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UREA BIOSYNTHESIS IN THE EARTHWORM, LUMBRICUS TERRESTRIS

by

STEPHEN H. BISHOP

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

Thesis Director's signature:

James W. Campbell

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INTRODUCTION

Free-living invertebrate animals are mainly "ammonotelic" or "uricotelic" with respect to their mode of elimination of nitrogenous end-products. The "uricotelic" organisms excrete uric acid or some purine (guanine, xanthine, adenine, hypoxanthine, etc.) while the "ammonotelic" organisms excrete ammonia (Prosser and Brown, 1961). Terrestrial invertebrates such as insects, some arachnids, and pulmonate snails are considered "uricotelic" while aquatic or semi-aquatic invertebrates are usually "ammonotelic". Some invertebrates do produce urea in their excreta but it rarely constitutes a major portion of the nitrogen in the excreta. Under conditions of starvation, some earthworm species undergo a transition from ammonotelism and become "ureotelic". In Lumbrix terrestris, the urea in the urine constitutes 80 to 90% of the nitrogen excreted when the animal is starved (Cohen and Lewis, 1949). Needham (1957) has confirmed the experiment of Cohen and Lewis for Lumbrix but has also demonstrated that not all earthworms produce high urea in their urine during starvation.

The generally accepted mechanism for urea biosynthesis in animals is the modification of the original Krebs and Henseleit ornithine cycle which now includes five separate enzymatic activities (Cohen and Brown, 1960). Krebs and Henseleit (1932) found ornithine to be catalytic in the
Chart 1

Outline of the Krebs and Henseleit cycle and the individual enzymatic activities operating in the ornithine cycle

1. Reaction I, citrulline synthesis \((\text{NH}_4^+ + \text{HCO}_3^- + \text{ornithine} \rightarrow \text{citrulline})\)

   a. Carbamyl phosphate synthetase
      \[
      2\text{ATP} + \text{NH}_4\text{HCO}_3 \xrightarrow{\text{Mg}^2+} \text{N-acetyl-L-glutamate} \xrightarrow{\text{Mg}^2+} \text{carbamyl phosphate} + \text{P}_1 + \text{ADP}
      \]

   b. Ornithine transcarbamylase
      \[
      \text{carbamyl phosphate} + \text{ornithine} \rightarrow \text{citrulline} + \text{P}_1
      \]

2. Reaction II, citrulline to arginine conversion

   a. Argininosuccinate synthetase (condensing enzyme)
      \[
      \text{citrulline} + \text{aspartate} + \text{ATP} \xrightarrow{\text{Mg}^2+} \text{argininosuccinate} + \text{AMP} + \text{PP}_1
      \]

   b. Argininosuccinase (cleavage (enzyme)
      \[
      \text{argininosuccinate} \rightarrow \text{arginine} + \text{fumarate}
      \]

3. Reaction III, arginase and urea formation

   \[
   \text{arginine} \xrightarrow{\text{Mg}^2+} \text{ornithine} + \text{urea}
   \]
production of urea in rat liver slices. Then, on the basis of the known arginase activity and the recently discovered amino acid, citrulline, they postulated the ornithine cycle. The original cycle had three basic steps and two intermediates, citrulline and arginine (Chart 1). The present modified cycle has four intermediate compounds, one of which (carbamyl phosphate) is unstable. By this mechanism, the bicarbonate carbon follows the pathway to the ureido-carbon of citrulline, through argininosuccinate to the amidine-carbon of arginine, and finally to the carbon of urea.

In microorganisms, the ornithine cycle is generally considered a mechanism for arginine synthesis. Bacteria lack arginase but fungi have both arginase and urease. Although arginase has been found in a number of invertebrates and most vertebrates (Brown and Cohen, 1960; Baldwin, 1936, 1957; Greenberg, 1951), the de novo biosynthesis of arginine and urea has been established only for amphibians, some fish, turtles, and mammals. Birds lack a citrulline synthetic mechanism (Bowers and Grisolia, 1962; Tamir and Ratner, 1936a, b). Both birds and insects have arginase but both seem to require arginine or citrulline for growth (Tamir and Ratner, 1963a; Gilmour, 1961). Dependency on dietary arginine for maximal growth is evident only in some mammals in the pre- and early post-natal periods (Kennan and Cohen, 1959). All
mammalian cells in tissue culture require arginine or citrulline for growth (Morgan et al., 1958; Eagle, 1963) and lack a citrulline synthetic mechanism (Jones et al., 1961; Schimke, 1964).

Few data have been available concerning the biosynthesis of either arginine or urea in invertebrates. Rogers (1952) found arginase in Ascaris lumbricoides and Ascaridia galli and a stimulation of urea production by incubating their gut tissue with citrulline and arginine in Ascaris and with ornithine, citrulline, and arginine in Ascaridia. Campbell (1963) has demonstrated arginase and ornithine transcarbamylase activity plus incorporation of label from $^{14}$C$\text{-HCO}_3$ into urea by the tapeworm, Hymenolepis diminuta. Campbell and Lee (1963) described arginase and ornithine transcarbamylase activities in a number of parasitic and free-living flatworms. All ornithine cycle activities except carbamyl phosphate synthesis were found in the land snail, Otala lactea (Linton and Campbell, 1962).

Lesser (1908) and Delaunay (1934), after isolating earthworms in large volumes of water and analyzing the fluid, reported ammonia as the major nitrogenous end-product. Bahl (1945) found about equal amounts of ammonia and urea in the urine of Pheritima posthuma. Bahl (1945,46) and Cohen and Lewis (1949) collected urine from earthworms under more
normal conditions by keeping them in an atmosphere saturated with water vapor rather than by keeping them immersed in water. This method seemed to reduce loss of nitrogen due to mucus secretion. The earthworm, *L. terrestris*, as mentioned above, becomes ureotelic upon starvation (Cohen and Lewis, 1949; Needham, 1957).

Bahl (1945, 46) found both urea and ammonia in all body parts and fluids of *Pheritima*. Heidemans (1937) reported the urea concentration of the gut of *Lumbricus* to be twice the ammonia concentration of the gut and ten times the urea concentration of the body wall. Haggag and El-Dueini (1959) found a great increase in the urea in the chloragogen cells during starvation of *Allolobophora caliginosa*. Laverack (1963) reports recent chromatographic studies of chloragogen cell extracts in which ornithine, glutamate, arginine, glycine, and kynurenine have been identified. Ornithine and glutamate disappear after a period of starvation and no citrulline was ever located. Campbell (1960, 63), however, has reported citrulline as a free amino acid in several cestodes.

Heidemans (1937) found a stimulation of urea production by incubating a gut tissue mince from *L. terrestris* with "peptone" but concluded that no ornithine cycle was operating because stimulation of urea production was not evident with ornithine or arginine. The demonstration of arginase activity
and the stimulation of urea production by feeding various amino acids with arginine and citrulline, led to the suggestion that the ornithine cycle might function at least in *Lumbricus* (Needham, 1960; Cohen and Lewis, 1949, 50). Upon starvation, both Needham (1960) and Cohen and Lewis, 1950) found a large increase in arginase activity which correlated with the increase in urea production in *Lumbricus*.

Evidence of the operation of a functional ornithine cycle in invertebrates and particularly earthworms is circumstantial. All the individual enzyme activities have not been demonstrated in a single invertebrate species. The lack of such data has even prompted the suggestion of an absence of any arginine and urea synthesizing system in invertebrates (Baldwin, 1957; Brown, 1962). The investigation to be reported had three purposes:

1. to establish the presence of an arginine and urea synthesizing system in *Lumbricus terrestris* tissues,

2. if present, to characterize the individual enzymatic steps and compare the activities with those reported from vertebrates, other invertebrates, and microorganisms,

3. and to quantitate the mode of urea formation and production in the transition from ammonotelism to ureotelism in *Lumbricus*. 
METHODS AND MATERIALS

General

*Lumbricus terrestris* were purchased from Caroline Biological Supply Co. or Lemberger and Co. and positively identified by reference to Oates (1942, 62). The worms were maintained in a box of dirt or peat moss until sacrificed. A few days prior to sacrifice, the worms were placed in plastic boxes lined with wet paper toweling. Under these conditions, the gut contents were usually eliminated in three days. Worms starved one or two weeks were routinely used for the enzyme assays. If the paper toweling was changed every day, there was no evidence of paper ingestion.

The mid-gut (gut) or intestinal region (Arthur, 1963) behind the crop and anterior to the rectum was dissected from worms narcotized by cooling on crushed ice (Bellamy, 1962). The tissue was kept on a watch glass placed on ice until enough tissue had accumulated for the enzymatic assay. The gut tissue (intestine) made up 11-12.5% of the total weight of worms weighing 3-5 grams. Needham (1960) found the gut to make up 12.5% and the body wall 60% of the worm weight when starved. Any residual material in the gut lumen was removed and the tissue washed in 0.44 M mannitol (0°C), blotted, and weighed. Tissue homogenates were prepared in TenBroek homogenizers. Acetone powders of the gut tissue were prepared
according to the method of Ratner (1955). Enzymatic activities were always assayed on tissue preparations of tissue pooled from several worms. Low speed centrifugations of less than 20,000G were executed at 0°C in an International Refrigerated Centrifuge, Model PR I using head #296 or HS296. High speed centrifugations (105,000G) were executed in a Spinco Model L refrigerated centrifuge with a #40 rotor.

Reagents

Tris (hydroxymethyl) aminomethane (Tris); 3-phosphoglyceric acid, barium salt; L-glycylglycine; NAD; glycine; malic acid; D-mannitol; L-glutamic acid; L-ornithine-HCL; L-citrulline; L-aspartic acid; L-arginine-HCL; N-acetyl-L-glutamic acid; carbamyl-DL-aspartate; dilithium, carbamyl phosphate (90%); adenosinetriphosphate (ATP), disodium tetrahydrate; L-glutamine; ethylenediaminetetraacetic acid (EDTA); and DEAE cellulose (medium mesh, 0.9meq/g) were purchased from Sigma Chem. Co. The dilithium carbamyl phosphate was prepared fresh at 0°C before use. Potassium-3-phosphoglycerate was prepared from the barium salt by the method of Ratner (1955).

Argininosuccinic acid, barium salt was purchased from California Biochem. Corp. as assaying at 1.56 umoles per mg of barium salt. The barium salt was converted to the potassium salt with K₂SO₄ (Ratner, 1955) at 0°C just before use. The arginine contamination was 0.028 to 0.03 umoles arginine per
umole of argininosuccinate. Chromatography revealed a slight aspartic acid contamination of the argininosuccinate.

α-Methyl-DL-aspartate was purchased from Cycle Chem Co.

Imidazole, hexadecyltrimethylammonium bromide (CTAB), 9-xanthenol, 1-phenyl-2,3-propanedione-2-oxime (PPO), 2,3-butanedione monoxime (BMO), and 2,4-dinitro-1-naphthol-7-sulfonic acid (flavianic acid) were purchased from Eastman Organic Chem.

Avidin was purchased from Nutritional Biochem. Co.

Inorganic chemicals and urea were purchased from Fisher Sci. Co. Sucrose was commercial sucrose. Solvents for chromatography were purchased from Mallinkrot Chem. Works or Fisher Sci. Co.

Radioisotopes

(14C)-NaHCO3 was purchased from Volk Radiochem. Co., Ureido-(14C)-L-citrulline and 5-(14C)-DL-ornithine were purchased from New England Nuclear Co. Some of the ornithine and citrulline were chromatographed and the chromatograms placed on x-ray film for two weeks. The developed film indicated several minor radioactive contaminating compounds with both compounds. A 2% (14C)-urea contamination with the citrulline was removed by treatment of the citrulline with urease. No arginine contamination was evident with either compound and the ornithine had no radioactive citrulline contamination.
Measurements of radioactivity were executed in a Nuclear Chicago gas-flow counter, Model 1040. The efficiency of the counting was uniform and about 22%. Samples in which the radioactivity was to be counted were plated on flat aluminum planchets (Nuclear Chicago, 31 mm diameter x 1 mm deep). In studies where \(^{14}\text{C}\)-urea was decomposed with urease or ureido-\(^{14}\text{C}\)-citrulline was arsennolyzed, the CO\(_2\) evolved was trapped in NaOH. The carbonate formed was then precipitated as BaCO\(_3\) in the presence of NH\(_4\)Cl, washed with hot water twice, then with ethanol twice. The washed BaCO\(_3\) was slurried in ethanol and plated on the flat aluminum planchets of known weight. They were dried, weighed again, counted in the gas-flow counter for radioactivity, and the counts per minute (CPM) corrected for self-absorption by reference to a standard curve.

Chromatography

Particular methods used for each experiment are described with the results. The solvent systems used were: 2-butanol, formic acid (88%) and water (75:15:10); ethanol, ammonia water; ethanol, 30% NH\(_4\)OH, water (80:5:15); and lutidine and water: 2,4-lutidine, water (80:20). Solvents were prepared by mixing the stated volume fractions. The spray reagents used were: ninhydrin from Sigma Chem. Co., p-dimethylaminobenzaldehyde, prepared after Pink et al. (1956) and the Sakaguchi reagent prepared after the method of Roche et al. (1957).
X-ray film was purchased from Ansco, catalog # 1405-009. The
radioautograms were prepared by stapling the x-ray film to the
developed dry chromatogram in the dark. After a suitable
period of setting under a weight in the dark, the x-ray film
was developed and the exposed areas on the film compared with
the areas on the chromatogram which may have reacted posi-
tively to one or more of the spray reagents.

Analytical procedures

1. Ammonia was liberated from solution with a saturated
$\text{K}_2\text{CO}_3$ solution and absorbed with $\text{H}_2\text{SO}_4$ in a microdiffusion
vial similar to that described by Segilson and Segilson (1951).
The absorbed ammonia was determined colorimetrically with
Nessler’s reagent in a Klett-Summerson colorimeter with a #50
filter.

2. Urea was estimated colorimetrically in the Klett-Summerson
colorimeter with a #54 filter on a sample of de-proteinized
solution according to the method of Archibald (1945) as modi-
fied by Ratner (1955) using PPO.

Urea was also, on occasion, determined indirectly by de-
composing the urea with urease and measuring ammonia formation
or by the difference in color development (as above) before
and after urease treatment.

In the radioisotope tracer experiments, ($^{14}\text{C}$)-urea was
isolated by co-precipitation with carrier urea as the di-
xanthylurea derivative (DXU) after the method of Greenberg
(1955). After addition of 9-xanthenol to the urea solution in 50% acetic acid, the precipitated DXU was washed with methanol, methanol and water, then crystalized from hot pyridine (constant boiling, 115°C) several times to a constant specific radioactivity as described by Sporn et al. (1959). The DXU decomposed, turned brown, at 261-267°C with slow heating on a Fisher Sci. Co. melting point block (uncorr.). Higher decomposition temperatures were obtained with more rapid heating or if the pyridine was not completely removed by a methanol wash. 9-Xanthenol was purified by the method of Greenberg (1955) before use. The DXU was determined colorimetrically (Engel and Engel, 1947). Samples of the dissolved DXU in pyridine were plated on flat aluminum planchets and the radioactivity counted in the gas-flow counter.

\(^{14}\text{C})\)-urea was also determined by trapping the CO\textsubscript{2} evolved after urease treatment in NaOH as described previously.

3. 

Citrulline was estimated colorimetrically in a Coleman Jr. Spectrophotometer at 490 nm in samples of de-proteinized solutions by the method of Archibald (1944) as modified by Ratner (1955) using BMO.

Arzenolysis of citrulline, to test for radiocarbon labeling of the ureido-carbon, was accomplished by a modification of the method of Reichard (1957) with purified rat liver OTCase and arsenate. Citrulline was incubated in 150 mM
arsenate, pH 5.8, with OTCase in a Warburg vessel. The reaction was stopped by the addition of H$_2$SO$_4$ from the side arm and the CO$_2$ evolved was trapped in the NaOH in the center well. The degree of arsenolysis was determined by the citrulline disappearance during arsenolysis and the specific radioactivity of the label in the ureido-carbon of citrulline calculated from the radioactivity in the carbonate. Non-radioactive citrulline was arsenolized in the presence of 1000 to 2000 CPM each of 1-(14C)-L-alanine, 1-(14C)-L-aspartate, and 1-(14C)-L-glutamate; no liberation of 14CO$_2$ was detected.


5. Arginine was determined colorimetrically on a sample of a de-proteinized solution by the method of Sakaguchi as modified by Wu (1959). Tris and imidazole were avoided in the solution when arginine was to be determined because they interfered with the color development. Samples to be analyzed were neutralized to pH 7 against pHydriion paper before estimation of the arginine content.

6. Protein content of the tissue preparations was estimated by the procedure of Lowry et al., (1951) using crystalline
serum albumin (Armour and Co.) as a standard.

**Isolation of free amino acids, citrulline and carbamyl aspartate**

In some experiments, the incorporation of label from \(^{14} \text{C})\text{-HCO}_3^-\) into citrulline and carbamyl aspartate was investigated. After completion of the incubation, the stopped reaction mixture was extracted with 4 volumes of ethanol. The precipitated protein and cellular debris were removed by centrifugation. The ethanolic supernatant fluid was extracted with 3 volumes of chloroform in a separatory funnel and the aqueous layer collected after 18 hours. The chloroform-ethanol layer was washed with 0.001 M HCl and the aqueous layer combined with the former aqueous layer. The combined aqueous layers were evaporated to dryness, and the residue was dissolved in a small volume of water.

