized extract of rabbit muscle prepared by the method of Racker 1947), 2.5 μmoles 3-phosphoglyceric acid), 2.5 mg. bovine arginase, 0.30 mg. urease and 100-500 μl. Otala S$_1$ fraction in a final volume of 2 ml. The reaction was started by blowing in the Otala enzyme preparation and after 60 minutes incubation at 25°C, was stopped with 5 ml 0.5 M HClO$_4$. A citrulline determination was carried out on a 100 ml. aliquot of the supernatant fluid after centrifugation to remove precipitated protein which interferes with the color reaction.

Duplicates are run with each assay system containing the complete
incubation mixture minus urease in order to determine urea production and thus estimate indirectly the action of the cleavage enzyme. As citrulline interferes with the colorimetric determination of urea, the standards from which the urea values are read must contain an amount of citrulline equal to that present in the sample aliquot.

Cleavage enzyme

The assay system for the cleavage enzyme (based on the methods of Brown and Cohen, 1959) consisted of 3 μmoles argininosuccinate (prepared from the barium salt by the method of Ratner, 1955), 50 μmoles of potassium phosphate pH 7.3, 2.5 mg. arginase and 400-500 μl. of Otala S fraction in a final volume of 1 ml. The reaction was initiated by the addition of the enzyme preparation. After 60 minutes incubation at 25°C, 5 ml. of 0.5 M HClO₄ was added to stop the reaction and, following centrifugation, the urea determination was carried out on a 1 ml. aliquot of the supernatant fluid.

Arginase

Arginase was determined by the modified assay system of Brown (1960) which was as follows: 85 μmoles L-arginine pH 9.5, 50 μmoles sodium glycinate buffer, pH 9.5, 15 μmoles MnCl₂, and 100 μl. of a 1:50 dilution with 0.02 M sodium glycinate buffer, pH 9.5 of an aliquot of crude 10% (v/v) CTB homogenate in a final volume of 1 ml. The reaction was started by the addition of the enzyme and after 30 minutes incubation at 25°C stopped with 5 ml. 0.5 M HClO₄. The protein precipitate that forms is removed by centrifugation and a urea determination carried out on a 1 ml. aliquot of the supernatant solution.
Although the crude homogenate is very stable, the activity of the enzyme at pH 9.5 declines rapidly and was used immediately after dilution.
Radioisotopic and Chromatograph Procedures

3, 4-C<sup>14</sup>-labeled glutamic acid was obtained from California Corporation for Biochemical Research. The specific activity was given as 10 μcuries per ml. C<sup>14</sup>-bicarbonate with a specific activity of 1 μcurie per ml. was obtained from Volk Radiochemicals.

The xanthylod for the precipitation of urea was prepared from commercial xanthone by the method of Greenberg (1955).

Helix saline was prepared according to Welsh and Smith (1960).

Incorporation of 3,4-C<sup>14</sup>-Glutamic Acid into Urea

1 μc. of 3,4 C<sup>14</sup>D, L-glutamic acid was injected into the foot of each of eight snails. At two, four and eight hour intervals two of the snails were killed and homogenized with four volumes of 5% chilled trichloroacetic acid and spun down at 8000 x gravity for 15 minutes. The precipitate was discarded and the supernatant recentrifuged for 15 minutes at 25,000 x gravity. Urea was isolated from the supernatant fluid according to the quantitative procedure of Fosse (1913). Two mg. of urea were added at this point to act as carrier for the labeled urea. The washed precipitate was dispersed in 1 ml. 2-ethoxyethanol and transferred to aluminum planchettes for counting. Samples were counted on a windowless flow counter.

