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METABOLIC ASPECTS OF THE AMMONOTELIC-UREOTELIC TRANSITION IN THE EARTHWORM, LUMBRICUS TERRESTRIS (L)

PAUL CARROLL MACDONNELL

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METABOLIC ASPECTS OF THE AMMONOTELIC—UREOTELIC TRANSITION
IN THE EARTHWORM, LUMBRICUS TERRESTRIS L.

by

PAUL CARROLL MACDONNELL

B.A., Northeastern University, 1968
M.S., University of New Hampshire, 1970

A THESIS

Submitted to the University of New Hampshire
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The Requirements for the Degree of

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Department of Zoology
September, 1972
This thesis has been examined and approved.

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ABSTRACT

METABOLIC ASPECTS OF THE AMMONOTELIC-UREOTELIC TRANSITION
IN THE EARTHWORM, LUMBRICUS TERRESTRIS L.

by

PAUL CARROLL MACDONNELL

The earthworm Lumbricus terrestris is ammonotelic while feeding but becomes ureotelic with fasting.

1. The metabolic sources of ammonia nitrogen in feeding worms have been examined. Two deaminating enzymes have been observed to be present in high activity, L-serine dehydratase and AMP deaminase. The latter enzyme was an order of magnitude more active than the former. While these two enzymes did not increase during fasting their possible role in ammonia production is discussed.

2. The activity of L-serine dehydratase was observed to be modified by theophylline. AMP deaminase was shown to be an allosteric enzyme; ATP functions in this system as a positive modulator. The positive allosteric effector increases the apparent Km of AMP deaminase while having no effect on the maximum velocity of the reaction.

3. The linear distribution of the ammonia-generating enzymes along the gut of Lumbricus was compared to the distribution of digestive enzymes. While digestive enzymes are situated predominantly in the anterior post-gizzard gut there appear to be two potential centers of ammonia production.
These two centers correspond to the anterior location of AMP deaminase and the specific hind-gut distribution of L-serine dehydratase.

4. Proteins of the gut and body wall undergo characteristic changes during ureotelism; proteins of the former tissue are more extensively degraded than of the latter.

5. The activity of a number of lysosomal enzymes was seen to change during the transition to ureotelism. Thus, cathepsin 5.2, neutral protease, and ribonuclease increased in activity in the intestinal tissue during fasting while concurrently cathepsin 2.8, cathepsin 5.2, and neutral protease activity decreased in the body wall.

6. A 12 fold increase in glutamate dehydrogenase activity was observed in the gut during ureotelism. It is suggested that this enzyme shunts metabolic ammonia into the urea cycle.

7. The effects of actinomycin D, puromycin, and cycloheximide on the transition to ureotelism were investigated. Actinomycin D prevents ammonotelic animals from becoming ureotelic while having little effect on animals already ureotelic. Cycloheximide and puromycin, in contrast, depress urea output during the ureotelic state.
CHAPTER I

INTRODUCTION

The ammonia-urea transition

Three principle forms of nitrogenous wastes exist among the various animal phyla. Where water is abundantly available many forms void nitrogen in the form of ammonia. The relative toxicity of ammonia, however, makes it unsuitable where water is limited; in such instances either urea or uric acid is the dominant nitrogenous waste. If water is extremely limited uric acid or other purine bases are excreted (Florey, 1967).

While one of the three major forms of nitrogen may be dominant in any one organism's excreta, typically one or both of the other two may be released as well, although these will be minor components. In man, for example, urea is the dominant form of nitrogenous waste, with ammonia serving a variable and minor role.

The predominant form of nitrogen waste may vary with the developmental stage, environment, or diet of the organism. The classical example is that of the metamorphosing tadpole which voids ammonia in its early stages but becomes ureotelic during metamorphosis (Cohen, 1966). Indeed, this transition in nitrogen excretion is correlated with the changes in both the organism's habitat and developmental state. The present study is concerned with a similar shift in nitrogen metabolism widely seen among the oligochaete annelids and which apparently depends upon the nutritional state.
The earthworm *Lumbricus terrestris* is ammonotelic when feeding but becomes ureotelic with fasting (Cohen and Lewis, 1949). There is no well established relationship between water availability and the dominant form of nitrogen waste as in other species (Tillinghast et al., 1969). The time between the initiation of fasting and the transition to ureotelism has been shown to be temperature dependent (Tillinghast et al., 1969). In contrast to the metamorphosing tadpole *Rana*, the transition to ureotelism in *Lumbricus* is reversible (Needham, 1958) and thus is similar to that pattern exhibited by the estivating lungfish *Protopterus* (Janssens, 1964a).

**Metabolic sources of ammonia nitrogen**

In a series of experiments on *Lumbricus* it was established that ammonia is voided via the gut whereas urea is voided by the nephridia (Tillinghast, 1967). Until now there has been no investigation of the metabolic origin of ammonia in terrestrial oligochaetes, although Bishop and Barnes (1971) have initiated studies on this problem in the aquatic polychaetes. They have presented evidence that AMP deaminase may be of importance in ammonia formation.