In the case of citrulline, the aqueous layer was passed through a Dowex-50W-12 (H\(^+\)) column. The column was washed to pH 5 with water and the amino acids were eluted with 2 N ammonia water. This eluate was evaporated to dryness and chromatographed or arslenolyzed for the identification of citrulline.

In the case of carbamyl aspartate, the aqueous layer was passed through a Dowex 2x-8 (formate) column and the acids eluted with formic acid after the method of Reichard and Hanshoff (1956). The eluate was evaporated to dryness, then
chromatographed for the identification and further isolation of carbamyl aspartate.

**Protein hydrolysis and arginine isolation**

In some experiments, the incorporation of the radiocarbon label into the protein arginine molecule was investigated. Upon completion of the experiment, the tissue was blended in 5 volumes of cold 5% TCA for 5 min. in a Serval Omnimix, then homogenized. The protein was isolated according to the method of Seikovitz (1952) using hot and cold TCA extractions and hot and cold ethanol-ether extractions, then dried. A 100 mg portion was hydrolyzed in 5 ml of 6 N HCl (constant boiling) by heating in a sealed tube for 24 hours at 117°C. The amino acid solution was cooled, the tube broken open, and 10 mg of arginine-HCl added. The solution was decolorized by treatment with 50 mg of activated charcoal (Darco) and filtered quantitatively through Whatman #5 filter paper. The filtrate was evaporated to dryness under infrared lamps and the residue dissolved in 10 ml of water. The arginine was precipitated from a 7.5 ml portion with an excess of flavianic acid (Greenstein and Winitz, 1961). Upon standing at room temperature, fine needle-like yellow crystals of arginine monoflavianate formed and were collected by centrifugation. Flat, orange, scale-like crystals of the arginine diflavianate formed after the first of three washings with 0.5% flavianic acid.
The crystals were washed twice with 95% ethanol and re-crystallized twice from 1 M ammonia water. The crystals were then washed again with ethanol, dissolved in ammonia water, and the flavianate decomposed by treatment with Dowex 2X-8 (Cl⁻) as described by Tamir and Ratner (1963b). The clear eluate was evaporated to dryness and the residue dissolved in 2.5 ml of water. Chromatograms of the eluate revealed a major and minor Sakaguchi positive spot. All the radioactivity was located in the major Sakaguchi positive spot which had the same Rf as arginine when the chromatograms were developed in butanol-formic acid-water and lutidine-water (ascending).

Enzyme assay procedures

1. Assays for enzymatic activity, in which the product formation or the substrate disappearance was measured colorimetrically, were executed in 12 ml conical centrifuge tubes. The reactions were terminated by the addition of 0.5 M or 1 M HClO₄ or 5% TCA (trichloroacetic acid), then the denatured protein removed by centrifugation. Radicisotope incorporation studies with cell-free extracts or tissue minces were executed in thick walled 25 ml or 50 ml Erlenmeyer flasks. Temperatures for incubations were controlled by immersing incubation vessels in a temperature controlled water bath. The pH of the reagents (substrates) was adjusted by titrating to the correct pH on a Beckman Zeromatic (Beckman Inst. Co.) with
a glass electrode (no correction for ionic strength). The pH of the reaction mixtures was checked on a Beckman G pH meter when the sensitivity of an enzyme activity to pH was determined. All substrates were adjusted to the pH of the buffer used in the reaction. Reaction mixtures and conditions peculiar to the assaying of each of the enzyme activities are described with the results.

2. Supplementary purified enzymes were included in a number of assays for enzymatic activity in *Lumbricus*. Beef liver arginase was purchased from Sigma Chem. Co. and assayed at 20 umoles/mg/hr. Jack bean urease (Type II) was purchased from Sigma Chem. Co. Ornithine transcarbamylase was purified from rat liver by the method of Caravaca and Grisolia (1960) and "fraction III" used. A 50-75% (NH$_4$)$_2$SO$_4$ fraction from rabbit muscle extract was prepared according to Racker (1947) and used with 3-phosphoglycerate as an ATP generating activity (pyruvic kinase). Aspartate transcarbamylase was prepared from *E. coli* after the method of Gerhart and Pardee (1962).

Specific activity with respect to enzymatic activity refers to synthesis as umoles/mg protein/hr.
RESULTS

I. **Biochemical Studies.**

A. **Studies in vivo or with cellular preparations.**

The experiment of Cohen and Lewis (1949) and Needham (1957) demonstrating increased urea production by *Lumbricus* with starvation were repeated and the findings of these authors confirmed qualitatively (Figure 15). The incorporation of label from $^{14}C$ labeled precursors into citrulline, arginine, and urea was investigated to test the occurrence and the labeling of the intermediates of the ornithine cycle in normally metabolizing worm tissue.

Table 1 demonstrates the incorporation of label from $^{14}C$-HCO$_3^-$ into the ureido-carbon of citrulline and into urea by a gut tissue preparation. *Lumbricus* gut tissue (500 mg) was incubated at 25$^\circ$C for varying time periods in 5 ml of saline containing, as mM: NaCl, 125; KCl, 2.7; CaCl$_2$, 1.8; MgCl$_2$, 0.4; NaHCO$_3$, 1; Na$_2$SO$_4$, 0.4; NaH$_2$PO$_4$, 1; (NH$_4$)$_2$SO$_4$, 1; L-ornithine, 2; and sodium succinate, 5; all at pH 7.5. The saline was sterilized by filtration and 0.7 mg penicillin G and 0.5 mg dihydrostreptomycin sulfate were added per ml. The reaction was started by the addition of $9.9 \times 10^5$ CPM of $^{14}C$-NaHCO$_3$ to each flask and stopped by the addition of 1 N H$_2$SO$_4$. The tissue debris and precipitated protein were neutralized to pH 7.0 and transferred to a closed tube, when
treated with 10 mg urease. The CO₂ evolved was trapped in NaOH and the radioactivity in the carbonate counted. After removal of the CO₂, 25 μmoles of L-citrulline were added to each vessel and the amino acids in each vessel isolated in the eluate from the Dowex 50W-12 column. The eluate was evaporated to dryness and the residues dissolved in a small volume of water. The solution was subjected to arsenolysis, the CO₂ evolved was trapped in NaOH and the radioactivity in the carbonate counted. The degree of arsenolysis was determined by measuring citrulline disappearance.

A significant incorporation of (¹⁴C)-HCO₃⁻ carbon occurs in both the ureido-carbon of citrulline and urea (Table 1). The incorporation into urea increases with time while the labeling of the ureido-carbon of citrulline decreases after the first hour. The incorporation was eliminated by boiling the tissue. The activity is present in the tissue rather than in the tissue wash as seen in Control 3 and Experimental 4 (Table 1). Control 3 was prepared by pre-incubating the tissue for one hour in 5 ml of saline less the (¹⁴C)-HCO₃⁻, then centrifuging the preparation gently (800G for 10 min.) in the cold. The supernatant fluid was incubated with (¹⁴C)-HCO₃⁻ as Control 3 while the tissue residue was resuspended in 5 ml saline and incubated with (¹⁴C)-HCO₃⁻ as Experimental 4. The greater incorporation of radiocarbon into urea and citrulline after 3 hours with the pre-incubated tissue (Experimental 4)
### Table 1

Incorporation of $(^{14}\text{C})$-HCO$_3$ label into citrulline and urea by *Lumbricus* gut tissue

<table>
<thead>
<tr>
<th>Flasks and Contents</th>
<th>Incubation time, hr.</th>
<th>CPM (BaCO$_3$) after treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Urease</td>
</tr>
<tr>
<td><strong>Controls:</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1. Complete</td>
<td>9</td>
<td>1.8</td>
</tr>
<tr>
<td>2. Complete (boiled tissue)</td>
<td>2</td>
<td>3.9</td>
</tr>
<tr>
<td>3. Supernatant saline*</td>
<td>3</td>
<td>4.9</td>
</tr>
<tr>
<td><strong>Experimental:</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4. Resuspended tissue (complete)*</td>
<td>3</td>
<td>414.4</td>
</tr>
<tr>
<td>5. Complete</td>
<td>1</td>
<td>125.5</td>
</tr>
<tr>
<td>6. Complete</td>
<td>1</td>
<td>169.1</td>
</tr>
<tr>
<td>7. Complete</td>
<td>3</td>
<td>190.0</td>
</tr>
</tbody>
</table>

*See text for explanation.

Urease treatment and arsenolysis of citrulline described in Methods section.
suggests that ornithine permeability limited citrulline and urea biosynthesis in this experiment.

Because of the results in Table 1, the labeling of protein arginine by injecting specifically labeled precursors was investigated (Table 2). The precursors were injected into the anterior body region of whole worms with a #27 needle and the worm kept moist in funnels covered with watch glasses. After 24 hours at room temperature (24-25°C), the worms were rinsed thoroughly with distilled water, cooled on crushed ice, any gut contents dissected out, and the worms cut into pieces. The pieces were blended in 4 volumes of cold 5% TCA, then homogenized. The protein was isolated, hydrolyzed, and the arginine isolated by precipitation with flavianic acid. The isolated arginine was dissolved in a small volume of water. One portion was plated on a planchet and the radioactivity counted. Another portion was spotted on Whatman 3mm paper and subjected to ascending chromatography, first in butanol-formic acid-water, then in lutidine-water. The dried chromatogram was placed on x-ray film for eight weeks. A third portion was added to a reaction mixture in a Warburg flask containing 50 units of arginase, 5 mg of urease, 50 μmoles of Tris-H₂SO₄ (pH 8), and 2.5 μmoles of CoCl₂ in 1.55 ml. The reaction was stopped after 3 hours at 38°C by tipping 0.45 ml of 5 N H₂SO₄ into the mixture from the side-arm. The CO₂ evolved was
Table 2
Incorporation of radiocarbon from labeled precursors into protein arginine

<table>
<thead>
<tr>
<th>Compound injected (CPM/g worm/10^5)</th>
<th>Worm wet wt. condition</th>
<th>CPM in arginine/100 mg protein</th>
<th>CPM in amidine-C of arginine from 100 mg protein</th>
<th>Incorporation Ratio*</th>
</tr>
</thead>
<tbody>
<tr>
<td>5-(14C)-ornithine</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>a. (3.54)</td>
<td>Fed 8.5</td>
<td>23,000</td>
<td>282</td>
<td>0.055</td>
</tr>
<tr>
<td>b. (3.87)</td>
<td>Starv. 7.75</td>
<td>17,500</td>
<td>110</td>
<td>0.038</td>
</tr>
<tr>
<td>Ureido-(14C)-citruline</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>a. (6.08)</td>
<td>Fed 7.85</td>
<td>24,500</td>
<td>22,767</td>
<td>0.034</td>
</tr>
<tr>
<td>b. (2.42)</td>
<td>Starv. 5.9</td>
<td>9,000</td>
<td>10,781</td>
<td>0.035</td>
</tr>
<tr>
<td>(14C)-NaHCO3</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>a. (4.02)</td>
<td>Fed 7.85</td>
<td>740</td>
<td>1,495</td>
<td>0.0016</td>
</tr>
<tr>
<td>b. (4.12)</td>
<td>Starv. 7.65</td>
<td>3,150</td>
<td>4,151</td>
<td>0.0065</td>
</tr>
</tbody>
</table>

*(CPM incorp./umole arg.) (umole arg./g worm)/(CPM injected/g worm) = ratio of isotope incorporation on a CPM/g worm basis.

Values in column where arginine was counted directly were not corrected for self-absorption while the values for the counts in the amidine-C are corrected for self-absorption.

After the urease-arginase treatment, only the ornithine experimentals retained radioactivity in the urease-arginase reaction mixture.

Procedure for isolation of arginine is described in the Methods section.
trapped in NaOH in the center well and the radioactivity in the carbonate counted. Arginine was measured at various stages to check recovery and to determine the degree of arginine decomposition by the arginase-urease treatment; all was decomposed.

Label from all three precursors was incorporated into the protein arginine (Table 2). After arginase-urease treatment, all the label incorporated into arginine from the ureido-carbon of citrulline and from the bicarbonate appeared in the amidine-carbon of the arginine molecule while little or none of the radiocarbon from the 5-(14C)-ornithine was evident in the amidine-carbon. The radioautogram revealed a single exposed region corresponding to the large Sakaguchi positive area on the chromatogram. Incorporation of carbon from HCO3- is enhanced while incorporation from citrulline and ornithine remains fairly constant or falls during starvation (Incorporation ratio in Table 2).

Foster et al. (1939) fed rats the then available (N15)-NH4Cl and found all the isotope incorporated into protein arginine was situated in the amidine moiety and none in the ornithine moiety. Clutton et al. (1940) found all deuterium labeled DL-ornithine injected into rats that was incorporated into protein arginine was located in the ornithine moiety of arginine. Delleuva and Wilson (1946) found that more label from 14CO2 is assimilated into the amidine-carbon of protein arginine
than into the 1-carboxyl carbon of protein glutamate or protein aspartate in rats. Swick and Handa (1956) found most of the label from $^{14}$CO$_2$ that was incorporated into protein arginine of rats was situated in the amidine-carbon rather than the carboxyl-carbon. Evans and Slotin (1940), in rat liver slice experiments with ($^{13}$C)-HCO$_3^-$, found an appreciable incorporation of label into the urea carbon. The same pattern of labeling has been established for _E. coli_ with chromatographic-radioautographic methods (Roberts et al., 1953). All these studies have established the transfer of carbon from bicarbonate to the amidine carbon of arginine and the incorporation of ornithine into the ornithine moiety of arginine in mammals and microorganisms. Data presented in Table 2 establishes the same pattern for _Lumbricus_.

The incorporation of label from ureido-($^{14}$C)-citrulline into protein arginine has been established for chicks (Tamir and Ratner, 1963b) and for rats and mammalian cells in tissue culture (Schimke, 1964). This conversion is established for _Lumbricus_ by the data in Table 2.

The identity of citrulline as an intermediate in arginine and urea biosynthesis was confirmed by Grisolia and Cohen (1948) when they demonstrated the labeling of the ureido-carbon by label from $^{14}$CO$_2$ in rats. The identity of citrulline as an intermediate for urea and arginine biosynthesis
is established for *Lumbricus* by the data in Tables 1 and 2.

The isolated protein from *Lumbricus* amounted to 70-80 mg of dry protein per gram wet weight of worm. The arginine content of the protein was 7.39% in fed worms and 8.93% in starved worms (Sakaguchi positive material in the protein hydrosates).

By combining the information in Tables 1 and 2, the biosynthesis of arginine and urea by a pathway similar to that found in mammals seemed a reasonable postulate. Experiments were undertaken to demonstrate the individual enzymatic reactions responsible for the transfer of carbon from bicarbonate to urea in cell-free extracts of *Lumbricus* tissue.
B. Studies with cell-free tissue extracts.

(1) Reaction I, citrulline biosynthesis.

Synthesis of L-citrulline from L-ornithine, ammonium ion, and bicarbonate ion was first achieved in a cell-free extract of rat liver tissue by Cohen and Hayano (1946). The synthesis was shown to be dependent on the presence of ATP, Mg$^{2+}$, L-glutamate, and O$_2$ (Cohen and Hayano, 1948). Cohen and Hayano (1948) separated the citrulline synthesizing activity from the citrulline utilizing activity by subjecting a 0.154 M KCL homogenate to centrifugation; citrulline synthesis was restricted to the residue while the citrulline to arginine to urea converting activity was localized in the supernatant fluid. Citrulline synthesis from ornithine and urea synthesis from citrulline was much faster in the fortified homogenates than in the tissue slice incubations suggesting that ornithine and citrulline permeability limited citrulline and urea synthesis respectively in the tissue slice studies (Cohen and Hayano, 1946).

Cohen and Grisolia (1948) found a two-to three-fold stimulation of citrulline synthesis from ornithine by adding N-carbamyl-L-glutamate to the reaction mixture containing the rat liver extract. They postulated N-carbamyl-L-glutamate (CG) as an intermediate compound in citrulline synthesis from ornithine, ammonia, and bicarbonate. Krebs (1936) had suggested a two-step citrulline synthetic reaction and postulated
carbamino-ornithine as an intermediate compound. Growth studies with *Neurospora crassa* by Srb and Horowitz (1944) had also suggested a two-step synthesis of citrulline from ornithine. Grisolia et al. (1951) were unable to demonstrate the actual labeling of CG from \(^{14}\text{C})\text{-HCO}_3^-\) and postulated that CG was active as a co-factor rather than as an intermediate in the reaction. Grisolia and Cohen (1953) tested many N-glutamyl substituted compounds and found N-acetyl-L-glutamate (AG) to be more active than CG. Grisolia and Cohen (1952) postulated the existence of an unstable intermediate in citrulline synthesis which accumulated when the reaction mixture was pre-incubated in the absence of ornithine; with ornithine addition, citrulline synthesis increased linearly as a function of the pre-incubation time period. Synthesis of the intermediate compound was dependent on the presence of ATP, \(\text{NH}_4^+\), \(\text{HCO}_3^-\), CG, and the rat liver enzyme. The initial reaction was a synthesis of the intermediate, compound X, while the second step was a reaction of compound X with ornithine to form citrulline. Compound X was precipitated as the tricyclohexylamine salt and partially characterized (Grisolia and Marshall, 1955).