Formation of Labeled Intermediates of the Urea Cycle from C<sup>14</sup>-Bicarbonate

The hepatopancreas was removed from 3 snails, slightly teased apart, and placed in a 25 ml. rubber stoppered Erlenmeyer flask in an incubation medium consisting of 4.5 ml. Helix saline, 5 μmoles citrulline, 20 μmoles ornithine, and 0.1 μcurie H<sub>2</sub>14CO<sub>3</sub>. The flask was incubated at 24.5°C with slight shaking for 3 hours, then four volumes of 95%
ethanol was added to stop the reaction and resultant solution homogenized in a Ten-Brock homogenizer at 3°, centrifuged, and partitioned with three volumes of chloroform at 3° and the upper layer retained according to the method of Awapara (1948). This was evaporated and the residue taken up in 1 ml. of 10% 2-propanol. This was chromatographed using the system of two-dimensional chromatography described by Campbell (1960).

Chromatographic autoradiography was carried out using no-screen x-ray film. Citrulline and urea were detected on the chromatographs with p-dimethylaminobenzaldehyde according to the method of Fink et al. (1956), urea by the phenol-hypochlorite method of Williams (1951), and arginine by the Sakaguchi reaction according to Roche et al. (1957).
EXPERIMENTAL

Carbamyl Phosphate Synthetase

All efforts to demonstrate carbamyl phosphate synthetase activity in the hepatopancreas of Otala lactea by both assay methods described have given negative results. The second assay method, when used to determine carbamyl phosphate synthetase activity in the rat liver, gave a value of 277 units as compared to the value of 599 units obtained by Brown and Cohen (1960b). Attempts to use glutamine as the ammonia source, as well as the addition or deletion of acetyl glutamate produced no detectible effects.

Although it has not been possible to show activity with CTB extracts, studies with C\textsuperscript{14}-bicarbonate and C\textsuperscript{14}-glutamate offer some indirect evidence for the presence of the enzyme. A 3 hour incubation of tissue brei with C\textsuperscript{14}-bicarbonate resulted in the formation of several labeled compounds. Chromatographic comparison of the compounds with amino acid standards, when correlated with radioautographic studies are consistent with the labeling of citrulline and possibly argininosuccinate. (Fig. 1, Table 1)

Precise chromatographic identification of argininosuccinate is difficult as the two-dimensional chromatograms of the "pure" barium argininosuccinate obtained commercially indicate the presence of several different compounds (Figure 2). Spots on Figure 2, on comparison with the Rf values of Westall (1960), are tentatively identified as argininosuccinate, the cyclic anhydride forms of argininosuccinate (B and C), and aspartate and ornithine. The presence of "B" and "C" may be attributed to the fact that argininosuccinate readily cyclizes in weakly aqueous solutions to form the anhydride. Although the anhydride forms cannot
Two-dimensional chromatogram of hepatopancreas extract following incubation in HCO$_3^-$.$^1$ Solid spots represent radioactivity; open circles refer to non-radioactive spots; "n" designates ninhydrin-positive; "s" sakaguchi reaction-positive; "pH" phenol-hypochlorite-positive; and "dmab" p-dimethylaminobenzaldehyde-positive. Urea and arginine were added as standards to determine their position on the chromatograms but were not detectable in the extracts with the color reactions used. CPB solvent, m-Cresol: phenol: 0.1 m borate buffer, pH 9.4 (60:30:15, w/w/v); BFW solvent, 2-butanol: 88% formic acid: water (75:15:10, by vol.). Whatman #3mm paper. 50 µl. of extract spotted at origin.

Ninhydrin-positive spots on a two-dimensional chromatogram of 5 µmoles commercial "Argininosuccinic acid" carried out as in Figure 1.
After incubation for 3 hours in medium containing C\textsuperscript{14}-bicarbonate, the snails were extracted and the radioactive compounds formed isolated by chromatography as described in the text. The radioactivity present in each compound was determined by eluting the compound from a 2-dimensional chromatograph and counting a sample of the eluate with a gas flow counter.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Radioactivity c/m (corrected for bkgd)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aspartate</td>
<td>579</td>
</tr>
<tr>
<td>Glutamate</td>
<td>669</td>
</tr>
<tr>
<td>Alanine</td>
<td>263</td>
</tr>
<tr>
<td>&quot;Succinate&quot;</td>
<td>2524</td>
</tr>
<tr>
<td>Arginine</td>
<td>00</td>
</tr>
<tr>
<td>Citrulline</td>
<td>91</td>
</tr>
<tr>
<td>&quot;Argininosuccinate&quot;</td>
<td>69</td>
</tr>
<tr>
<td>Urea</td>
<td>00</td>
</tr>
</tbody>
</table>
FORMATION OF C$^{14}$-UREA FROM C$^{14}$-GLUTAMATE IN OTALA LACTEA