We have examined earthworm tissues for the presence and activity of this enzyme as well as a variety of other ammonia-generating enzymes. Because ammonia production in feeding animals may be either physiologically related to neutralization of the gut contents, or possibly involved in base conservation, as is well established in mammals (reviewed by Lotspeich, 1967), we have determined the activity of various ammonia-generating
enzymes in specific areas along the gut of *Lumbricus* using the distribution of selected digestive enzymes as a frame of reference. A preliminary report has already appeared (MacDonnell and Tillinghast, 1971).

**Metabolic sources of urea nitrogen**

Studies on urea synthesis in *Lumbricus* have been more numerous than those on ammonia production. The early work of Heidermanns (1937) established that intestinal homogenates were capable of forming urea from peptone. This work was extended by Cohen and Lewis (1950) who were able to locate arginase in gut tissue rather than in the body wall. Needham (1962) observed that arginase activity increases from anterior to posterior along the linear gut. The work of Bishop and Campbell (1965) demonstrated the existence of all five urea cycle enzymes in *Lumbricus* and showed that they increased in activity with fasting.

The sources of nitrogen for urea biosynthesis have not been studied until the present work. Although Cohen and Lewis (1949) suggested that urea nitrogen may arise from protein catabolism, experimental data were not presented. That mobilization of the body wall proteins may be involved is suggested by the histological studies of Cooper and Baculi (1968) who observed a decrease in the body wall tissue when earthworms were maintained on a synthetic, and apparently inadequate, diet. This possibility is further strengthened by the observations of Heran (1956) who noted that proteolytic activity increased in the gut during fasting.
In the present study we have sought to observe changes in body wall and gut proteins, and to correlate these changes with the activities of lysosomal and gluconeogenic enzymes during fasting.

The control of the ammonia-urea transition

Few studies have been done on the mechanisms controlling the ammonia-urea transition in Lumbricus. This situation suffers by comparison with work on metamorphosing amphibians where it was established that a causal relationship existed between the thyroid hormone and this event (reviewed by Gorbman and Bern, 1962). The subsequent work of Cohen and his coworkers (Nakagawa et al., 1967; Nakagawa and Cohen, 1967; and Unsworth and Cohen, 1968) have clearly documented the thyroid hormone as instrumental in increasing the activity of the urea cycle enzymes.

On the basis of bisection data Needham (1958) proposed that the ammonia-urea transition was controlled by two centers, each promoting the synthesis of one of these nitrogenous wastes. Which center was dominant, it was suggested, depended upon the activity of the gut. While later studies did not support Needham's hypothesis of a dual control mechanism they did not exclude an hormonal involvement (Tillinghast and Janson, 1971).

In the present study we have assumed that the ammonia-urea transition is regulated by hormones and have investigated the role of transcription and translation during the transition to ureotelism.
CHAPTER II

MATERIALS AND METHODS

Maintenance of animals

Mature earthworms were collected locally and identified as *Lumbricus terrestris* L. Feeding individuals were those used within 24 hours of collection. For fasting studies earthworms were starved for two weeks during which time they were maintained individually in screw-cap jars with 2 ml of distilled water. The animals were rinsed with tap water daily and replaced in clean containers with 2 ml of distilled water. Regardless of their dietary regime all earthworms were maintained at 11 to 13°.

Tissue preparation

The gut from the gizzard to the anus was rapidly excised, cut longitudinally, and rinsed with a stream of distilled water in order to remove fecal material. A 10% (w/v) aqueous extract was prepared by homogenizing the tissue with a Potter-Elvehjem ground glass homogenizer at 4°. Depending upon the enzyme under investigation, aliquots of the homogenate were either used immediately or centrifuged for 30 minutes at 27,000 x g in a Sorvall Refrigerated Centrifuge, Model RC2-B. The supernatant was used immediately for enzyme assays.

A portion of the adjacent body wall was prepared by the same procedure except that the homogenate was always centrifuged at 27,000 x g for 30 minutes. Homogenates of other
tissues were prepared as described above with the exception of the nervous system (supra- and subesophageal ganglia, ventral nerve cord) and the seminal vesicles, which were not centrifuged.

Analysis of the distribution of gut enzymes was performed by dividing the post-gizzard gut into four sections. Section 1 was from the gizzard to the posterior clitellum segment (segments 19 through 37) while sections 2 (segments 38 to 70) and 3 (segments 71 through 115) were equal divisions of the remaining gut to the end of the chloragogen-containing region. Section 4 was the terminal chloragogen-free region of the gut (segments 116 to the anus).