Jones et al. (1955) synthesized carbamyl phosphate from isocyanate and phosphate then demonstrated its activity in the formation of citrulline when incubated with ornithine and an extract of rat liver mitochondria. The identity of compound
X as equivalent to carbamyl phosphate was established by Hall and Cohen (1957). The superiority of AG as a co-factor in the synthesis of compound X and carbamyl phosphate was established by Marshall et al. (1955).

The mammalian carbamyl phosphate synthesizing activity was quite easily denatured during purification. Marshall et al. (1958) purified the more stable activity from frog liver (Rana catesbiana). The activity, carbamyl phosphate synthetase, requires a divalent cation and AG as co-factors, uses two moles of ATP per mole of carbamyl phosphate formed, and has a high sensitivity to ammonium ion (K_m 0.7mM) (Cohen, 1962). The synthetase activity in the frog can use Mn^{2+}, Mg^{2+}, Co^{2+}, and Ni^{2+} as co-factors but the latter two are not as active as the former (Metzenberg et al., 1961). Mn^{2+} has a much higher affinity for ATP and the enzyme than Mg^{2+}, thereby lowering the K_m for ATP below that found when Mg^{2+} is used as a co-factor. The K_m for AG (1.3 mM) is also lower with Mn^{2+} than with Mg^{2+}. The maximal activity was obtained at 5 mM ATP and 5 mM MnCl_2; MnCl_2 concentrations greater than the ATP concentration were inhibitory while ATP concentrations greater than the MnCl_2 concentration did not enhance the activity (Caravaca and Grisolia, 1960; Marshall et al., 1961). The maximal activity obtained under optimal conditions with Mg^{2+} and ATP was about 75% of the activity under optimal conditions with Mn^{2+} and ATP.
The mechanism of the synthetase reaction is obscure in that the role of AG has not been elucidated. Metzenberg et al. (1961) and Marshall et al. (1958) have suggested a stimulation or "activating" effect on both steps of a two-step reaction: (a) the formation of an "active $\text{CO}_2$" and (b) the reaction of this "active $\text{CO}_2$" with ATP then ammonium ion to form ADP and carbamyl phosphate. One mole of ATP is hydrolyzed in the formation of the "active $\text{CO}_2$" while the other is used essentially in a phosphorylation reaction. The second step is reversible in that one mole of carbamyl phosphate in the presence of the enzyme and $\text{Mg}^{2+}$ will yield one mole of ATP. Jones and Lipmann (1960) have suggested a more direct participation of AG in which the "active $\text{CO}_2$" is attached to the N inside the actyl group, then the complex reacts with ATP and ammonium ion. Recently, Schooler et al. (1963) have been able to obtain activity by replacing the AG with 2-acetoxyglutarate. These data and earlier data on N-glutamyl substituted derivatives apparently casts some doubt on the mechanism proposed by Jones and Lipmann (1960).

Carbamyl phosphate synthesis in the mushroom, *Agaricus bisporus* utilizes the amide group of glutamine as the amino donor rather than ammonium ion (Levenberg, 1962). The activity requires ATP, $\text{HCO}_3^-$, and $\text{Mg}^{2+}$ as a co-factor but has no AG requirement and has not been purified. A similar activity has been described in mutant strains of *E. coli*
(Prerard and Wiame, 1964).

In most microorganisms, the synthesis of carbamyl phosphate involves the fixing of $\text{HCO}_3^-$ in the presence of high $\text{NH}_4^+$ concentrations ($K_m$ 67 mM) and ATP. The small amount of spontaneously formed carbamion is phosphorylated by a transfer of phosphate from ATP to form ADP and carbamyl phosphate. The reaction requires $\text{Mg}^{++}$ as a co-factor, is completely reversible, proceeds optimally at pH 8.5, and is referred to as a carbamyl phosphokinase (Grisolia et al., 1962; Cohen, 1962).

Carbamyl phosphate is a precursor in the biosynthesis of several compounds of biological importance (Jones, 1963). With respect to the ornithine cycle, it reacts with L-ornithine in the presence of ornithine transcarbamylase to form L-citrulline (Reichard, 1957; Burnett and Cohen, 1957). It serves as a precursor to pyrimidine biosynthesis in that it reacts with L-aspartate in the presence of aspartic transcarbamylase to form carbamyl-L-aspartate (Reichard and Hanshoff, 1956; Lowenstein and Cohen, 1956). Recently, Bojanowski et al. (1964) have described the reaction of oxamate with carbamyl phosphate to form carbamyl oxamate in the presence of oxamic transcarbamylase from Streptococcus allantoicus. Jones (1963) also reports a personal communication from Lynen stating that carbamyl phosphate is a precursor in the synthesis of the biotin molecule.
Table 3

Sensitivity of the carbamyl phosphate synthesizing activity in *Lumbricus* gut tissue extracts to various homogenizing solutions

<table>
<thead>
<tr>
<th>Homogenizing solution</th>
<th>Net citrulline synthesis: umoles/ml extract/15 min.</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.38 M mannitol</td>
<td>0.20</td>
</tr>
<tr>
<td>0.38 M mannitol, 0.025 M K₂SO₄</td>
<td>0.28</td>
</tr>
<tr>
<td>0.38 M mannitol, 0.02 M MgSO₄</td>
<td>0.11</td>
</tr>
<tr>
<td>0.38 M mannitol, 0.002 M ATP</td>
<td>0.10</td>
</tr>
<tr>
<td>0.38 M mannitol, 0.005 M N-acetyl-L-glutamate</td>
<td>0.06</td>
</tr>
<tr>
<td>0.154 M KCl</td>
<td>0.10</td>
</tr>
<tr>
<td>0.1% CTAB, 0.002 M ATP, 0.05 M K₂SO₄</td>
<td>0.17</td>
</tr>
<tr>
<td>Distilled water</td>
<td>0.12</td>
</tr>
</tbody>
</table>

Assay: reaction mixture, 1 ml, ingredients in umoles: KHCO₃ 10; N-acetyl-L-glutamate 10; MgSO₄ 10; ATP 5; K-phosphoglycerate 10; NAD 1; L-ornithine 20; OTCase; rabbit muscle enzyme 0.3 mg; earthworm gut extract (homogenate) 0.3 ml; imidazole HCl, pH 7.8, 100.

Earthworm gut extract prep.: 25% homogenates prepared individually in the above solutions with tissue from a pool of worm guts. Worms starved one week.

Incubation temp: 30°C.

Reaction started by adding homogenate; stopped with 1 ml 1 M HC10₄.
(a) **Carbamyl phosphate synthesis.**

Carbamyl phosphate synthesis was measured indirectly as either citrulline or carbamyl aspartate when *Lumbricus* gut extracts were incubated with ATP, NH$_4^+$, and HCO$_3^-$ in the presence of added purified ornithine transcarbamylase (OTCase) and L-ornithine or aspartic transcarbamylase (ATCase) and L-aspar- tate. The method of assay was adapted from Brown and Cohen (1959). Conditions peculiar to each assay for enzymatic activity are described in the legends of the figures and tables.

Initial investigations indicated that the carbamyl phosphate synthesizing activity in *Lumbricus* gut was easily denatured. The sensitivity of the activity to various solutions used for homogenization is described in Table 3. Homogenization of the gut tissue in 0.38 M mannitol seemed to yield a fair amount of activity. Addition of K$_2$SO$_4$ to the mannitol solution further improved the yield while addition of MgSO$_4$, ATP, or AG depressed the recovered activity. Homogenization of the gut tissue in KCl, water, or a CTAB solution depressed the recovered activity. In other experiments, buffering the mannitol-K$_2$SO$_4$ solution with K-glycylglycine at pH 7.5 seemed to stabilize the activity. The activity was more stable if 5 mM MnSO$_4$ was included in the homogenizing solution. The gut tissue activities were extremely low if the gut tissue was homogenized in more than 4 ml of homogenizing solution per
gram of tissue. Attempts to isolate the activity by acetone or ammonium sulfate precipitation resulted in loss of activity. The combination of 0.4 M mannitol, 0.1 M K₂SO₄, and 0.015 M K-glycylglycine at pH 7.5 in a 25% homogenate was found to be most effective in stabilizing the activity for characterization. The instability of the worm gut activity seems similar to the instability reported for the dog liver activity (Metzenberg et al., 1957).

In an attempt to improve the specific activity, the formed elements of the tissue homogenate in the buffered-mannitol-salt solution were removed by centrifugation. Most of the activity was in the supernatant fluid from a 15,000G centrifugation (S-II) (Table 16) with a specific activity of 0.05 umoles/mg protein/hour.*

Because of the low activity observed, a clearer definition of the requirements for carbamyl phosphate synthesis was obtained by studying the incorporation of (¹⁴C)-HCO₃⁻ into citrulline (Figure 1). After completion of the incubation, 10 umoles of carrier citrulline were added and the amino acids collected in the eluate from the Dowex 50 W-12 (H⁺) column. The eluate was evaporated to dryness and the residue dissolved in 1 ml of water. One portion was arsenolyzed, the CO₂ evolved, trapped, and the radioactivity in the carbonate counted. Another portion

* The diverse variety of compounds present in some of the homogenizing solutions of some experiments was part of an initial attempt to find solutions which would stabilize the easily denatured activity.
was spotted on Whatman 3 mm paper. The paper was subjected to ascending chromatography two times in butanol-formic acid-water in one dimension and one time in ethanol-ammonia-water in the other dimension. The dried chromatogram was placed on x-ray film for three months. The single exposed area on the radioautogram corresponded to the p-dimethylaminobenzaldehyde spot on the chromatogram having the same $R_f$ as citrulline. The specific radioactivity of the citrulline formed was calculated from the counts in the carbonate and the citrulline disappearance due to arsenolysis.

Citrulline synthesis increased linearly with time in the complete assay system (Figure 1). Omission of ATP or addition of EDTA completely depressed the activity. In both controls, boiled extract and zero time incubations, there was no incorporation of radioactivity and the carbonate counted at background. Omission of the ATP generating system (rabbit muscle enzyme, 3-phosphoglycerate, NAD) depressed incorporation of the activity 70% in 30 minutes. In an attempt to establish ammonium ion dependence, the OTCase was dialyzed before use and ammonium ion omitted from the reaction mixture; 55% of the activity still prevailed due to the ammonium ion content of the rabbit muscle preparation (3 umoles). The experiment established ATP and divalent ion dependency for maximal activity, suggested a high affinity for ammonium ion by the activity, and described the linear increase in
Figure 1

The incorporation of radiocarbon from $^{14}\text{C}-\text{HCO}_3^-$ into the ureido-carbon of citrulline by cell-free extracts of *Lumbricus* gut tissue.

Assay: reaction mixture in 5 ml, ingredients in umoles: L-ornithine 37.5; N-acetyl-L-glutamate 50; Imidazole-HCl, pH 7.8, 500; ATP 25; ($\text{NH}_4)_2\text{SO}_4$ 50; K-phosphoglycerate; $^{14}\text{C}-\text{NaHCO}_3$ (1 ycurie) 50; rabbit muscle enzyme 1.5 mg; dialyzed OTCase;

Reaction started by adding 1 ml of gut enzyme extract, stopped with 5 ml 5% TCA.

Gut extract prep.: 25% homogenate in 0.4 M mannitol, 2.5 mM MnSO$_4$, 5 mM MgSO$_4$, 2.5 mM N-acetyl-L-glutamate, 0.1 mM ATP, 35 mM K$_2$SO$_4$, pH 7.4, was centrifuged at 6000G for 10 min. The supernatant was used as the enzyme source (extract) in the assay.

Incubation temp. 28$^\circ$C.

- Complete system
- Less ($\text{NH}_4)_2\text{SO}_4$
- Less ATP
- Less ATP generating system
- Plus EDTA
incorporation of label from $^{14}\text{C}}^{-}\text{HCO}_3^-$ into the ureido-carbon of citrulline with time.

Table 4 best illustrates the requirements for maximal activity. Upon termination of the incubations, the amino acid isolation was carried to the aqueous layer following ethanol-chloroform partitioning. In the case of citrulline isolation, the aqueous extract was streaked on Whatman 3 mm paper and developed twice in butanol-formic acid-water. Radioautograms were made of the dry chromatograms and the radioactive citrulline was identified by comparing the exposed area on the radioautogram with the $p$-dimethylaminobenzaldehyde area of the chromatogram. The citrulline was eluted from the paper, the citrulline content was determined, samples were plated on flat aluminum planchets, and the radioactivity was determined. In the case of carbamyl aspartate, the aqueous extract was eluted from the Dowex 2X-8 (formate) column and the eluate streaked on Whatman 3 mm paper. The chromatogram was developed twice in butanol-formic acid-water and a radioautogram of the dried chromatogram prepared. The exposed area of the radioautogram was compared to the $p$-dimethylaminobenzaldehyde positive area of the chromatogram which gave a negative ninhydrin test. The carbamyl aspartate was eluted from the paper and the specific radioactivity determined. The incorporation into carbamyl aspartate and into citrulline were separate experiments with
Table 4

Incorporation of $^{14}$C-HCO$_3^-$ label into citrulline and carbamyl aspartate by cell-free extracts of Lumbricus gut tissue.

<table>
<thead>
<tr>
<th>Assay modification</th>
<th>Incubation time (min.)</th>
<th>Total incorporation of label in CPM:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>citrulline</td>
</tr>
<tr>
<td>None</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>None</td>
<td>15</td>
<td>18,250</td>
</tr>
<tr>
<td>None</td>
<td>30</td>
<td>38,200</td>
</tr>
<tr>
<td>None (boiled extract)</td>
<td>30</td>
<td>97</td>
</tr>
<tr>
<td>Less ATP</td>
<td>30</td>
<td>108</td>
</tr>
<tr>
<td>Less ATP generating system</td>
<td>30</td>
<td>-</td>
</tr>
<tr>
<td>Less N-acetyl-L-glutamate</td>
<td>30</td>
<td>795</td>
</tr>
<tr>
<td>Less MnSO$_4$</td>
<td>30</td>
<td>1,265</td>
</tr>
<tr>
<td>Less MnSO$_4$, plus MgSO$_4$ (50 umoles)</td>
<td>30</td>
<td>2,330</td>
</tr>
<tr>
<td>Plus ZnCl$_2$ (25 umoles)</td>
<td>30</td>
<td>-</td>
</tr>
<tr>
<td>Plus avidin (1.25 units)</td>
<td>30</td>
<td>-</td>
</tr>
</tbody>
</table>

Assay: (a) Incorporation into citrulline, reaction mixture in umoles: NaH$^{14}$CO$_3$ (100,000 CPM/umole) 50; (NH$_4$)$_2$SO$_4$ 20; MnSO$_4$ 50; imidazole HCl, pH 7.9, 500; L-ornithine 70; N-acetyl-L-glutamate 50; ATP 25; 3-phosphoglycerate 30; NAD 5; rabbit muscle enzyme 0.9 mg; OTCase; earthworm gut extract 1.0 ml; reaction volume 5.0 ml.

(b) Incorporation into carbamyl aspartate reaction mixture same as for citrulline except: NaH$^{14}$CO$_3$ (10,000 CPM/umole) 50; (NH$_4$)$_2$SO$_4$ 10; Tris HCl, pH 7.8, 250; MnSO$_4$ 25; ATP 25; K-aspartate 50; N-acetyl-L-glutamate 25; ATCase; earthworm gut extract 1.0 ml; omit OTCase and ornithine; reaction volume 5.0 ml.

Extract preparation: Supernatant from 25% homogenate of gut tissue in 0.4 M mannitol, 0.1 M K$_2$SO$_4$, 0.015 M K-glycylglycine, pH 7.5, centrifuged at 15,000G for 8 min. at 0°C (S-II fraction).

Gut tissue used in citrulline incorporation experiment from worms starved one week and in carbamyl aspartate incorporation experiment, from worms starved two weeks.

Reaction started by adding gut extract; stopped by adding 5 ml 5% TCA

Incubation temp., 30°C.

*CA" carbamyl aspartate
differing amounts of radioactivity and with differing sources of worm gut extract.

Maximal synthesis of citrulline and carbamyl aspartate were dependent upon the presence of ATP, an ATP generating system, MnSO₄, N-acetyl-L-glutamate, and an extract of worm gut tissue in the reaction mixture. Substitution of 50 μmoles of MgSO₄ for MnSO₄ gave double the activity without added divalent cations but less than 6% of the activity with MnSO₄. Avidin in low concentration inhibited about 40% and the addition of 5 mM ZnCl₂ completely abolished the activity. Since the incorporation of label from (¹⁴C)-HCO₃⁻ into citrulline and into carbamyl aspartate had the same requirements for maximal incorporation, the formation of both compounds is probably dependent on the synthesis of the same intermediate, carbamyl phosphate. Both ATCase and OTCase require carbamyl phosphate in the synthesis of carbamyl aspartate from aspartate and citrulline from ornithine respectively.

The basic requirements for the carbamyl phosphate synthesis activity have been established with the radiocarbon tracer method. A series of experiments was undertaken using the more rapid but less sensitive colorimetric method for determining citrulline formation (Figure 2, Tables 5, 6, 7).

Citrulline synthesis was linear with time for short time periods in the absence of an ATP generating system (Figure
Carbamyl phosphate synthesis by Lumbricus gut extracts with time in the absence of an ATP generating system.

Assay: see 2c, use S-II extract 0.3 ml.

Gut extract prep.: see 2c
Incubation temp. 28°C

Carbamyl phosphate synthesis by Lumbricus gut extracts with time in the absence of an ATP generating system.

Assay: see 2c, use S-I extract 0.3 ml.