At 2, 4, and 8 hour intervals after injection of 1 uc 3,4-C$^{14}$-glutamate, the snails were extracted and urea isolated by the Fosse method as described in text. The radioactivity present was determined by counting the urea with a gas flow counter.

<table>
<thead>
<tr>
<th>No. of hours after injection</th>
<th>Radioactivity c/m/gm. (corrected for bkgnd)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>10.0</td>
</tr>
<tr>
<td>4</td>
<td>8.7</td>
</tr>
<tr>
<td>8</td>
<td>405.9</td>
</tr>
</tbody>
</table>
be distinguished by chemical methods from the substrate, they are metabolically inert.

Studies with C\textsuperscript{14} labeled glutamate also offers indirect support for the presence of the enzyme. Urea isolated by the Fosse method from trichloroacetic acid extracts of the complete snail 8 hours after the injection of 3,4\textsuperscript{14}C-glutamate into the foot was shown to possess considerable activity (Table 2).

Ornithine Transcarbamylase

CTB extracts of the hepatopancreas of Otala form citrulline when incubated in an assay mixture containing ornithine and carbamyl phosphate at pH 8.0 for 15 minutes. Both ornithine and carbamyl phosphate are required, only minimal activity being obtained when either was omitted from the assay mixture (Figure 3). The activity of ornithine transcarbamylase as determined by the method of Brown and Cohen (i.e., the acid stopped 0 time used as control) is 235 units; assay systems with heat-killed enzyme serving as control give 170 units, each unit representing the production of 1 \textmu mole of citrulline per gram wet weight per hour. The use of the heat-killed enzyme system as the control makes it possible to correct for nonenzymatic transcarbamylation and the effect of protein. This gives more accurate results as nonenzymatic transcarbamylation can introduce a serious error at low levels of activity. The specific activity as calculated from the first system is .93 per mg. protein, from the second .732 per mg. protein. These values may be compared to the 22,800 units and specific activity of 331 obtained for rat liver by Brown and Cohen (1960).

Linearity of enzyme activity as a function of protein concentration
TABLE 3
ORNITHINE TRANSCARBAMYLASE ACTIVITY IN CTB EXTRACTS OF THE HEPATO-PANCREAS OF OTALA LACTEA

In system corrected by zero time each tube contained 20 \( \mu \)m. L-Ornithine, pH 8.0, 20 \( \mu \)m Carbamyl phosphate, 90 \( \mu \)m Sodium glycylglycinate, pH 8.0 and 100 \( \mu \)l. Otala combined \( S_1 / S_2 \). Incubation is for 15 min. at 25°C.

In system corrected by heat-killed enzyme control each tube contained 29 \( \mu \)m. L-Ornithine pH 8.0, 20 \( \mu \)moles Carbamyl phosphate, 100 \( \mu \)moles Sodium glycylglycinate pH 8.2 or 8.4 and 100 \( \mu \)l. Otala combined \( S_1 / S_2 \) in a total volume of 1 ml. Incubation is for 15 min. at 25°C.

<table>
<thead>
<tr>
<th>Expt. No.</th>
<th>Activity (corr. for 0)</th>
<th>Specific Activity for H-K</th>
<th>Specific Activity for H-K</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>288.08</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>149.59</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>288.4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>298.4</td>
<td>0.958</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>151.16</td>
<td>0.902</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>177.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>164.0</td>
<td>0.759</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>170.5 \pm 9.19</td>
<td>0.930 \pm 0.040</td>
<td>0.732 \pm 0.038</td>
</tr>
</tbody>
</table>

Ave. 235.12 \pm 47.66 170.5 \pm 9.19 0.930 \pm 0.040 0.732 \pm 0.038
Enzyme activity vs. protein concentration with hepatopancreas ornithine transcarbamylase of *Otala lactea*. Assay system at pH 8.4 contained 0.59 mg. protein per 100 microliter. Incubation at 25°C for 15 minutes. • = total citrulline synthesized. ▲ = citrulline synthesized by heat-killed control. 0 = net synthesis of citrulline.
Enzyme activity vs. time with hepatopancreas ornithine transcarbamylase of Otala lactea. Assay system described in text contained 0.55 mg. protein. Incubation for 15 minutes at 25°C. \( \Theta \) = total um. citrulline formed. \( \Delta \) = Citrulline formed in heat-killed system. \( \bigcirc \) = total um. citrulline formed minus citrulline formed in heat-killed system = net synthesis of citrulline.
pH optimum of *Otala lactea* hepatopancreas ornithine transcarbamylase. Assays contained 0.55 mg protein. Buffer used at indicated pH. Ornithine was adjusted to the corresponding pH before use.
was obtained between the range 0.00 and 0.59 mg.; higher concentrations appeared to inhibit the enzyme (Figure 3). The enzyme activity is also linear with respect to time between 0 and 15 minutes, the rate falling off slightly with longer periods of incubation (Figure 4). The pH optimum of the ornithine transcarbamylase system was determined in tris-HCl and in sodium glycyglycinate buffer as both buffers have been used in previous studies (Reichard, 1957; Burnett and Cohen, 1957). As shown in Figure 5, the optimum pH for the ornithine transcarbamylase in tris-HCl at 25° was around 7.4, that in sodium glycyglycinate range from pH 7.8 to 8.3. Tris-HCl buffer, even at optimum pH, appeared to be inhibitory to the enzyme system; this agreed with the results of Burnett and Cohen (1957), in studies of beef liver ornithine transcarbamylase. However, this effect was not noted by Reichard (1957) in his study of the enzyme of rat liver, although the pH optimum in tris-HCl for rat and Otala enzyme are comparable. The pH optimums of the rat liver purified as described in methods section was around 7.4-7.5 in tris-HCl. Using sodium glycyglycinate, the optimum pH value 7.8-8.3 for Otala is comparable to the pH optimum of 8.0-8.5 recorded by Burnett and Cohen for the beef liver enzyme.

Arginine Synthetase System

A low but fairly consistent level of arginine synthetase activity (Table 4) has been calculated from data obtained by the incubation of CTB extract with an assay mixture containing citrulline, aspartate, magnesium and ATP at pH 7.5 and 25°. The activity of both enzymes was determined indirectly from the overall system. The disappearance of citrulline indicates the level of enzyme activity of the condensing
TABLE 4

ARGININE SYNTHETASE ACTIVITY IN CTB EXTRACTS
OF THE HEPATOPANCREAS OF OATA LACTEA

Assay system 1 contained in a final volume of 1 ml. 5 µm. citrulline pH 7.0, 5 µmoles aspartate pH 7.0, 50 µm. potassium phosphate buffer, pH 7.0, 10 µm. ATP, 2.5 mg. bovine arginase, 0.30 mg. urease and 200 µl. (0.985 mg. protein) Oata S₁ fraction. Incubated at 25° for 60 min.

Assay system 2 contained in a final volume of 2 ml. 10 µm. citrulline pH 7.0, 10 µm. aspartate pH 7.0, 50 µm potassium phosphate buffer, pH 7.0, 7 µm. MgSO₄, 4 µm. ATP, 1 mg. lyophilized extract rabbit muscle, 2.5 µm. 3-phosphoglyceric acid, 2.5 mg. bovine arginase, 0.30 mg. urease, and 300 µl. (1.4 mg. protein) Oata S₁ fraction. Incubated at 25° for 60 min.