Gut extract prep.: see 2c
Incubation temp. 28°C

Carbamyl phosphate synthesis by Lumbricus gut extracts with extract concentration in the absence of an ATP generating system.

Assay: reaction mixture in 1 ml, ingredients in μmoles: imidazole-HCl, pH 7.8, 50; NaHCO₃ 10; NH₄Cl 10; N-acetyl-L-glutamate 5; MnSO₄ 5; ATP 5; L-ornithine 20; OTCase; gut extract, S-I.

Gut extract prep.: 25% homogenate in 0.4 M mannitol, 0.1 M K₂SO₄, 0.015 M K-glycylglycine, pH 7.5 of gut tissue, centrifuged at 6000G for 10 min. (supernatant = S-I). S-I centrifuged at 60000G for 20 min. (supernatant = S-II).

Incubation temp. 28°C.
- Complete
- Boiled extract
Table 5
Requirements for carbamyl phosphate synthesizing activity in *Lumbricus* gut tissue homogenates.

<table>
<thead>
<tr>
<th>Assay modification</th>
<th>Net citrulline synthesis(\text{umoles/ml extract/40 min.})</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>0.46</td>
</tr>
<tr>
<td>Less ATP</td>
<td>0.23</td>
</tr>
<tr>
<td>Less OTCase</td>
<td>0.16</td>
</tr>
<tr>
<td>Less L-glutamine</td>
<td>0.46</td>
</tr>
<tr>
<td>None (boiled extract)</td>
<td>0.0</td>
</tr>
</tbody>
</table>

Assay. Reaction mixture 1 ml, ingredients in \(\text{umoles}:\) \(\text{NaHCO}_3, 50;\ \text{ATP} 5; \text{NH}_4\text{Cl}, 50; \text{MnCl}_2 10; \text{N-acetyl-L-glutamate} 10; \text{Imidazole HCl}, \text{pH} 7.8, 100; \text{L-glutamine} 10; \text{L-ornithine} 20; \text{OTCase}.

Earthworm gut extract prep.: 25% homogenate in solution of 3.5 \(\text{mM}\) \(\text{KCl}, 5 \text{mM} \text{K}_2\text{SO}_4, 1 \text{mM} \text{ATP}; 1 \text{mM} \text{N-acetyl-L-glutamate}, 2.5 \text{mM} \text{MgSO}_4; \) Non-starved worms.

Incubation temp. 28°C. Control, boiled extract. Start reaction by adding extract, stop reaction with \(\text{HClO}_4\).

---

Table 6
The effect of di-valent ions and \text{N-acetyl-L-glutamate} on citrulline synthesis in *Lumbricus* gut tissue extracts.

<table>
<thead>
<tr>
<th>Co-factor added (\text{umoles})</th>
<th>Net citrulline synthesis(\text{umoles/ml extract/20 min.})</th>
</tr>
</thead>
<tbody>
<tr>
<td>(\text{MnSO}_4) 10 (\text{MgSO}_4) acetylglutamate</td>
<td>10</td>
</tr>
<tr>
<td>10 - -</td>
<td>0.38</td>
</tr>
<tr>
<td>10 10 10</td>
<td>0.34</td>
</tr>
<tr>
<td>- 10 10</td>
<td>0.10</td>
</tr>
<tr>
<td>- - 10</td>
<td>0.0</td>
</tr>
</tbody>
</table>

Assay. Reaction mixture 1 ml, ingredients in \(\text{umoles}:\) \(\text{NaHCO}_3 10; (\text{NH}_4)\text{SO}_4 10;\ \text{ATP} 10; \text{L-ornithine} 10; \text{Imidazole-HCl, pH} 7.8, 50; \text{NAD} 1; \text{K-phosphoglycerate} 20; \text{rabbit muscle enzyme} 0.3 \text{mg}; \text{OTCase}; \text{worm gut extract} 0.25 \text{ml}.

Reaction started by adding extract; stopped by adding 1 ml \(1\ \text{M} \text{HClO}_4\).

Earthworm gut extract prep.: as in Table 4 (S-II).

Incubation temp. 30°C.
2a, 2b). The presence of mitochondria in the gut extract (S-I) seemed to enhance the net citrulline synthesis (Figure 2b). The mitochondria which probably have some ATP generating capacity are removed by the 6000G for 20 min. centrifugation.

Net citrulline synthesis at low extract concentrations (Figure 2c) in the absence of an ATP generating system was linear with extract concentration. In other experiments, linear synthesis with extract concentration was lost at or above 0.4 ml of the 25% homogenate of gut tissue in a 1 ml reaction mixture whether an ATP generating system was included or not.

Dependence on added OTCase for maximal activity and a lack of dependence is shown in Table 5. The lack of complete dependence on ATP may be attributed to the inclusion of some ATP in the homogenizing solution and the use of a crude homogenate as the enzyme source.

Table 6 describes the dependence on MnSO₄ and AG for maximal citrulline synthesis when citrulline is determined colorimetrically. Substitution of MgSO₄ for MnSO₄ under these circumstances (10 mM ATP) depressed the activity 75%.

When the pH of the incubation medium, buffered with 0.1 M K-glycylglycine, was varied, optimal activity for a 15 min. incubation was achieved at pH 7.9-8.0 with 50% of the activity at pH 7.5 and pH 8.5. With 0.1 M imidazole-HCl buffer, maximal activity for a 20 min. incubation was obtained between
Table 7a

Effect of various co-factors and inhibitors on carbamyl phosphate synthesis by an extract of *Lumbricus* gut tissue

<table>
<thead>
<tr>
<th>Sample Number</th>
<th>Additions to the reaction mixture</th>
<th>Net citrulline synthesis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>umoles</td>
<td>MnSO₄</td>
</tr>
<tr>
<td>A</td>
<td>12.5</td>
<td>-</td>
</tr>
<tr>
<td>B</td>
<td>12.5</td>
<td>10</td>
</tr>
<tr>
<td>C</td>
<td>25.0</td>
<td>10</td>
</tr>
<tr>
<td>D</td>
<td>12.5</td>
<td>20</td>
</tr>
<tr>
<td>E</td>
<td>12.5</td>
<td>-</td>
</tr>
<tr>
<td>F</td>
<td>12.5</td>
<td>-</td>
</tr>
<tr>
<td>G</td>
<td>12.5</td>
<td>-</td>
</tr>
<tr>
<td>H</td>
<td>12.5</td>
<td>-</td>
</tr>
<tr>
<td>I</td>
<td>12.5</td>
<td>-</td>
</tr>
<tr>
<td>J</td>
<td>-</td>
<td>10</td>
</tr>
<tr>
<td>K</td>
<td>-</td>
<td>10</td>
</tr>
<tr>
<td>L</td>
<td>12.5</td>
<td>10</td>
</tr>
<tr>
<td>M</td>
<td>12.5</td>
<td>10</td>
</tr>
<tr>
<td>N</td>
<td>-</td>
<td>10</td>
</tr>
</tbody>
</table>

Assay: reaction mixture in 1 ml, ingredients in umoles: L-ornithine 10; imidazole-HCl, pH 7.9, 50; KHCO₃ 10; (NH₄)₂SO₄ 5; ATP 5; K-phosphoglycerate 10; NAD 1; rabbit muscle enzyme 0.3 mg; OTCase; gut extract from S-I or S-II, 0.25 ml.

Control: complete reaction mixture with boiled extract for each variation on the table. Incubation time, 30 min. at 30°C.

*Lumbricus* gut extract prep.: 25% homogenate of *Lumbricus* gut tissue in 0.4 M mannitol, 0.1 M K₂SO₄, 0.015 M K-glycylglycine, pH 7.5; S-I = supernatant fluid from 6000G for 10 min. centrifugation. S-II = supernatant fluid from 10,000G for 15 min. centrifugation, Mito. = mitochondria or 10,000G for 15 min. centrifugation residue from 4 g tissue in a 2 ml volume of fluid. Gut tissue from worms starved three weeks on filter paper.
pH 7.8 and pH 8.05 with 50% of the activity at pH 6.8. The dependence of maximal activity on pH could not be established in the absence of an ATP generating system between pH 6.8 and pH 8.4 with 30 min. incubations in Tris HCl.

Two experiments were undertaken to test the sensitivity of the *Lumbricus* gut carbamyl phosphate synthesizing activity to the co-factors and some inhibitors (Tables 7a; 7b).

Increased concentration of MnSO₄ (Table 7a, A-D) to 4 times the ATP concentration depressed citrulline synthesis about 25%, while high AG concentrations had little effect on the activity. Increasing the MgSO₄ concentration (Table 7a, E-G) stimulated the low activity but to only 30% of the activity with MnSO₄. The addition of 2.5 mM ZnCl₂ depressed the activity 75% (H). Cobalt ions at 25 mM seemed to either inhibit the activity or have no activity as a co-factor (I). Experiments J-N were designed to test whether the mitochondria might possibly account for the lack of complete dependence of the gut activity on added AG in some cases. The mitochondria in the extract (S-I) (Figure 2a, 2b) may have some ATP generating capacity, but in these experiments (Table 7a), an ATP generating system was included in the reaction mixture during the assay. Substitution of mitochondria for AG did stimulate citrulline synthesis over that with added boiled mitochondria (J-K). Little carbamyl phosphate synthesizing
Table 7b

Effect of various co-factors and inhibitors on carbamyl phosphate synthesis by an extract of *Lumbricus* gut tissue

<table>
<thead>
<tr>
<th>Additions to the reaction mixture</th>
<th>Net citrulline synthesis</th>
</tr>
</thead>
<tbody>
<tr>
<td>umoles</td>
<td>units</td>
</tr>
<tr>
<td>MnSO₄, A⁰</td>
<td>avidin</td>
</tr>
<tr>
<td>10</td>
<td>-</td>
</tr>
<tr>
<td>-</td>
<td>10</td>
</tr>
<tr>
<td>2.5</td>
<td>10</td>
</tr>
<tr>
<td>5.</td>
<td>10</td>
</tr>
<tr>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>10</td>
<td>5.</td>
</tr>
<tr>
<td>10</td>
<td>2.5</td>
</tr>
<tr>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>10</td>
<td>10</td>
</tr>
</tbody>
</table>

Assay: as in Table 7a: substitute Tris HCl, pH 7.8, 50, for imidazole. Control: boiled extract. Gut tissue was from worms starved two weeks.
activity was associated with the mitochondria themselves (L). When the S-I fraction containing intact mitochondria was substituted for the S-II fraction (M, N), AG dependence was still evident. Comparing the carbamyl phosphate synthesizing activity of S-I(M) with that of S-II(B) indicates a 25% stimulation of the activity by removal of the mitochondria. The stimulating effect by substitution of mitochondria for AG was apparently due to a heat labile factor (J, K), but it is impossible to evaluate the data without more information on more purified preparations.

Maximal carbamyl phosphate synthesis was obtained at 2.5 mM AG and MnSO₄ (Table 7b). MnSO₄ concentrations greater than the ATP concentration seemed to inhibit the activity. Avidin inhibition at avidin concentrations greater than those in Table 4 inhibited the activity to a greater degree (Table 7b).

**Discussion of the carbamyl phosphate synthesizing activity.**

Citrulline synthesis from HCO₃⁻, NH₄⁺, ATP, ornithine and added OTCase has been demonstrated in *Lumbricus* gut tissue extracts. The heat labile activity requires MnSO₄ and N-acetyl-L-glutamate for maximal activity and operates maximally at low (10 mM) ammonium ion concentrations. Carbamyl aspartate synthesis from NH₄⁺, HCO₃⁻, ATP, aspartate, and added ATCase has been demonstrated in *Lumbricus* gut extract and has the same co-factor requirements as the citrulline synthesis.
These characteristics identify the activity as a carbamyl phosphate synthetase activity similar to the activity described from frog and mammalian liver tissue. The lack of glutamine dependence eliminates the activity described by Levenberg (1962) from discussion. The carbamyl phosphokinase activity is probably not operating because the Lumbricus activity has a high affinity for ammonium ion and requires AG for activity. Additional evidence for the operation of a synthetase rather than a kinase is found in the behavior of the activity in the presence of ZnCl₂. Caravaca and Grisolia (1960) found strong inhibition of the frog activity at very low ZnCl₂ concentrations (50% at 0.3 mM) while inhibition of the kinase was evident only above 5 mM Zn²⁺. The worm gut was completely inhibited by 5 mM ZnCl₂ (Table 4) while 75% inhibition was evident at 2.5 mM (Table 8b).

The major difference between the Lumbricus gut activity and the frog liver activity is the behavior with respect to Mn²⁺ and Mg²⁺. The Lumbricus gut activity has a much lower relative activity with MgSO₄ possibly indicating that MnSO₄ is the more suitable co-factor.

The inhibition of the activity with avidin was surprising because there is no report of a direct biotin dependency for either the kinase (Grisolia et al., 1962) or the synthetase (Cohen and Hayano, 1948). MacLeod et al.
(1949) found that the citrulline synthesizing activity in liver tissue from biotin deficient rats had one-half the activity per gram of tissue of that found in the controls (non-biotin deficient rats). However, there was no stimulation of citrulline synthesis by the biotin deficient rat liver tissue when 80 μgrams of biotin were added to the reaction mixture during assay. In an earlier experiment, MacLeod and Lardy (1949) found a six-fold depression of label incorporation from \(^{14}\text{C}\)-HCO\(_3\) \(^{-}\) into the amidine carbon of protein arginine in biotin deficient rats. Feldott and Lardy (1951) found that CG or high glutamate concentrations in the assay mixture tended to restore citrulline synthesis by the liver tissue extract in biotin deficient rats to that of the controls. They concluded that biotin was involved in the formation of CG which was then considered an intermediate compound in citrulline synthesis. More recent data since the purification of the carbamyl phosphate synthetase and the identification of the actual intermediate compound, carbamyl phosphate, is lacking. In order to evaluate the unexpected avidin inhibition of carbamyl phosphate synthesis in *Lumbricus* gut tissue in a proper manner, boiled avidin, serum albumin, and biotin addition control preparations should be included with the assay for inhibition of the enzyme activity.

These experiments are the first demonstration of a
carbamyl phosphate synthetase activity in an invertebrate animal. The *Lumbricus* gut activity varied from 1.5 to 2.5 in fed worms and 4 to 6.5 in starved worms (umoles/g tissue/hr. at 30°C).

(b) Ornithine transcarbamylase.

The ornithine transcarbamylase (OTCase) activity was assayed according to the method of Brown and Conen (1959) using *Lumbricus* tissue extracts. Citrulline synthesis was measured by reaction of commercially available carbamyl phosphate with L-ornithine in the presence of a cell-free *Lumbricus* tissue extract. CTAB (hexadecyltrimethylammonium bromide) extracts of worm tissue were prepared by homogenizing the tissue at room temperature in a 0.1% CTAB (aqueous) solution (9 ml of solution/g tissue). The homogenate was centrifuged at 0°C at 6000G for 20 min. and the supernatant fluid (*S*₁) saved. The residue was homogenized in 0.1% CTAB (10 ml/g), centrifuged, and the supernatant fluid (*S*₂) combined with *S*₁. The *S*₁-*S*₂ extract was used as the enzyme source and considered a 5% tissue extract.

Table 8 describes the OTCase activity in the *S*₁-*S*₂ extracts of both body wall and gut tissue. Since only 10 umoles of carbamyl phosphate were included in the assay, the extremely active gut activity converted about half of the carbamyl phosphate in the first 10 min. and 80% in the first
Table 8

Requirements of ornithine transcarbamylase activity in *Lumbricus* tissue extracts

<table>
<thead>
<tr>
<th>Assay modification</th>
<th>Time (min.)</th>
<th>Umoles citrulline/0.2 ml extract</th>
<th>gut</th>
<th>body wall</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>0</td>
<td>0.04</td>
<td>0.04</td>
<td>0.04</td>
</tr>
<tr>
<td>None</td>
<td>5</td>
<td>3.0</td>
<td>0.069</td>
<td>0.098</td>
</tr>
<tr>
<td>None</td>
<td>10</td>
<td>4.6</td>
<td>0.138</td>
<td>0.15</td>
</tr>
<tr>
<td>None</td>
<td>20</td>
<td>8.0</td>
<td>0.073</td>
<td>0.06</td>
</tr>
<tr>
<td>None</td>
<td>60</td>
<td>10.0</td>
<td>0.073</td>
<td>0.04</td>
</tr>
<tr>
<td>None (boiled extract)</td>
<td>60</td>
<td>0.11</td>
<td>0.073</td>
<td>0.04</td>
</tr>
<tr>
<td>Less L-ornithine</td>
<td>60</td>
<td>0.07</td>
<td>0.04</td>
<td>0.04</td>
</tr>
<tr>
<td>Less carbamyl phosphate</td>
<td>60</td>
<td>0.04</td>
<td>0.073</td>
<td>0.04</td>
</tr>
<tr>
<td>Less tissue extract</td>
<td>60</td>
<td>0.0</td>
<td>0.073</td>
<td>0.04</td>
</tr>
</tbody>
</table>

Assay: reaction mixture in 1 ml, ingredients in umoles: carbamyl phosphate, dL1, 10; L-ornithine 20; Tris HCl, pH 7.8, 100; 31-32 tissue extract (5% for gut tissue, 20% for body wall). 0.2 ml. Incubated in 12 ml conical centrifuge tubes.

Reaction started by adding carbamyl phosphate, stopped by adding 1 ml 1 M HClO4.