<table>
<thead>
<tr>
<th>Expt.</th>
<th>Assay 1</th>
<th>Assay 2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>um. cit.</td>
<td>um. urea</td>
</tr>
<tr>
<td>1</td>
<td>7.1</td>
<td>6.4</td>
</tr>
<tr>
<td>2</td>
<td>5.1</td>
<td>4.7</td>
</tr>
<tr>
<td>3</td>
<td>4.6</td>
<td></td>
</tr>
</tbody>
</table>
TABLE 5

REQUIREMENTS FOR ACTIVITY
OF ARGinine SYNTHETASE SYSTEM
IN OTAIA LACTEA

Assay system 1 contained in a final volume
of 1 ml. 5 µm. citrulline pH 7.0, 5 µm. aspartate
pH 7.0, 50 µm. potassium phosphate buffer, pH 7.0,
10 µm. ATP, 2.5 mg. bovine arginase, 0.30 mg.
urease and 200 µl. (0.985 mg. protein) Otala S1
fraction. Incubated at 25° for 60 min.

<table>
<thead>
<tr>
<th>Citrulline Disappearance (µm/60 min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complete system</td>
</tr>
<tr>
<td>citrulline omitted</td>
</tr>
<tr>
<td>aspartate omitted</td>
</tr>
<tr>
<td>ATP omitted</td>
</tr>
<tr>
<td>Enzyme omitted</td>
</tr>
<tr>
<td></td>
</tr>
</tbody>
</table>
enzyme; activity of the cleavage enzyme may be suggested by the production of urea (not an accurate representation because the amount of argininosuccinate, i.e., the activity of the condensing enzyme, will be rate-limiting). The level of activity of the system using ATP as the energy source was 5.6 units in terms of citrulline disappearance, 5.0 units of urea were produced. After several determinations to show the correlation between citrulline disappearance and urea production (Table 4), only citrulline utilization was used to calculate the activity of the overall system.

The presence of an ATP-generating system, which was found by Ratner (1951) to yield higher activities, apparently resulted in more nearly optimum enzyme conditions as shown by the level of activity in Table 4.

Although attempts to study linearity of enzyme activity as a function of protein concentration did not give clear cut results, it will be noted from Table 6 that there is a definite trend toward increasing enzyme activity with increasing protein concentration. This difficulty has been discussed by Ratner (1960).

Cleavage Enzyme

Attempts to demonstrate separately the activity of the cleavage enzyme have also produced negative results. This is due partially to the difficulty of preparing the substrate, argininosuccinate, so that it remains in the metabolically active form. At the present time the only method of preparing this compound is biosynthetically (Ratner, 1953, Westall, 1960). Figure 2, a two-dimensional chromatograph of the commercial "argininosuccinate" available shows that there was obviously more than one compound present due perhaps to contamination of the original material or
### Activity of the Arginine Synthetase System

**as a function of protein concentration**

Assay system 1 contained in a final volume of 1 ml. 5 μm. citrulline pH 7.0, 5 μm. aspartate pH 7.0, 50 μm. potassium phosphate buffer, pH 7.0, 10 μm. ATP, 2.5 mg. bovine arginase, 0.30 mg. urease, and Otala S1 fraction (0.985 mg. protein per 200 μl.)

<table>
<thead>
<tr>
<th>Protein conc. (μl.)</th>
<th>Citrulline Disappearance (μm/60 min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>50</td>
<td>0.090</td>
</tr>
<tr>
<td>100</td>
<td>0.070</td>
</tr>
<tr>
<td>200</td>
<td>0.180</td>
</tr>
<tr>
<td>300</td>
<td>0.195</td>
</tr>
<tr>
<td>400</td>
<td>0.210</td>
</tr>
<tr>
<td>500</td>
<td>0.240</td>
</tr>
</tbody>
</table>
its partial degradation during chromatography. The spots are tentatively identified as aspartate, ornithine, and compounds "B" and "C" (Westall, 1960), two metabolically inactive forms of the cyclic anhydride.