Incubation temp. 28°C.

Worms starved two weeks.
Figure 3

The ornithine transcarbamylase activity in a partially purified preparation from Lumbricus gut tissue extracts.

Assay: reaction mixture in 1 ml, ingredients in umoles: Tris-HCl, pH 7.8, 100; carbamyl phosphate 10; L-ornithine 20; 0.1 ml enzyme solution.

Reaction started by adding carbamyl phosphate, stopped with 1 ml of 1 M HClO₄.

Incubation temp. 33°C.

Control was a boiled enzyme incubation.
20 min. Citrulline synthesis is dependent on L-ornithine and carbamyl phosphate and is eliminated by boiling the extract. Under the conditions of Table 8, the activity in umoles synthesized per gram tissue per hr. was 1680 for the gut tissue and 29 for the body wall with reference to the five and ten min. incubations respectively. Considering that the gut tissue makes up 12.5% and the body wall 60% of the body weight, then the gut has better than ten times the citrulline synthetic capacity of the body wall.

A partially purified preparation was used for the characterization of the OCCase activity in *Lumbricus* gut tissue. The purification scheme is described in Table 9. A 5%, S₁-S₂ extract was brought to 50% saturation by adding an equal volume of saturated (NH₄)₂SO₄ solution, pH 5.5, with stirring over a 10 min. period at 0°C. The preparation was brought to 64°C by immersing the tube in a 64°C water bath and kept at 64°C for 5 minutes. The tube was removed, cooled to 0°C in an ice bucket, and kept for 2 hours. The tube was centrifuged at 10,000G for 15 min. and the supernatant fluid poured into another tube. An equal volume of saturated (NH₄)₂SO₄ solution, pH 7.4, was added to the supernatant bringing the saturation to 75%. The solution was kept at 0°C for 2 hours and centrifuged at 6000G for 15 min. The residue was dissolved in 2 ml of water and dialyzed against
against 0.01 M Tris HCl, pH 8.0, for 18 hours at 0°C with frequent change of the dialyzing fluid, then centrifuged. The supernatant fluid was passed through a 10 cm DEAE cellulose column (Medium mesh, 0.9 meq/g) which had been equilibrated against 0.01 M Tris HCl, pH 8.0. The column contents were washed with a retention volume of 0.01 M Tris HCl, pH 8.0 (Fraction 1), then with two retention volumes of 0.05 M K-phosphate, pH 7.4 (Fraction 2). Fraction 2 contained 80-90% of the activity originally put on the column. If K-glycylglycine was substituted for Tris HCl during dialysis and K₂SO₄ included in the dialyzing fluid, there was no loss of activity. The partial purification procedure (Table 9) yielded a 45-50 fold increase in the specific activity of the enzyme and a 20% recovery of the total activity. A two-fold purification of the DEAE cellulose column eluate (Fraction 2) was obtained with an acetone extraction but the per cent recovery was very low. Citrulline synthesis increased linearly with time for short time periods with a partially purified gut extract preparation (Figure 3).

The crude S₁-S₂ extract was heated for short time periods at various temperatures to obtain some indication of the heat stability of the Lumbricus gut OTCase activity (Table 10). After heating, the denatured protein was removed by centrifugation in the cold. The supernatant fluid was
Figure 4

Variation of ornithine transcarbamylase activity with the pH of the reaction mixture.

Assay: conditions same as in Figure 3.
Incubation temp. 28°C.
Control was a boiled extract incubation for each point.
assayed for OTCase activity and protein content. The heat stability is similar to that reported for the rat and ox liver activity (Caravaca and Grisolia, 1960; Burnett and Cohen, 1957; Reichard, 1957). The crude S1-S2 extract lost 50% of the OTCase activity with freezing while the activity was stable for months at -20°C when precipitated with (NH4)2SO4.

The pH for optimal OTCase activity (Figure 4) in 0.1 M Tris HCl was 7.6-7.7 which is similar to that reported for the rat and ox liver activities (Reichard, 1957; Burnett and Cohen, 1957) but lower than that reported for the cestode, Hymenolepis diminuta (Campbell, 1963) and the snail, Otala lactea (Linton and Campbell, 1962). The optimal pH for the worm gut OTCase was very much lower than that for the bacteria, Streptococcus lactus (Ravel et al., 1959) and Escherichia coli (Rogers and Novelli, 1962). When the activities in Tris HCl, imidazole-HCl, and K-glycylglycine were compared at pH 7.7, Tris seemed to inhibit about 40% and the values with K-glycylglycine were about 10% lower than with imidazole. The pH for optimal activity was pH 7.7 with 0.1 M imidazole-HCl and pH 7.9 with 0.1 M K-glycylglycine.

The activation energy, calculated from initial reaction velocities at 28°C, 33°C, and 38°C, was 13.63 x 10³ calories per mole in 0.1 M Tris HCl at pH 7.8. This value is
Table 9

Partial purification of the ornithine transcarbamylase activity from Lumbricus gut tissue

<table>
<thead>
<tr>
<th>Preparation</th>
<th>Total activity</th>
<th>Specific activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>5% S1-S2 extract</td>
<td>1478</td>
<td>19</td>
</tr>
<tr>
<td>(NH₄)₂SO₄, fraction I, supernat.</td>
<td>1030</td>
<td>26.8</td>
</tr>
<tr>
<td>Heat step, supernat.</td>
<td>800</td>
<td>60</td>
</tr>
<tr>
<td>(NH₄)₂SO₄, fraction II, supernat.</td>
<td>725</td>
<td>220</td>
</tr>
<tr>
<td>After dialysis</td>
<td>384</td>
<td>264</td>
</tr>
<tr>
<td>DEAE cellulose eluate, fraction II</td>
<td>310</td>
<td>448</td>
</tr>
</tbody>
</table>

Assay: reaction mixture in 1 ml, ingredients in μmoles: Same as in Table 8. Control was a boiled extract incubation.
Incubation time was 10 min. at 28°C.
See the text for details of purification procedure.

Table 10

Heat stability of ornithine transcarbamylase in Lumbricus gut tissue extracts

<table>
<thead>
<tr>
<th>Heat Treatment</th>
<th>pre-incubation time, min.</th>
<th>% of total activity recovered</th>
<th>Specific activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>0°C</td>
<td>60</td>
<td>100</td>
<td>24.2</td>
</tr>
<tr>
<td>50°C</td>
<td>20</td>
<td>75</td>
<td>44.4</td>
</tr>
<tr>
<td>60°C</td>
<td>10</td>
<td>63</td>
<td>46.</td>
</tr>
<tr>
<td>60°C</td>
<td>20</td>
<td>60</td>
<td>47.</td>
</tr>
<tr>
<td>65°C</td>
<td>5</td>
<td>53</td>
<td>48.</td>
</tr>
</tbody>
</table>

Assay: reaction mixture in 1 ml, ingredients in μmoles: Same as in Table 8. Control was a boiled extract incubation.
Incubation time was 10 min. at 28°C.
equivalent to the activation energies reported for the OTCase from the ox (Joseph et al., 1963), the frog (Rana catesbiana) (Brown and Cohen, 1959), the frog (Rana temporaria), and the shark (Scylliorhium caniculum) (Joseph et al., 1963) but is much lower than that reported for Streptococcus lactus (Ravel et al., 1959).

The characteristics of the Lumbricus gut OTCase activity identify it as a typical animal ornithine transcarbamylase similar in most characteristics to the activity described in vertebrates and other invertebrates but differing somewhat from that described in bacteria. The level of gut tissue activity (μmoles/g tissue/hr., 26°C) varied from 400-700 in non-starved worms to 1600-2300 in worms starved more than a week.

(2) Reaction II, arginine synthesis from citrulline.

The conversion of citrulline to arginine was originally thought to be an ammonium ion condensation on the ureido-carbon of citrulline in an energy requiring reaction (Krebs and Henseleit, 1932). Borsook and Dubnoff (1941), studying citrulline to arginine conversion in rat kidney slices, suggested a "transimination reaction" with a dicarboxylic amino acid (aspartate or glutamate) as the imino group donor. Gornall and Hunter (1943) found that citrulline could replace ornithine as a "catalyst" in urea biosynthesis, that
citrulline accumulated in the liver slices when the tissue was incubated with high concentrations of ornithine, and that the arginine hydrolyzing activity was more active than the arginine synthesizing activity in liver slices. From these observations, Gornall and Hunter (1943) concluded that the citrulline to arginine conversion limited de novo urea biosynthesis in rat liver slices.

Cohen and Hayano (1946, 48) demonstrated that the citrulline to arginine conversion by cell-free extracts of rat liver tissue required the presence of Mg$^{+2}$, ATP, glutamate, cytochrome c, and O$_2$ and that the activity was localized in the soluble fraction of the cell. Aspartate could replace glutamate but at only 20% effectiveness.

Ratner and Pappas (1949a) isolated the activity in an acetone powder extract of hog kidney. The activity was a two-step reaction requiring citrulline, Mg$^{+2}$, ATP, aspartate and an ATP generating system while the second was a cleavage (hydrolysis) in which arginine and malate were products. The superior activity with glutamate in crude homogenates (Cohen and Hayano, 1946) was attributed to an increased ATP generating potential (Ratner and Pappas, 1949b). The idea of a simple "transamination reaction" was abandoned. Identification of the intermediate was restricted until fumarate rather than malate was shown to be the actual product of the
cleavage reaction (Ratner and Petrack, 1953). Using a purified condensing enzyme, the intermediate compound was synthesized, precipitated as the barium salt in ethanol, and identified as argininosuccinic acid (Ratner et al., 1953b). Petrack and Ratner (1958) showed that the condensing activity could be stimulated by adding inorganic pyrophosphatase and that pyrophosphate and AMP rather than ADP and phosphate were the actual by-products of the condensation. The forward reaction (citrulline utilization) was shown to proceed optimally at pH 8.5 while the weaker reverse reaction proceeded optimally at pH 6.0 (Schuegraf et al., 1960). From O₁₆ transfer studies, Rochovansky and Ratner (1961) demonstrated the transfer of oxygen from the ureido-carbon of citrulline to AMP during the condensation step; the actual reaction mechanism is still doubtful.

The cleavage activity was found to be inhibited by any accumulation of the reaction products, fumarate or arginine (Ratner et al., 1953a). When assaying the cleavage enzyme or the overall synthesis of arginine from citrulline, arginase is usually added and urea formation measured. Fumarase must be added to measure the cleavage activity if a purified enzyme is assayed (Ratner et al., 1953a). Pyrophosphatase must also be included when measuring the overall reaction if purified preparations are used (Schuegraf et al.,
1960). In the overall synthesis of arginine from citrulline with crude preparations such as homogenates or acetone powder extracts of tissues, only arginase and an ATP generating activity need be added to the reaction mixture.

The condensing activity is referred to as "argininosuccinate synthetase" and the cleavage activity as "argininosuccinase". The activities are found in most non-muscular or non-connective tissues of most vertebrates and are found in microorganisms (Ratner, 1962; Cohen and Brown, 1960). The snail, Otala lactea, is the only invertebrate reported to have the activities (Linton and Campbell, 1962).

(a) **Overall synthesis of arginine from citrulline.**

The experiment in Table 2 has established that the ureido-carbon of citrulline was incorporated into the amidine-carbon of arginine by the whole animal. The assay method for demonstrating the overall arginine synthesis from citrulline with cell-free extracts was executed after the method described by Brown and Cohen (1959) with certain modifications. The system assays the consecutive enzymatic reactions catalyzing the condensation of aspartate and citrulline to form argininosuccinate and the cleavage of the latter. The assay system contained citrulline, aspartate, ATP, MgSO₄, and the earthworm gut extract plus an excess of arginase to convert the arginine formed to urea. Because of the
endogenous chromogenic material in the gut tissue, the differential colorimetric determination of urea in the presence of citrulline (Ratner, 1955) was impractical. An extremely sensitive system using ureido-(\(^{14}\)C)-citrulline and measuring the (\(^{14}\)C)-urea formed was employed.

Acetone powders of gut tissue were prepared after the method of Ratner (1955). The powders were dried briefly (15 min.) at room temperature, then extracted with constant gentle stirring at 0°C in 0.02 M MgSO\(_4\) and 0.02 M K-phosphate at pH 7.5. Activity in the acetone powders was lost upon standing overnight at 0°C or 20°C. The extraction of the powders was very critical. Activity was lost if the MgSO\(_4\) was omitted from the extraction fluid and reduced if the volume of extracting fluid exceeded 3 ml per gram of wet gut tissue originally used in the preparation of the acetone powders. The powders were extracted for 30 min., centrifuged (800G for 15 min.), and the supernatant fluid used as the enzyme.

The (\(^{14}\)C)-urea formed was precipitated as dixanthylurea (DXU) after the addition of 30 _umoles of carrier urea, then washed to constant specific radioactivity by recrystallizing the DXU from hot pyridine (see Methods and Materials).

Since the cleavage enzyme is usually present in excess of the condensing enzyme and the overall reaction is usually run near the optium pH for the cleavage enzyme, no purified
cleavage enzyme was added to the reaction mixture (Brown and Conen, 1959; McLean and Gurnery, 1963). Arginase was added to the assay mixture since the endogenous activity was low at pH 7.5 in phosphate buffer. Under the conditions in Table 11 using a one hour incubation, 18% of the label from ureido-\(^{(14}\text{C})\)-citrulline was incorporated into urea for a net urea synthesis of 1.9 \text{umoles}. The incorporation was inactivated by boiling the extract, entirely ATP dependent, and seemed slightly inhibited (10% by \(\alpha\)-methylaspartate at 5 \text{mM}.

Braunstein (1956) using \(\alpha\)-methylaspartate in a similar system with rat liver extracts, found no inhibition at 33 \text{mM}, 20% to 30% inhibition at 67 \text{mM}, and 60% to 80% inhibition at 133 \text{mM}. It would seem under these circumstances that the \textit{Lumbricus} gut activity is more sensitive to the inhibitor than the rat liver activity.

Lower enzyme concentrations and ureido-\(^{(14}\text{C})\)-citrulline of a lower specific radioactivity were used to characterize the activity (Table 12). When twice as much extract was used, urea formation doubled. Omission of aspartate from the reaction mixture depressed the urea formation more than 50%. No determination of aspartate in the acetone powder was undertaken, but Bellamy (1962) reports 2.3 \text{umoles} per gram of dried mitochondria from \textit{Lumbricus}, and Raghupathiramireddy and Rao (1963) report 0.8 mg per gram wet tissue in
Table 11

Incorporation of radiocarbon from ureido (14C)-citrulline into urea by a Lumbricus gut extract

<table>
<thead>
<tr>
<th>Assay modification</th>
<th>Incubation time, min.</th>
<th>DXU total CPM/10^3</th>
<th>umoles urea synthesized</th>
</tr>
</thead>
<tbody>
<tr>
<td>None (boiled extract)</td>
<td>60</td>
<td>12.0</td>
<td>0.075</td>
</tr>
<tr>
<td>Less ATP</td>
<td>60</td>
<td>14.6</td>
<td>0.083</td>
</tr>
<tr>
<td>None</td>
<td>60</td>
<td>322.9</td>
<td>2.01</td>
</tr>
<tr>
<td>Plus 25 umoles methyl-DL-aspartate</td>
<td>60</td>
<td>292.0</td>
<td>1.82</td>
</tr>
</tbody>
</table>

Assay: reaction mixture in 4.5 ml, ingredients in umoles: K-aspartate 25; K-phosphate, pH 7.5, 150; K-phosphoglycerate 30; NAD 4; ATP 15; rabbit muscle enzyme 1 mg; arginase 140 units; citrulline (1.6 x 10^2 CPM/umole) 11.2; 2 ml gut acetone powder extract.

Incubation temp. 30°C.

Earthworm gut extract prep.: acetone powders of the gut tissue were extracted in 0.02 M MgSO_4, 0.02 M K-phosphate, pH 7.5, as described in text.

Table 12

Characteristics of the incorporation of radiocarbon from ureido-(14C)-citrulline into urea by a Lumbricus gut extract.

<table>
<thead>
<tr>
<th>Assay modification</th>
<th>Incubation time, min.</th>
<th>ml of extract added</th>
<th>umoles urea synthesized</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>0</td>
<td>0.25</td>
<td>0.0158</td>
</tr>
<tr>
<td>None</td>
<td>45</td>
<td>0.25</td>
<td>0.0930</td>
</tr>
<tr>
<td>None</td>
<td>45</td>
<td>0.50</td>
<td>0.185</td>
</tr>
<tr>
<td>Less ATP</td>
<td>45</td>
<td>0.25</td>
<td>0.0193</td>
</tr>
<tr>
<td>Less aspartate</td>
<td>45</td>
<td>0.25</td>
<td>0.0530</td>
</tr>
<tr>
<td>Plus 25 umoles EDTA</td>
<td>45</td>
<td>0.25</td>
<td>0.0152</td>
</tr>
<tr>
<td>Plus 10 mg urease</td>
<td>45</td>
<td>0.25</td>
<td>0.0073</td>
</tr>
<tr>
<td>None (boiled extract)</td>
<td>45</td>
<td>0.25</td>
<td>0.0126</td>
</tr>
</tbody>
</table>

Assay: reaction mixture in 2 ml, ingredients in umoles: K-aspartate 10; ATP 5; K-phosphate, pH 7.5, 150; K-phosphoglycerate 20; rabbit muscle enzyme 0.6 mg; arginase 50 units; (14C)-citrulline (3.7 x 10^5 CPM) 10; gut acetone powder extract as indicated.