Arginase

CTB extracts of the hepatopancreas of Otala form urea when incubated in an assay mixture containing arginine and manganous chloride at pH 9.5 and 25°C. In the presence of excess enzyme there was essentially a mole for mole conversion of arginine to urea by the arginase system under the conditions employed (Table 7). Activity calculated as micromoles urea per gram of tissue per hour is 18,659 units (Table 8). Specific activity is 285 umoles urea per mg. protein.

Linearity of enzyme activity as a function of protein concentration was obtained between the range of 0.0 to .048 mg. protein (Figure 6); higher concentrations seemed to inhibit the enzyme. Linearity of enzyme activity with time was also noted under assay conditions (Figure 7).

Activation studies (Table 9) show that only manganous and cadmium ions give 100% activation after incubation at 50°C for 30 minutes. No activation, actually a slight inhibition was noticed with cobaltous, vanadyl, zinc, cuprous, ferrous, nickelous, and magnesium ions under the same conditions. Omission of Mn²⁺ from the reaction mixture produced no appreciable change in the level of activity. The $K_m$ was calculated to be $2.77 \times 10^{-3}$ M as compared to a $K_m$ value of $2.6 \times 10^{-3}$ M for horse liver arginase (Greenberg, 1956), under the same conditions of temperature and pH.
## TABLE 7

ARGINASE - STOICHIOMETRY

Each tube contained in a final volume of 1 ml.
85 µmoles L-Arginine, 50 µmoles Glycine-NaOH buffer, 
pH 9.5, 15 µmoles MnCl₂, and 500 µl. 1-10 diluted 
CTB crude homogenate. Incubation was at 25°C for 
30 minutes. Assay system contained 0.410 mg. protein.

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>µm. Arginine (disappeared)</th>
<th>µm. Urea (produced)</th>
</tr>
</thead>
<tbody>
<tr>
<td>00</td>
<td>00.0</td>
<td>00.0</td>
</tr>
<tr>
<td>11</td>
<td>42.0</td>
<td>42.0</td>
</tr>
<tr>
<td>15</td>
<td>47.4</td>
<td>46.2</td>
</tr>
<tr>
<td>30</td>
<td>68.4</td>
<td>63.0</td>
</tr>
<tr>
<td>60</td>
<td>81.9</td>
<td>81.0</td>
</tr>
<tr>
<td>75</td>
<td>85.5</td>
<td>84.0</td>
</tr>
</tbody>
</table>
ARGINASE ACTIVITY IN CTB EXTRACTS OF
THE HEPATOPANCREAS OF OTALA LACTEA

Each tube contained in a final volume of 1 ml.
85 μm. L-Arginine, 50 μm. Glycine-NaOH buffer pH 9.5,
15 μm. MnCl₂, and 100 μl. of 1-50 dilution (with .02
M Glycine buffer pH 9.0) of 1-10 CTB crude homogenate.
Incubation is for 30 minutes at 25°C.

<table>
<thead>
<tr>
<th>Expt. No.</th>
<th>Activity (μm/gm/hr)</th>
<th>Specific Activity (μm/mg Protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>17,400</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>17,329</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>17,803</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>18,963</td>
<td>310.0</td>
</tr>
<tr>
<td>5</td>
<td>22,640</td>
<td>370.6</td>
</tr>
</tbody>
</table>
ION ACTIVATION STUDIES ON ARGINASE IN CTB EXTRACTS OF THE HEPATOPANCREAS OF OTALA LACTEA

Preincubation was carried out for 30 minutes at 50°C in a volume of .9 ml. containing 50 um. Glycine-NaOH buffer pH 9.5, 100 ul. 1-50 dilution Otala CTB extract and 100 um. ion. Arginine was then added (.1 ml. of .85 M Solution pH 9.5). Incubation was for 30 minutes at 25°C. Assay system contained 0.023 mg. protein.

<table>
<thead>
<tr>
<th>Ion</th>
<th>Activity (um./ml./min.)</th>
<th>Activation %</th>
<th>Inhibition %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mn</td>
<td>.220</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>Mg</td>
<td>.070</td>
<td>0</td>
<td>26</td>
</tr>
<tr>
<td>Zn</td>
<td>.070</td>
<td>0</td>
<td>26</td>
</tr>
<tr>
<td>Fe</td>
<td>.060</td>
<td>0</td>
<td>37</td>
</tr>
<tr>
<td>Cu</td>
<td>.060</td>
<td>0</td>
<td>37</td>
</tr>
<tr>
<td>Ni</td>
<td>.070</td>
<td>0</td>
<td>26</td>
</tr>
<tr>
<td>Cd</td>
<td>.210</td>
<td>95.5</td>
<td>0</td>
</tr>
<tr>
<td>Co</td>
<td>.075</td>
<td>0</td>
<td>21</td>
</tr>
</tbody>
</table>

No preincubation: .095 um/ml/min
Fig. 6

Enzyme activity vs. protein concentration with hepatopancreas arginase of *Ottala lactea*. Assay system described in text. Incubation was at 25°C. for 30 minutes.
Enzyme activity vs. time with hepatopancreas arginase of Otala lactea. Assay system described in text. Assay contained 0.021 mg. protein. Incubation at 25°C for length of time indicated.
Fig. 8

Line-Weaver-Burke graphic representation of rate vs. substrate concentration data for *Obala lactea* hepatopancreas arginase system. Incubation for 30 minutes at 25°C. The assay system contained 0.027 mg. protein. \( V = \text{umoles urea per mg. protein per minute.} \)
\[ \frac{1}{V_m} = 0.312 \]
\[ V_m = 3.205 \]

SLOPE = \( 8.65 \times 10^{-4} \)

\[ K_m = 2.77 \times 10^{-3} \text{ M} \]
DISCUSSION

Direct evidence for the presence of carbamyl phosphate synthetase in the hepatopancreas of Otala is not available as the in vitro synthesis of carbamyl phosphate from ammonia and carbon dioxide was not demonstrated. Indirect evidence of its presence was given in the form of radioisotopic and chromatographic studies showing the in vivo incorporation of C\textsuperscript{14}-bicarbonate into citrulline and possibly argininosuccinate, and the in vivo formation of C\textsuperscript{14}-urea from injected 3,\textsuperscript{14}-C\textsuperscript{14}-glutamate. However, as CO\textsubscript{2} fixation through pathways not involving the urea cycle is not uncommon among invertebrates (Hammon and Osborne, 1959), this cannot be taken as absolute proof of the presence of the enzyme.

Further indirect support for carbamyl phosphate synthetase activity was the in vitro formation of citrulline by CEB extracts of the hepatopancreas when incubated with ornithine and carbamyl phosphate. As the enzyme mediating this reaction, ornithine transcarbamylase, requires carbamyl phosphate for activity (Figure 3) the ability of the tissue to form carbamyl phosphate is indicated. The activity of ornithine transcarbamylase at 25\degree when determined by the method of Brown and Cohen (i.e., the use of the acid-stopped zero time as control) is 235.12 units as compared to the value of 22,800 units obtained for rat liver (Brown and Cohen, 1960) at 38\degree. However, experiments using the complete system with heat-killed enzyme as controls yield more accurate results in that a correction is made for nonenzymatic transcarbamylation and the effect of protein on the system, which at low levels of activity introduce a considerable error. The error due to nonenzymatic transcarbamylation is increased with increasing temperature.

pH studies of ornithine transcarbamylase (Figure 5) give an optimum
pH 7.8-8.3 in sodium glycylglycinate buffer that is comparable to the pH optimum of 8.0-8.5 noted by Burnett and Cohen (1953) with beef ornithine transcarbamylase and by Brown and Cohen (1960) with liver preparations of *Squalus suckleyi*.