Incubation temp. 30°C.

Earthworm gut extract prep.: acetone powders of gut tissue were extracted in 0.05 M MgSO_4, 0.02 M K-phosphate.
Figure 5

Overall synthesis of arginine from citrulline by an extract of *Lumbricus* gut tissue.

Assay: conditions were the same as in Table 12.
Lampito mauritii, an Indian earthworm. Addition of 25 μmoles of EDTA (Table 12) completely inhibited the activity and urease destroyed the product.

Under the conditions of Table 12, urea formation from citrulline was linear with time between 15 and 45 min. (Figure 6) after initiation of the reaction but lagged during the first 15 min. This lag, also noted by Brown and Cohen (1959) when measuring the overall reaction for the frog liver activity, can be attributed to the accumulation of the intermediates, argininosuccinate and arginine.

Urea formation under the conditions described is taken as a direct indication of arginine synthesis. Arginine synthesis from citrulline in Lumbricus gut tissue is similar in most characteristics to the activity reported by Brown and Cohen (1959) for the frog, by Schimke (1962) for rat liver, by Ratner and Pappas (1949) for hog kidney, and by Brown and Cohen (1960) for a great variety of vertebrates. The overall synthetic rate by this method was about 4 μmoles synthesized per gram equivalent of acetone powder of gut tissue per hour at 30°C from worms starved five days.

(b) Argininosuccinase (cleavage enzyme).

Argininosuccinase is usually assayed by measuring urea formation from argininosuccinate in the presence of added arginase and tissue extract. The Lumbricus gut extracts
Table 13

Characteristics of the argininosuccinase (cleavage enzyme) activity in a *Lumbricus* gut tissue extract

<table>
<thead>
<tr>
<th>Assay modification</th>
<th>Incubation time, min.</th>
<th>Urea synthesis umoles/0.1 ml extract</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>0</td>
<td>0.092</td>
</tr>
<tr>
<td>None</td>
<td>30</td>
<td>0.240</td>
</tr>
<tr>
<td>None (boiled extract)</td>
<td>30</td>
<td>0.128</td>
</tr>
<tr>
<td>Less extract, plus urease</td>
<td>30</td>
<td>0.02</td>
</tr>
<tr>
<td>Less extract</td>
<td>30</td>
<td>0.104</td>
</tr>
<tr>
<td>Less arginase</td>
<td>30</td>
<td>0.04</td>
</tr>
<tr>
<td>Less argininosuccinate</td>
<td>30</td>
<td>0.04</td>
</tr>
<tr>
<td>Plus urease</td>
<td>30</td>
<td>0.03</td>
</tr>
</tbody>
</table>

**Assay:** reaction mixture in 1 ml, ingredients in umoles: K-argininosuccinate; K-phosphate, pH 7.5, 50; arginase 50 units; gut extract; (urease when added, 10 mg).

**Incubation temp.** 28°C.

**Reaction mixture was pre-incubated for 2 min. prior to addition of the gut tissue extract.**

**Earthworm gut extract prep.:** supernatant from a 105,000G for 60 min. centrifugation of a 10% 0.4 M mannitol homogenate was used as the enzymatic activity.
Figure 7

Urea formation from argininosuccinate in the presence of varying concentrations of a *Lumbricus* gut tissue extract (argininosuccinase activity).

Assay: reaction mixture in 1 ml, ingredients in umoles:
- Tris-HCl, pH 7.6, 50; argininosuccinate-K £ 4;
- arginase 50 units; gut extract.

Incubation temp. 28°C.

Gut extract prep.: supernatant fluid from a 105,000G for 60 min. centrifugation of a 20% homogenate in 0.4 M mannitol.

- Complete
- Boiled extract

Figure 8

Urea formation from argininosuccinate in time of incubation in the presence of a *Lumbricus* gut tissue extract (argininosuccinase activity).

Assay: same conditions as in Figure 7 using 0.1 ml gut extract.

Other conditions were the same as in Figure 7.

Control was a boiled extract incubation.
were prepared by homogenizing the tissue in 0.4 M mannitol, subjecting the homogenate to differential centrifugation (Table 18), and using the supernatant fluid as the source of enzyme activity.

Maximal urea formation from argininosuccinate depended on the addition of _Lumbricus_ gut extract and purified arginase to the reaction mixture (Table 13). Spurious results were obtained if the reaction mixture was not pre-incubated for a few minutes before addition of the _Lumbricus_ gut extract due to the arginine contamination of the argininosuccinate.

Figure 7 describes the net increase in urea formation with gut extract concentration. Extrapolation of the boiled extract control curve and experimental curve to zero extract concentration results in a yield of 0.11 μmoles of urea from 4 μmoles of argininosuccinate. This intercept agrees with the value obtained in Table 13 for the less extract control preparation (the arginine contamination). Urea formation from argininosuccinate increased linearly with time (Figure 8) and had an optimal activity at pH 7.8-7.9 in 0.1 M Tris-HCl (Figure 9). In phosphate buffer, the pH for optimal activity was about pH 7.6. Tris HCl (0.1 M) inhibited the activity 10 to 20%.

Extracts of acetone powders from _Lumbricus_ gut tissue,
Figure 9

Urea formation from argininosuccinate with varying pH in the presence of a Lumbricus gut tissue extract (argininosuccinate activity).

Assay: conditions same as in Figure 7. Use 0.1 ml extract and 100 μmoles Tris-HCl buffer. Other conditions were the same as in Figure 7. Control was a boiled extract incubation.

Figure 10

Arginine formation from argininosuccinase with time of incubation in the presence of a partially purified (arginase free) Lumbricus gut tissue extract (argininosuccinase activity).

Assay: reaction mixture in 1 ml, ingredients in μmoles:
K-phosphate, pH 7.5, 50; argininosuccinate-K 4; extract 0.1 ml.
Incubation temp. 28°C. Control was a boiled extract incubation. Reaction started by adding extract, stopped by adding 1 ml 2% TCA.
prepared according to the method of Ratner (1955) in 0.02 M K-phosphate, pH 7.5, were brought to 30% (NH₄)₂SO₄ saturation and the 50% of the activity precipitated at the 20 to 30% (NH₄)₂SO₄ saturation step. The precipitate was dissolved and dialyzed overnight against 0.02 M K-phosphate, pH 7.5. No arginase activity was evident in the preparation; argininosuccinase activity was measured directly as arginine formation and found to increase linearly with time (Figure 10). The product formed in the reaction was destroyed by the purified arginase.

The argininosuccinase activity described for Lumbricus gut tissue is similar in most characteristics to that described for vertebrates (Ratner, 1962). The activity (expressed as umoles/g gut tissue/hr. at 28°C) was 25 for non-starved worms and 40-50 for starved worms. This tissue activity is five to ten times higher than that described for snail (Otala lactea) hepatopancreas (Linton and Campbell, 1962). It can be concluded that the condensing activity rather than the cleavage activity limits arginine synthesis from citrulline in Lumbricus.

(c) **Condensing enzyme.**

The condensing activity can be assayed by measuring citrulline disappearance from the reaction mixture used for the demonstration of the overall arginine synthesis system if
Figure 11

Condensing enzyme activity (argininosuccinate synthetase measured as citrulline disappearance in the presence of a Lumbricus gut tissue extract.

Assay: reaction mixture in 1 ml, ingredients in umoles:
- K-aspartate 5
- ATP 3
- K-phosphate, pH 7.5, 50
- citrulline 2
- NAD 1
- K-phosphoglycerate 10
- arginase 50 units
- rabbit muscle enzyme 0.3 mg
- urease 10 mg
- gut extract 0.4 ml

Gut extract prep.: same as for table 41.
Reaction started by adding extract, stopped by adding 5 ml 0.5 M HClO4.
Incubation temp. 30°C.

x Less ATP
● Less aspartate
○ Complete system.
urease is included (Tamir and Ratner, 1963a; Linton and Campbell, 1962). By using low citrulline substrate levels, citrulline disappearance with time was slightly hyperbolic (Figure 11) when an acetone powder extract of *Lumbricus* gut tissue was used as a source of enzyme activity (powders prepared as described previously). To obtain the zero time incubation, the reaction mixture less citrulline was incubated for 20 min., the perchloric acid added, then the citrulline added. The boiled extract controls were equal in citrulline content to the zero time incubations; neither used any citrulline. Maximal citrulline disappearance was dependent upon the presence of both ATP and aspartate (Figure 11).

The gut tissue condensing activity as measured by this method at pH 7.5 was about 6 umoles per gram per hour at 30°C in worms starved one week.

(3) **Reaction III, arginase.**

Arginase activity in *Lumbricus* tissues has been described by Lewis and Cohen (1950) and Needham (1960) in phosphate buffer at pH 7.5 to 8. Vertebrate arginase activities are generally inhibited by phosphate and have high pH optimaums (Greenberg, 1951, 1960). In order to better evaluate the *Lumbricus* gut activity, a S1-S2 extract (see OTCase preparation) was assayed in sodium glycine buffer at pH 9.5 with
MnCl₂ as a co-factor. The activity (expressed as umoles/g gut tissue/hr. at 28°C) was 300-600 in non-starved worms and 2000 in worms starved one week. The activity, assayed in this manner, was eight to ten times as great as that formerly reported for Lumbricus.

The activity was partially purified using the scheme outlined in Table 14a and the activity was shown to require arginine and MnCl₂ for activity (Table 14b).

A 10% homogenate of Lumbricus gut tissue was prepared in 0.44 M mannitol, centrifuged at 15,000G for 20 min., and the supernatant fluid collected. Two volumes of cold acetone were added to the supernatant fluid and the precipitated protein removed by centrifugation. The residue was dissolved in 10 ml of water per gram of gut tissue originally used, then brought to 50% (NH₄)₂SO₄ saturation by adding crystalline (NH₄)₂SO₄. The residue was removed by centrifugation and the supernatant fluid brought to 80% (NH₄)₂SO₄ saturation. The solution was centrifuged and the residue dissolved in 3 ml of water. An equal volume of cold acetone was added to the dissolved residue and the precipitated protein removed by centrifugation. The residue was dissolved in 5 ml of water and heated for 3 min. at 55°C. The tube was cooled to 0°C, centrifuged, and the supernatant fluid used as the enzyme (IV). All operations except the heat step were executed
Table 14a

Partial purification and some properties of arginase activity in the gut tissue of Lumbricus

<table>
<thead>
<tr>
<th>Purification stage</th>
<th>Specific Activity</th>
<th>% of original activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>S-II (supernatant fluid)</td>
<td>17</td>
<td>100</td>
</tr>
<tr>
<td>Acetone fraction I</td>
<td>30</td>
<td>65</td>
</tr>
<tr>
<td>50-80% (NH₄)₂SO₄ fraction</td>
<td>75</td>
<td>65</td>
</tr>
<tr>
<td>Heat step supernatant fluid (IV)</td>
<td>130</td>
<td>30</td>
</tr>
<tr>
<td>MnCl₂ activation</td>
<td>520</td>
<td></td>
</tr>
</tbody>
</table>

Table 14b

<table>
<thead>
<tr>
<th>Assay modification</th>
<th>Urea formation umoles/80 mg protein/15 min.</th>
</tr>
</thead>
<tbody>
<tr>
<td>None (boiled enzyme)</td>
<td>0.0</td>
</tr>
<tr>
<td>None</td>
<td>1.75</td>
</tr>
<tr>
<td>Less MnCl₂</td>
<td>0.0</td>
</tr>
<tr>
<td>Less arginine</td>
<td>0.0</td>
</tr>
</tbody>
</table>

Assay: reaction mixture in 1 ml, ingredients in umoles: arginine-HCl 85; MnCl₂ 0.5; Na-glycine, pH 9.5, 50; enzyme solution 0.1 ml.
Incubation temp. 25°C.
Reaction started by adding enzyme, stopped by adding 5.0 ml 0.5 M HClO₄.
Figure 13

Urea formation from arginine in the presence of varying amounts of *Lumbricus* gut tissue extract.

Assay: Conditions same as in Table 14.

Figure 12

Urea formation from arginine with time in the presence of a *Lumbricus* gut tissue extract.

Assay: Conditions same as in Table 14.
at 0°C and all centrifugations were at 800G for 15 min. Incubation of the partially purified preparation (IV) with 0.05 M Tris maleate, pH 7, and 0.01 M MnCl₂ at 55°C for 12 min. raised the activity four-fold (Table 14).

Figures 12 and 13 describe the increase in urea formation with time and enzyme concentration with the partially purified preparation (Table 14, IV). There was a slight change in the slope (10%) between 20 and 40 min. in the time course (Figure 12). The pH for optimal activity in Na-glycine buffer with MnCl₂ is probably about 10.5-11 for both the "Mn activated" and the non-activated enzyme (Figure 14).

The "Mn activation", carried out according to the method of Roholt and Greenberg (1956), was temperature-time dependent. Fifty per cent of the activation occurred within the first 5 min. at 50°C and at about 2 hours at 25°C, while little or no activation was evident at 0°C after 4 hours of pre-incubation. The activity seemed quite stable at 50°C in the absence of MnCl₂ in that activity began to decline only after an hour of heating. The activation was dependent on the presence of MnCl₂. Lewis and Cohen (1950) reported an activation of the Lumbricus arginase activity by a pre-incubation with cobalt ions. When CoCl₂ was substituted for MnCl₂ in the pre-incubation with the enzyme used in this study, the activation was less than that with MnCl₂. The "Mn
Figure 14

Variation of "Mn activated" and non-activated arginase activity from Lumbricus gut tissue extracts with the pH of the reaction mixture.

Assay: reaction mixture in 1 ml, ingredients in umoles: L-arginine 85; Na-glycine 50; MnCl₂ 0.5; gut extract 0.1 ml. pH of mixture adjusted to the various pHs indicated on figure.
Incubation temp. 25°C.

x "Mn activated" extract (extract heated for 12 min. at 55°C in 0.01 M Tris maleate 0.005 M MnCl₂, pH 7 before assay.

• Non-activated.
activation" is similar to that described for mammalian arginase activities (Ronolt and Greenberg, 1956; Mohammed and Greenberg, 1945).

Both Cohen and Lewis (1950) and Needham (1960) report most of the arginase activity localized in the *Lumbricus* gut tissue. Needham (1962) found the greatest concentration of activity in the region behind the chloragogen area of the intestine and anterior to the rectum. This region of the gut makes up less than 5% of the gut tissue weight and had only twice the arginase activity of the more anterior region of the gut. From the economy of the worm, the quantitatively significant arginase activity seems localized in the more anterior chloragogen area of the gut. Abdel-Pattah (1955) found more arginase activity in chloragogen cells isolated from the gut tissue proper than in the gut tissue with the chloragogen cells removed.

The arginase activity described for *Lumbricus* gut tissue is similar in most characteristics to that reported for vertebrates (Greenberg, 1951; Cohen and Brown, 1960) and for other invertebrates (Baldwin, 1936; Campbell, 1963; Linton and Campbell, 1962; Campbell and Lee, 1963). It differs in heat stability from the activity reported from the shark, *Mustelus canus* (Campbell, 1961) and the cestode, *Calliobothrium* (Campbell and Lee, 1963).
(4) Other enzyme activities.

(a) Urease

Because of reports by various authors of a urease activity in *Lumbricus* (Needham, 1960; Laverack, 1963), and the reports of urease in the snails, *Otala lactea* (Linton and Campbell, 1962) and in some cestodes (Simmons, 1961), urease activity in both the body wall and gut tissue were assayed. Ammonia formation was measured when 20 μmoles of urea were incubated with 50 μmoles of Tris-maleate, pH 7, in 1 ml at 28°C for 30 min. in the presence of 0.2 ml of a 20% homogenate of *Lumbricus* tissue. Ammonia production was never greater than that in the boiled enzyme controls or the less urea controls. It is concluded that urease was not present in detectable quantities.

(b) Arginine desiminase

Hammen et al., (1962) have reported arginine desiminase (normally a bacterial enzyme) in two bivalve molluscs and the brachiopod, *Lingula reevi*. Ammonia production was measured when arginine was incubated at pH 6.8 in 0.1 M K-phosphate in the presence of 0.2 ml of a 20% homogenate of either the body wall or gut tissue. Ammonia production was never greater than the boiled homogenate controls or the less arginine controls. It is concluded that arginine desiminase if present is below detectable limits (1 μmole/g tissue/hr. at 28°C).
(c) Aspartic transcarbamylase

The most important alternate pathway for carbamyl phosphate utilization, from a quantitative point of view, is the pyrimidine synthetic pathway (Cohen and Marshall, 1962). The determination of the magnitude of any aspartic transcarbamylase (ATCase) activity in Lumbricus gut tissue was necessary if a quantitative estimation of the control of urea biosynthesis was to be obtained.

The ATCase activity was determined by measuring carbamyl aspartate synthesis from the reaction of commercially available carbamyl phosphate with L-aspartate in the presence of a Lumbricus gut extract. The assay was modified from that described by Bresnick (1963). The Lumbricus gut ATCase activity is described in Table 15 in both bicarbonate and Tris-H₂SO₄ buffers. Bicarbonate buffer seemed to be inhibitory. Glycine buffer normally used at pH 9 to 9.8 gave spurious results due to high spontaneous carbamylation in the boiled extract controls. Bresnick (1963) has also noted the rapid spontaneous carbamylation at high pHs. When the activity was assayed in K-glycylglycine buffer at pH 7.8, the activity in the experimental barely exceeded the boiled extract controls. The activity in rat liver has a high pH for optimal activity, pH 9.3, (Bresnick, 1963; Lowenstein and Cohen, 1956) while the optimal pH for the
Table 15

Requirements for aspartic transcarbamylase activity in homogenates of *Lumbricus* gut tissue

<table>
<thead>
<tr>
<th>Assay modification</th>
<th>Carbamyl aspartate umoles/0.1 ml extract/20 min.</th>
</tr>
</thead>
<tbody>
<tr>
<td>KHCO$_3$, 0.1 M, pH 9.3</td>
<td></td>
</tr>
<tr>
<td>None (boiled homogenate)</td>
<td>0.284</td>
</tr>
<tr>
<td>Less aspartate</td>
<td>0.280</td>
</tr>
<tr>
<td>None</td>
<td>0.448</td>
</tr>
<tr>
<td>Tris-H$_2$SO$_4$, 0.1 M, pH 9.05</td>
<td></td>
</tr>
<tr>
<td>None (boiled extract)</td>
<td>0.292</td>
</tr>
<tr>
<td>None</td>
<td>0.520</td>
</tr>
</tbody>
</table>

Assay: reaction mixture in 1 ml, ingredients in umoles:
- K-aspartate 10; buffer as in table; carbamyl phosphate 10; gut extract.

Gut extract prep.: 20% homogenate in 0.4 M mannitol.

Incubating temp. 28°C.

Reaction started by adding carbamyl phosphate, stopped by adding 1.0 ml 1 M HClO$_4$. 
activity in \textit{E. coli} is about 7.5 (Reichard and Hanshoff, 1956). Since the activity in \textit{Lumbricus} is optimal at higher pHs, it would seem to be more similar to the activity described in rat liver. The worm gut tissue ATCase activity varied from 16 to 20 (umoles/g tissue/hr. at 28^\circ C) in Tris-H_2SO_4 for both starved and non-starved worms. This activity is one to ten per cent as great as the ornithine transcarbamylase activity of \textit{Lumbricus} gut tissue and should not interfere significantly in urea biosynthesis.

These experiments on aspartic transcarbamylase are the first demonstration of this activity in an invertebrate animal. The demonstration of this activity and carbamyl phosphate synthesis in \textit{Lumbricus} is critical if the generalizations, derived from study of mammals and microorganisms, concerning pyrimidine biosynthesis are to be confirmed for all animal species. The absence of a carbamyl phosphate synthesis in birds has led to various suggestions of alternate pathways of pyrimidine precursor biosynthesis (Bowers and Grisolia, 1962; Cohen and Marshall, 1962; Reichard, 1959).
II. **Physiological studies**

A. **Intracellular localization of the ornithine cycle enzyme activities.**

The intracellular localization of the various activities were resolved by differentially centrifuging tissue homogenates which had been prepared in sucrose or mannitol solutions. The method used was a modification of that described by Dounce et al. (1959) and is outlined in Chart 2. After each centrifugation step, the residue was suspended in a known volume of 0.154 M KCl or the homogenizing solution. Samples of each fraction separated were kept until completion of the centrifugation steps. When samples were centrifuged at 105,000G for more than 30 min., these supernatant fluids and residues were assayed separately. The 600G residue is referred to as "cellular debris and nuclei". The 6000G for 20 min. (soft-long) centrifugation or the 15,000G for 8 min. (hard-short) centrifugation isolate the mitochondria in the residue. Van Lancker and Holtzer (1959) have a discussion of the force-time relationship when using differential centrifugation to isolate the mitochondria and microsomes. All the force-time relationships used give the same results. The 105,000G residue is referred to as the microsomal fraction. The supernatant fluid from the mitochondrial or microsomal centrifugations is considered the
Chart 2

Centrifugation procedure used in differential centrifugation of *Lumbricus* tissue homogenates

<table>
<thead>
<tr>
<th>homogenate</th>
<th>600G for 10 min.</th>
</tr>
</thead>
<tbody>
<tr>
<td>residue (R-I)</td>
<td>supernatant (S-I)</td>
</tr>
<tr>
<td>a. 6,000G for 20 min.</td>
<td></td>
</tr>
<tr>
<td>b. 10,000G for 12 min.</td>
<td></td>
</tr>
<tr>
<td>c. 15,000G for 8 min. (use one force-time)</td>
<td></td>
</tr>
<tr>
<td>supernatant (S-II)</td>
<td>residue (R-II)</td>
</tr>
<tr>
<td>105,000G for 30 to 90 min.</td>
<td></td>
</tr>
<tr>
<td>residue (R-III, microsomes)</td>
<td>supernatant (S-III, soluble fraction)</td>
</tr>
</tbody>
</table>

mitochondria
"soluble fraction". All centrifugations were carried out at 0°C.

Both mannitol and sucrose solutions were employed when homogenizing the tissue. Mannitol at 0.44 M or 0.4 M was found to be most satisfactory. Sucrose gave some interference with the color reaction for both the citrulline and urea determinations. The gut tissue also has an invertase (Laverack, 1963) which can interfere with the maintenance of a uniform osmotic strength when sucrose was used. Low concentrations (0.25M) are usually employed for mouse or rat liver studies, but Dounce et al. (1955) suggests using higher concentrations of non-electrolytes when homogenizing tissues for differential centrifugation. A 25% homogenate is one gram of tissue homogenized in three ml of fluid while a 10% homogenate is one gram of tissue homogenized in nine ml of fluid.

1. Carbamyl phosphate synthetase

The 0.4 M mannitol-salt solution (see Table 4) in a 25% homogenate of the gut tissue was used for localization of the carbamyl phosphate synthesizing activity. The results (Table 16) are expressed as a net synthesis of both carbamyl aspartate and citrulline in duplicate assays of the same extract. Equal amounts of citrulline and carbamyl aspartate were synthesized. Little or no activity was present in the
<table>
<thead>
<tr>
<th>Cell fraction</th>
<th>Total umoles/hr. of carbamyl phosphate synthesis as:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>carbamyl aspartate</td>
</tr>
<tr>
<td>25% homogenate</td>
<td>4.92</td>
</tr>
<tr>
<td>R-I</td>
<td>0.0</td>
</tr>
<tr>
<td>S-I</td>
<td>4.90</td>
</tr>
<tr>
<td>R-II (mitochondria)</td>
<td>0.02</td>
</tr>
<tr>
<td>S-II (soluble fraction)</td>
<td>3.78</td>
</tr>
</tbody>
</table>

*Isolated, not assayed.

Assay: reaction mixture 1 ml, ingredients in umoles: Same as in Table 7a; for carbamyl aspartate substitute L-aspartate for L-ornithine and ATCase for OTCase. 

Control: boiled extract.

Incubation time, 30 min.; temp. 28°C.
mitochondria. Most of the activity was found in the soluble fraction indicating a soluble rather than a particulate enzyme. The differential centrifugation was repeated five times; twice the cell debris contained 25% of the activity, none was ever recovered in the mitochondria, and 60-90% was always recovered in the soluble fraction.

Grisolia and Cohen (1953) and Cohen and Hayano (1948) have reported that citrulline synthesis is limited to the particulate fraction, the mitochondria, of liver tissue from several vertebrate species. Jones et al., (1955), originally described the mammalian carbamyl phosphate synthesizing activity from rat liver mitochondria.

(2) Ornithine transcarbamylase

The intracellular localization of the OTCase activity in Lumbricus tissue is described in Table 17. The mitochondria were resuspended in 0.154 M KCl and recentrifuged at 15,000G for 8 min. to remove the sucrose or mannitol. The other cell fractions were diluted with two volumes of 0.154 M KCl and the dilutions used in the assay for activity. In the sucrose fractionations a good deal of the total activity was lost. Possibly it was extracted by the 0.154 M KCl wash but this is unlikely because in the mannitol preparations there was a high percentage of the activity recovered in the various fractions. The high specific activity in the
Table 17

Intracellular localization of ornithine transcarbamylase activity in tissue from Lumbricus

<table>
<thead>
<tr>
<th>Tissue Preparation (Homogenizing solution)</th>
<th>10% homogenate SA **</th>
<th>600G supernat. % Total activity</th>
<th>10,000G supernat. % Total activity</th>
<th>10,000G residue % Total activity</th>
<th>105,000G supernat. % Total activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body wall, 0.25 M sucr.</td>
<td>0.68</td>
<td>0.16 100</td>
<td>-</td>
<td>0.72 22</td>
<td>0.16 33</td>
</tr>
<tr>
<td>Gut, 0.25 M sucr.</td>
<td>6.3</td>
<td>16.3 100</td>
<td>-</td>
<td>25.2 20</td>
<td>14.4 43</td>
</tr>
<tr>
<td>Gut, 0.44 M sucr.</td>
<td>6.4</td>
<td>11.3 95</td>
<td>4.7 32</td>
<td>28.25 24</td>
<td>-</td>
</tr>
<tr>
<td>Gut, 0.44 M mannitol</td>
<td>4.7</td>
<td>7.2 76</td>
<td>4.3 32</td>
<td>30.4 43</td>
<td>-</td>
</tr>
<tr>
<td>*Gut, 0.44 M sucr.</td>
<td>9.6</td>
<td>22.1 100</td>
<td>9.7 70</td>
<td>44.4 15</td>
<td>-</td>
</tr>
<tr>
<td>*Gut, 0.44 M mannitol</td>
<td>11.7</td>
<td>22.8 88</td>
<td>12.4 70</td>
<td>38.3 16</td>
<td>-</td>
</tr>
</tbody>
</table>

Assay in mM: Same as in Table 8.

*Worms starved on cellulose filter paper for 2 weeks; other worms not starved.

Centrifugations carried out in refrigerated PR I with #269 head, 15,000g and 600g centrifugations lasted 8 and 15 minutes respectively; 105,000g centrifugation was carried out in Spinco L, using #40 rotor for 90 minutes; see Chart 2 for explanation.

**SA = Specific Activity (umoles/mg protein/hr.)
mitochondria indicated a possible particulate activity but
the high percentage of the total activity recovered in the
soluble fraction suggested a cytoplasmic activity. The soft-
long centrifugation gave the same distribution as the hard-
short centrifugation indicating that the mitochondria were
probably not ruptured during treatment. In starved worms,
the specific activity of the homogenates and mitochondria
was high, but the per cent activity recovered in the mito-
chondria was low. Either some mitochondria were extremely
fragile or the activity is actually present in both the solu-
ble fraction and the mitochondria. If the activity is pre-
sent in both fractions, then during starvation, there would
seem to be a greater increase in the soluble activity than
in the mitochondrial activity. The localization of the
body wall activity is similar to that of the gut activity
(Table 19).

Ornithine transcarbamylase is generally considered a
mitochondrial activity in vertebrates (Cohen and Marshall,
1962). Campbell (1963), using 0.33 M sucrose, found the
activity localized in the mitochondria of the cestode,
*Hymenolepis diminuta*.

(3) **Aspartate transcarbamylase**

The ATCase activity was localized in the soluble frac-
tion of the cell when a 20% homogenate in 0.4 M mannitol
Table 18

Intracellular localization of aspartic transcarbamylase, argininosuccinase, and arginase activities in the gut tissue of Lumbricus

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Aspartic Transcarbamylase</th>
<th>Argininosuccinase</th>
<th>Arginase</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Activity u-moles/ml extract</td>
<td>% Total Activity</td>
<td>Specific Activity</td>
</tr>
<tr>
<td>Homogenate</td>
<td>0.77</td>
<td>100</td>
<td>0.2</td>
</tr>
<tr>
<td>600g Residue</td>
<td>0.0</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td>600g Supernatant</td>
<td>0.89</td>
<td>115</td>
<td>-</td>
</tr>
<tr>
<td>15,000g Residue</td>
<td>0.0</td>
<td>0</td>
<td>0.0</td>
</tr>
<tr>
<td>15,000g Supernatant</td>
<td>1.35</td>
<td>175</td>
<td>0.56</td>
</tr>
<tr>
<td>105,000g Residue</td>
<td>-</td>
<td>-</td>
<td>0.14</td>
</tr>
<tr>
<td>105,000g Supernatant</td>
<td>1.35</td>
<td>175</td>
<td>0.95</td>
</tr>
</tbody>
</table>

Assays: ATC assay as in Table 1 with bicarbonate buffer; 20 min. incubation; boiled ext. control. Temp. 30°C, tissue homogenized in 0.44 M mannitol, buffered with citrate to pH 6.0, 4 ml/gm tissue.
Argininosuccinase assay as in Table 1 with phosphate buffer; 15 min. incubations, control, 0 time pre-incubated for 5 minutes before adding extract; tissue homogenized as in ATC preparation but 9 ml/gm tissue; incubation temp. 28°C (worms starved 1 wk.).
Arginase assay as in Table 1 with Na-glycine buffer; control, 0 time; 15 min. incubations; temp. 28°C; tissue homogenized in 0.44 M mannitol, 9 ml/gm tissue; worms starved 3 days.
Centrifugations at 600g and 15,000g for 10 and 8 minutes respectively; Centrifugation at 105,000g: 30 min. for ATCase, 60 min. for argininosuccinase, 90 min. for arginase.
- indicates fraction isolated but not assayed for activity.
was subjected to differential centrifugation (Table 15). A 75% stimulation of the activity was achieved with removal of the particulate elements. Bresnick (1963) has demonstrated a soluble activity in rat liver homogenates and a 9% stimulation upon removal of the particulate elements of the cell. The stimulation may be attributed to a removal of a mitochondrial "carbamyl phosphate phosphatase" activity (Bishop and Campbell, 1963) or to the removal of nucleic acids (Bresnick, 1963).

(4) Argininosuccinase (cleavage enzyme)

All the argininosuccinase activity was localized in the soluble fraction of a 10% gut tissue homogenate (Table 1). Removal of the particulate fractions increased the activity (27 to 68%). Neither Ratner (1962) nor Schimke (1962) mention a stimulation of the activity by removal of the particulate elements, but both report the activity in the soluble fraction of rat liver and hog kidney homogenates.

(5) Arginase

Sucrose interferes with the colorimetric determination of urea and gave spurious results when the various fractions from differential centrifugation of gut tissue homogenates were assayed for arginase activity. The activity had a random distribution in that some activity was associated with all particulate fractions when sucrose was used.
Rosenthal et al. (1956) reported an isolation of arginase activity in the microsomal fraction of rat liver homogenates when homogenized in 0.25 M sucrose. Rosenthal removed the activity by washing the microsomes with 0.15 M KCl and attributed the removal of the activity to an ionic solubilization of the arginase protein from the microsomes. In *Lumbricus* gut tissue, when homogenates were made in sucrose, the activity was removed from the particulate fractions with a 0.154 M KCl or 0.4 M mannitol wash with equal success. When a mannitol homogenate of *Lumbricus* gut activity was recovered in the soluble fraction. The same results were obtained with 0.154 M KCl homogenates of gut tissue. No activity was ever found in the 6000G residue (nuclei); Dounce and Beger (1948) found a nuclear arginase activity in rat liver. No stimulation of the worm gut arginase activity was evident with the removal of the particulate fractions containing nucleic acids. Some investigators have reported an inhibition of rat liver arginase activity by nucleic acids (Moss, 1952). Fisher and Chesko (1959) have reported inhibition of chick arginase activity by nucleic acids but this report has been discounted by Eliasson (1962a,b).

(6) Discussion of the intracellular localization of the activities

All the gut tissue activities except those concerned
with citrulline synthesis have the same intracellular localization as those reported from vertebrate liver tissue. Condensing enzyme was not localized. Carbamyl phosphate synthesis was entirely soluble while OTCase had a major portion of its cellular activity in the soluble fraction. This distribution may not be unusual because Mattisson (1961b), Tappel (1960), and Pablo and Tappel (1961) found great variations both quantitatively and qualitatively in the cytochrome and flavin content of mitochondria from invertebrates. Mattisson (1961a) was unable to demonstrate cytochrome c and only a weak cytochrome $a_1+a_2$ in *Lumbricus* mitochondria. Garfinkel (1963), studying electron transport systems in microsomes, found a greater variation in the activity from tissue to tissue in a single species than in a single type of tissue from various vertebrate species. In these studies with *Lumbricus*, gut tissue rather than true "liver" tissue was used in most cases possibly explaining some of the differences.

The distribution of arginase activity when sucrose was used suggests that sucrose sticks the activity to the particulate elements of the cell homogenate. Any solution, electrolyte or non-electrolyte, removed the activity from the particles.
B. **Quantitation of urea production**

The experiment of Cohen and Lewis (1949) and Needham (1957) demonstrating increased urea production with starvation was repeated and their data confirmed (Figure 15). Ten worms were washed, weighed, and placed in a large Buchner funnel at room temperature (24-26°C). The funnel was covered with a petri dish lined with a moist paper towel and the worms rinsed with de-ionized water every eight hours. The washings were collected under toluene in a graduated cylinder and after 24 hours, the urea and ammonia content determined. The ammonia excretion increased during the first days of starvation then decreased to or below the original level after 5 days (Figure 15). Urea excretion increased rapidly with starvation and leveled off at about five to six times the original level after a week of starvation. Both Needham (1957) and Cohen and Lewis (1949) report lower urea production by feeding *Lumbricus* than is reported in Figure 15. There data are probably more correct since the first value obtained in Figure 15 was after one day of starvation. The drop in urea production on the sixth and twelfth days may be attributed to the death of two worms on each of these days.