Levels of activity, measured by the disappearance of citrulline, of the overall arginine synthetase system (condensing and cleavage enzyme) are relatively low. The calculation of 5.6 enzyme units when ATP is added as the energy source or of 9.5 units in systems containing the ATP-generating source compare in magnitude to those obtained by Ratner (1960) with crude homogenates of rat brain. The appearance of urea when correlated with the disappearance of citrulline, indicates the presence of the cleavage enzyme, which splits the argininosuccinate formed by the condensation of citrulline and aspartate to give arginine and fumarate. However, these values are probably not a true measure of the activity of the cleavage enzyme, as the amount of argininosuccinate formed by the condensing enzyme (which is normally the rate limiting reaction) limits the formation of arginine and fumarate. Direct measurement of the activity of the splitting enzyme is lacking due to the difficulty of obtaining the metabolically active form of the substrate.

Data obtained from the present study agrees with that of early investigators (Baldwin and Needham, 1945) in that the level of *Otala* arginase activity, 18,659 μmoles of urea produced per gram per hour, is comparable to the levels found in the livers of many vertebrates.

Activation studies point out a dissimilarity between the mammalian enzyme and that of *Otala*. *Otala* arginase is maximally activated by manganous and cadmium ions, with the cobaltous ion having an inhibitory effect. Only cobaltous and manganous ions result in maximal activation of the mammalian
enzyme; cadmium has little or no effect.

The $K_m$ value of $2.77 \times 10^{-3}$M compares with that of $2.6 \times 10^{-3}$M obtained by Greenberg (1956) with mammalian enzyme under the same conditions of assay.

A level of arginase far above that needed to handle the amount of arginine that could theoretically be produced by the other enzymes of the urea cycle has led some workers to investigate the possibility that arginase in the snail may play some part in other metabolic processes. Baldwin and Needham (1934) noted a positive correlation between the level of arginase present in the hepatopancreas and the amount of uric acid excreted. However, the pathway of synthesis of uric acid in invertebrates has not been elucidated and the understanding of a possible role of arginase in the cycle must await further study.

This study has presented evidence of the presence in the hepatopancreas of *Otala* of four of the urea cycle enzymes. This does not serve as proof of a functional urea cycle in the land snail but indicates that further studies must be made before the presence or absence of the cycle is established.
SUMMARY

1. A study was made of the urea cycle enzymes present in the hepatopancreas of *Otala lactea*.

2. Indirect evidence of the presence of carbamyl phosphate synthetase was obtained through chromatographic and isotopic studies. The incorporation of C\(^{14}\)-bicarbonate into citrulline and possibly argininosuccinate and the formation of labeled urea from 3,4-C\(^{14}\)-glutamate was demonstrated.

3. *Otala* ornithine transcarbamylase when studied in a crude extract of the hepatopancreas has no apparent cofactor requirement but requires ornithine and citrulline for maximal activity. The pH optimum in sodium glycylglycinate is pH 7.8-8.3.

4. Arginine synthetase activity for CTB extracts of hepatopancreas was 5.6 units using added ATP as the energy source, 9.5 units with an ATP-generating system.

5. Arginase activity of a crude homogenate of the hepatopancreas of *Otala* is shown to be 18,659 units. The enzyme is activated by preincubation with Mn\(^{2+}\) and Ca\(^{2+}\) but inhibited by Co\(^{2+}\), V\(^{2+}\), Zn\(^{2+}\), Cu\(^{2+}\), Fe\(^{2+}\), Ni\(^{2+}\), and Mg\(^{2+}\). The Km value for arginine is 2.77 x 10\(^{-3}\)M.