From the studies on cell-free extracts, all enzymatic activities concerned with the **de novo** biosynthesis of urea
Figure 15

Ammonia and urea production by *Lumbricus* during starvation.

- urea
- ammonia
Table 19
Incorporation of label into urea from $^{14}\text{C}-\text{HCO}_3^-$ and ureido-$^{14}\text{C}$-citrulline injected into 
*Lumbricus*

<table>
<thead>
<tr>
<th>Compound injected</th>
<th>Worm wet wt.(g)</th>
<th>Urea synthesis</th>
<th>CPM incorporated</th>
<th>Incorp. Ratio*</th>
</tr>
</thead>
<tbody>
<tr>
<td>(CPM/g worm/10^5)</td>
<td>condition</td>
<td>umoles/g/day</td>
<td>into urea</td>
<td></td>
</tr>
<tr>
<td>Ureido-$^{14}\text{C}$-citrulline</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(a) (6.08)</td>
<td>Non-starv.</td>
<td>7.85</td>
<td>250,000</td>
<td>0.053</td>
</tr>
<tr>
<td>(b) (2.42)</td>
<td>Starv.</td>
<td>5.9</td>
<td>153,000</td>
<td>0.107</td>
</tr>
<tr>
<td>$^{14}\text{C}$-NaHCO$_3$</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(a) (4.02)</td>
<td>Non-starv.</td>
<td>7.85</td>
<td>10,525</td>
<td>0.0034</td>
</tr>
<tr>
<td>(b) (4.12)</td>
<td>Starv.</td>
<td>7.65</td>
<td>258,300</td>
<td>0.083</td>
</tr>
</tbody>
</table>

*CPM incorporated/umole urea) (umoles urea synthesized/g worm) = ratio of isotope incorporation on a CPM/g basis*
seem present in L. gut tissue. The two "synthetase" activities seem to have the lowest tissue activities. In order to test which step was limiting during starvation and feeding and to confirm urea biosynthesis from citrulline and HCO₃⁻ in vivo, (¹⁴C)-HCO₃⁻ and ureido-(¹⁴C)-citrulline were injected into the anterior "hearts" region of starved and non-starved worms (Table 19). The worms were put in funnels covered with watch glasses lined with wet paper toweling and washed with de-ionized water every six hours. The washings were collected in a graduated cylinder and after 24 hours, the urea and ammonia content determined. The washings were then evaporated to dryness and the residue taken up in a ml of water, transferred to a test tube, and incubated at pH 6.8 with urease. The reaction was stopped with H₂SO₄ and the CO₂ evolved trapped in NaOH. The specific radioactivity of the urea was calculated from the urea disappearance and the radioactivity in the carbonate.

Less ureido-(¹⁴C)-citrulline was injected into the starved worms than into the non-starved worms and the starved worms produced more urea than the non-starved worms (Table 19). Since urea is an end-product, the data on urea can be treated as a yield. The incorporation ratio is an expression of the total radioactivity incorporated into urea per gram of animal. The ratio can be used to compare animals of different
Table 20

Effect of starvation on citrulline synthetic activities in *Lumbricus* gut tissue

<table>
<thead>
<tr>
<th>Days of starvation</th>
<th>umoles/ml extract/20 min.</th>
<th>CPM/ml extract/20 min.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ATCase (CA)</td>
<td>OTCase (citrulline)</td>
</tr>
<tr>
<td>0</td>
<td>2.24</td>
<td>23.6</td>
</tr>
<tr>
<td>2</td>
<td>2.0</td>
<td>47.5</td>
</tr>
<tr>
<td>5</td>
<td>2.0</td>
<td>84.0</td>
</tr>
<tr>
<td>8</td>
<td>2.4</td>
<td>93.0</td>
</tr>
</tbody>
</table>

**Assay:**
- **ATCase (aspartic transcarbamylase):** Same as in Table 15, use 0.1 M Tris-H$_2$SO$_4$; control was boiled extract.
- **OTCase (ornithine transcarbamylase):** Same as in Table 8, use K-glycylglycine; control was boiled extract.
- **CPSase (carbamyl phosphate synthetase):** Same as in Table 4, use imidazole-HCl; 1 ycurie of (14C)-NaHCO$_3$. Reaction stopped with 5% TCA and CO$_2$ bubbled through the mixture to remove all radioactive CO$_2$. The denatured protein was removed by centrifugation and a sample of the supernatant fluid plated on a stainless steel planchet and evaporated to dryness. The acid stable radioactivity was taken as a measure of incorporation into citrulline.
- **Method adapted from Davis (1963).**
- **Earthworm extract prep.:** Tissue from 5 worms was pooled on each day and homogenized in 0.4 M mannitol, 0.015 M K-glycylglycine, pH 7.5, and 0.1 M K$_2$SO$_4$ (3 ml/g tissue).
body weights which were injected with varying amounts of radioactivity from various sources and which produced different amounts of urea per gram of body weight.

In the starved worms, incorporation of label from citrulline into urea was twice that of the non-starved worms while the incorporation from bicarbonate into urea was 25 fold greater in starved worms than in non-starved worms (Table 19). Incorporation of label from citrulline into urea was 13 times greater than the incorporation from bicarbonate in non-starved worms. In starved worms, however, the incorporation of label from bicarbonate was almost equal to that from citrulline. Therefore, during feeding, the formation of citrulline limits urea biosynthesis; during starvation, citrulline utilization limits urea biosynthesis. The citrulline synthesizing activities must be activated with starvation.

To measure the activation of the citrulline synthesizing system during starvation, the relative activities of ATCase, OTCase, and carbamyl phosphate synthetase were compared on different days following the onset of starvation (Table 20). The ratio of OTCase to ATCase increased from 10:1 in non-starved worms to 40:1 in worms starved eight days. Carbamyl phosphate synthetase activity increased 50% after five days of starvation and 70% by the eighth day of starvation. The rise in carbamyl phosphate synthetase activity was concomitant
with the decrease in ammonia excretion during starvation (Table 20, Figure 15). By multiplying the increased carbamyl phosphate synthetase activity by the increased OTCase activity, the citrulline synthesizing capacity of the gut tissue increases 6.8 times with eight days of starvation. The urea excretion during the same eight day period increased five-to six-fold (Figure 15). This close correlation between the fall in ammonia production, increased ammonia fixation and citrulline synthesizing capacity, and the actual rise in urea excretion during starvation suggests that urea biosynthesis is a truly adaptive mechanism for elimination of ammonia during starvation by Lumbricus. Lumbricus becomes "ureotelic" when the citrulline synthesizing capacity exceeds the citrulline utilizing ability.
DISCUSSION AND CONCLUSIONS

The characteristics peculiar to each of the individual enzyme activities associated with the ornithine cycle in *Lumbricus* have been discussed previously. Their activities are summarized in Table 21. The level of the activities in the gut tissue of *Lumbricus* is about one order of magnitude less than that found in mammalian liver tissue and ureotelic amphibian liver tissue (see Conen and Brown, 1960). The level of the activity limiting urea biosynthesis in *Lumbricus* approximates the level of oxygen consumption (metabolic rate) of *Lumbricus* of about 0.2 ml O\(_2\) per gram per hour at 21\(^{\circ}\)C reported in Laverack (1963).

Investigations of the mechanism of urea biosynthesis and excretion as a method of nitrogenous waste elimination have been restricted to vertebrates. The quantitatively significant urea biosynthesis in mammals was first localized in the liver tissue of dogs from Eck fistula experiments (see Bollman, 1961), although in all of these studies, some extrahepatic urea biosynthesis always occurred. Urea biosynthesis is generally thought to be an ammonia detoxication mechanism. Citrulline biosynthesis, from enzymatic assays, seems confined to the liver in mammals (Jones et al., 1961; Hall et al., 1960) even though Hall et al. did find a low but significant carbamyl phosphate synthesis in the intestinal mucosal tissue.
Table 21

Summary of ornithine cycle enzyme activities from *Lumbricus* gut tissue

<table>
<thead>
<tr>
<th>Enzyme Activity</th>
<th>Activity in umoles/g/hr. at 25°C</th>
<th>Non-starved Worms</th>
<th>Starved Worms</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carbamyl phosphate syn.</td>
<td>1.5-2.5</td>
<td></td>
<td>4-5</td>
</tr>
<tr>
<td>Ornithine transcarbamylase</td>
<td>300-700</td>
<td>1600-2300</td>
<td></td>
</tr>
<tr>
<td>Aspartate transcarbamylase</td>
<td>16-20</td>
<td>16-20</td>
<td></td>
</tr>
<tr>
<td>Condensing enzyme</td>
<td>3</td>
<td>4-5</td>
<td></td>
</tr>
<tr>
<td>Cleavage enzyme</td>
<td>25</td>
<td>35-50</td>
<td></td>
</tr>
<tr>
<td>Arginase</td>
<td>300-600</td>
<td>2000</td>
<td></td>
</tr>
</tbody>
</table>

Values taken from data already presented.
of the rat. Jonson et al. (1962a,b) have noted variable serum OTCase levels which are related to the degree of liver damage in man and dogs. The arginine synthetase activities may be high in various tissues in most vertebrates (Ratner, 1962; Tamir and Ratner, 1963a; Cohen and Brown, 1960; Schimke, 1964). Arginase activity may be high in various tissues of most vertebrates (Campbell, 1961; Robinson and Schmidt-Nielsen, 1963; Cohen and Brown, 1960; Baldwin, 1960, 36; Sporn et al. 1959). In vertebrates which synthesize urea as the major nitrogenous waste product, the full complement of ornithine cycle activities seems restricted to the liver tissue.

All the ornithine cycle enzyme have been located in Lumbricus gut tissue. If the quantitatively significant urea biosynthesis by the worm is restricted to the gut tissue, then the gut tissue activities should be able to account for the actual urea production by the whole worm. During starvation, urea production is highest and the two synthetase activities seem about equal (from enzymatic assays, Table 21). If 3.5 umoles per gram gut tissue per hour is used as a minimal activity during starvation and the gut makes up 12% of the worm body weight, then the activity can produce 10.1 umoles per day. The average urea production during starvation was five to six umoles per gram per day (Figure 15). The
minimal activity can account for almost twice the actual average production during starvation.

The location of the major urea biosynthetic mechanism in the gut tissue of *Lumbricus* may represent an early stage in the evolutionary appearance of the ornithine cycle enzymes in the intestinal cell prior to their association with a hepatic diverticulum or liver as in the vertebrates. The "hepatic functions" of the earthworms gut are generally associated with the loosely organized chloragogen cells of the gut (Roots, 1960; Abdel-Fattah, 1955; Laverack, 1963).

In all the ureotelic animals investigated (Cohen and Brown, 1960), the enzymatic reaction limiting urea biosynthesis is the arginino-succinate synthetase or condensing activity. All the activities in rat liver can be raised or lowered by high or low protein diets (Schimke, 1962a), starvation (Schimke, 1962b), cortisone administration or adrenalectomy (McLean, 1961; McLean and Gurney, 1963; Schimke, 1963), and carbon tetrachloride poisoning (Rossi and McLean, 1961). In each case, the condensing activity limited urea biosynthesis. Only incomplete data are available in the case of mammals under meat intoxication or ammonia intoxication brought on either by an Eck fistula or ammonium acetate injections (Brown, 1963; Salvatore et al., 1961,63; Cittodini et al., 1964).
In the transition from ammonotelism to ureotelism in the developing frog, the increase in urea excretion is a function of the increase in the condensing enzyme activity (Brown and Cohen, 1958; Brown et al., 1959). In the ureotelic amphibians, *Necturus* and *Amblystoma*, where urea excretion is always low (10% of urine-N), the condensing activity limits urea biosynthesis (Brown et al., 1959). In the developing pig and rat, carbamyl phosphate synthetase limits urea biosynthesis rather than the condensing activity for prior to and for a short time after gestation (Kennan and Cohen, 1959). The rat carbamyl phosphate synthetase activity rises sharply to the adult level within 24 hours after birth (Jones et al., 1961) while the pig activity rises more slowly after birth (Kennan and Cohen, 1959). In the adult pig and rat, the condensing enzyme limits urea biosynthesis and they are ureotelic. No data are available on the urea and ammonia composition of piglet urine. The limitation of urea biosynthesis by the condensing enzyme activity might explain Cedrango's (1958) results concerning a stimulation of urea formation when rat liver slices are incubated with various amino acids and no stimulation when incubated with added ammonium ion. Citrulline formation is never a limiting activity in urea biosynthesis in a ureotelic animal unless ornithine is lost from the cycle (Gornall and Hunter, 1943).
The feeding *Lumbricus* is ammonotelic and urea biosynthesis is limited by the ammonia fixing activity, carbamyl phosphate synthetase. The starved *Lumbricus* is ureotelic and limited by the citrulline utilizing activity, the condensing enzyme. *Lumbricus* becomes ureotelic when the ammonia fixing and citrulline synthesizing activity exceeds the citrulline utilizing ability. It would seem then, for an animal to be ureotelic, all enzymes for urea biosynthesis must be present in a single tissue (hepatic) and argininosuccinate synthetase (condensing enzyme) must be the rate-limiting reaction in the pathway.

Control of urea biosynthesis in the rat has been examined by Schimke (1963) and McLean and Gurney (1963). Adrenalectomy lowered all the activities of the ornithine cycle while cortisone replacement therapy restored all the activities. Cortisone injection had little effect on the activity levels if the adrenals were left intact. Schimke (1963) could find no change in the endogenous free amino acid levels of citrulline, arginine, or ornithine under the various stresses imposed on the rats. He concluded, from the effect of arginine free diets and adrenalectomy, that the arginase activity was under a control separate from the other activities and that regulation of urea biosynthesis must be from an extracellular rather than from an intracellular control mechanism. McLean
and Gurney's (1963) results support Schimke's (1963) data on adrenalectomy and cortisone replacement and they concluded that there is an adrenal control of urea biosynthesis by a regulation of the condensing enzyme activity. Mammalian liver cells and tumor cells in culture lose their ability to synthesize citrulline (Jones et al., 1961; Schimke, 1964). Schimke (1964) has very good evidence that arginine levels in the medium control the levels of condensing and cleavage activities, thus regulating arginine biosynthesis from citrulline. The change in biosynthetic capacity and regulatory function for cells in tissue culture suggests that the citrulline biosynthetic activities might have a regulatory mechanism differing from that regulating citrulline utilization. Regulation of urea biosynthesis would seem to operate by regulation of the argininosuccinate synthetase activity in ureotelic animals. In non-ureotelic animals, limitation of urea and arginine synthesis is probably obtained by regulation of another activity such as carbamyl phosphate synthetase in the ammonotelic Lumbricus.

The problem of regulation in Lumbricus is not control of ureotelism as such, but rather of the control of the transition from ammonotelism to ureotelism. In the forced transition, the citrulline synthetic capacity increased markedly and is similar in some respects to the situation found in the
developing frog. Faik and Cohen (1960) and Metzenberg et al. (1961) have found a specific stimulation of carbamyl phosphate synthetase and acceleration of the onset of ureotelism by treating developing tadpoles with thyroxine. The increased carbamyl phosphate synthetase activity is due to an actual increase in the amount of synthetase protein by an activation of the protein synthesizing system (RNA) for this protein. Dolphin and Freeman (1955) demonstrated a stimulation of arginase in tadpoles treated with thyroxine which suggests that the thyroxine effect may not be as specific as suggested by Metzenberg et al. (1961). In Lumbricus, Needham (1957) cut worms in half and starved the halves separately. The anterior half became ureotelic while the posterior half remained ammonotelic, as in the feeding worms. Needham (1958) interpreted these results as indicating a "metabolic gradient (axial field)" and studied regeneration or wound healing in the various sections of the worms when cut in fifths. Laverack (1963) and Aros and Vigh (1962) have described the neurosecretory apparatus of Lumbricus as existing mainly in the anterior segments, three through seven. Cutting the worm in half removes any neurosecretory or humoral control from the posterior end. A re-examination of Needham's (1957, 58) data on urea and ammonia excretion by the various body parts suggests to me that there is a
humoral control of the transition from ammonotelism to ureotelism that may be analogous to that found in the frog.

An interesting corollary to this study was the elucidation of the arginine biosynthetic mechanism in Lumbricus (Tables 2, 21) and the arginine content of the total body protein. The value of 70-80 mg protein per gram of tissue was obtained from fully hydrated worms possibly accounting for the somewhat lower values than those obtained by other investigators (80-140 mg/g) (see Laverack, 1963). Florkin (1957) found 10.07 mg of arginine per 100 mg of Lumbricus hemoglobin which is fairly close to the 7.39% and 8.93% obtained for the total body protein in this study. It may be concluded from the levels of the enzymatic activities, that arginine is probably not a dietary requirement for Lumbricus terrestris.

These studies on urea biosynthesis in Lumbricus terrestris have demonstrated for the first time the actual biosynthesis of urea and arginine in an invertebrate animal and should eliminate doubts as to the existence of the ornithine cycle in invertebrate animals. Carbamyl phosphate synthetase and aspartic transcarbamylase were also demonstrated for the first time in an invertebrate animal. The demonstration of these two activities is critical if the generalizations concerning pyrimidine and nucleic acid synthesis are to be applied to and confirmed for all animal species.
LITERATURE CITED


Schimke, R. T. 1963. Studies on factors affecting the levels of urea cycle enzymes in rat liver. J. Biol. Chem. 238: 