**Measurement of excretory ammonia and urea**

The amount of ammonia and urea excreted was assayed by diluting the washings collected the preceding 24 hours to 10 ml with distilled water. A sample of the washings was divided into two aliquots of 0.5 ml each. Ammonia was determined in one aliquot by the phenate-hypochlorite reagent (Kaplan, 1965). Urea was enzymatically hydrolyzed to ammonia in the second sample by the addition of an excess of urease (0.1 unit of Type V urease, Sigma Chemical Co.) prior to ammonia determination. The difference in absorbance at 560 nm between the two samples was proportional to the amount of urea originally present (Kaplan, 1965).
Metabolic sources of ammonia nitrogen

The possible metabolic source of ammonia was investigated by incubating naturally occurring nitrogenous compounds with gut homogenates.

Deamination of nitrogenous substrates was assayed by a modification of the procedure of Janicki and Lingis (1970). An aliquot of the gut homogenate was incubated with 5 micromoles of the compound under investigation (or 10 micromoles of DL-amino acids), 5 micromoles MgSO$_4$, and 67 micromoles of potassium phosphate buffer pH 7.0 for 1 hour at 37° in Conway microdiffusion chambers. The reaction was terminated by the addition of 1.0 ml saturated potassium carbonate. The chambers were incubated an additional hour at 37° at which time the ammonia which was produced during the reaction was trapped by 1.0 ml of 0.1 N HCl, previously placed in the center well. Ammonia was measured by the phenate-hypochlorite reagent (Kaplan, 1965).

The enzymic activity of serine dehydratase (L-serine hydrolyase, E.C. 4.2.1.13) was assayed by a modification of the method of Bresnick et al. (1971). An aliquot of the homogenate was incubated with 100 micromoles L-serine, 100 millimicromoles pyridoxal 5'-phosphate, and 67 micromoles potassium phosphate buffer pH 7.0 in a total volume of 1.0 ml for 20 minutes at 37°. At the end of the incubation period 5.0 ml of cold 10% trichloroacetic acid (TCA) was added and pyruvic acid determined in an aliquot of the protein-free supernatant by the method of Friedemann and Haugen (1943).

The assay for adenosine deaminase (adenosine aminohydrolase, E.C. 3.5.4.4) and 2'-deoxyadenosine deaminase was
performed by a modification of the procedure of Janicki and Lingis (1970). Appropriate dilutions of the homogenate were incubated with 5 micromoles MgSO₄, 67 micromoles potassium phosphate buffer pH 7.0, and 20 micromoles of the respective purine derivative in a total volume of 1.0 ml for 1 hour at 37°. Ammonia was determined using the Conway microdiffusion technique as described above.

Adenosine 5'-monophosphate deaminase (AMP aminohydrolase, E.C. 3.5.4.6) was assayed by a modification of the method of Makarewicz (1969). Twenty micromoles of 5'-AMP, 200 micromoles KCl, 200 micromoles potassium succinate buffer pH 6.54, and appropriate dilution of the homogenate were incubated at 37° for 30 minutes. One ml of cold 10% TCA was added to terminate the reaction and precipitate the proteins. A 1.5 ml aliquot of the protein-free supernatant was neutralized by the addition of 0.4 ml of 1.0 N KOH. Ammonia was measured by the phenate-hypochlorite reagent.

**Distribution of enzymes along the intestine**

Sections of the linear gut of *Lumbricus* as described above were assayed for the following enzymes:

Alpha-mannosidase (alpha-D-mannoside mannohydrolase, E.C. 3.2.1.24), alpha-glucosidase (alpha-D-glucoside glucohydrolase, E.C. 3.2.1.20) and alpha-galactosidase (alpha-D-galactoside galactohydrolase, E.C. 3.2.1.22) were assayed by the procedure of Li and Shetlar (1965). The assay employed for beta-glucuronidase (beta-D-glucuronide glucuronohydrolase, E.C. 3.2.1.31) was that of Plaice (1961). Acid and alkaline
phosphatases (orthophosphoric monoester phosphohydrolase, E.C. 3.1.3.2) were assayed by the method of Arsenis et al. (1970). Trypsin (E.C. 3.4.1.4) was determined by the procedure of Hummel (1959) using the synthetic substrate p-toluenesulfonyl-L-arginine methyl ester HCl (TAME). Amylase was assayed by the amyloclastic method from the Sigma Technical Bulletin No. 700, Sigma Chemical Co. General proteolytic activity was measured by the method of Bewley and DeVillez (1968).

Tissue samples were assayed for the activity of phosphorylase (alpha-1,4-glucan: orthophosphate glucosyltransferase, E.C. 2.4.1.1) by a modification of the method of Sutherland and Wosilait (1956). An aliquot of the homogenate was added to 0.1 ml of a reaction mixture which contained the following: 0.1 M glucose-1-phosphate, 2% glycogen, 0.2 M NaF and when present, 0.003 M 5'-AMP. The mixture was incubated at 37° for 30 minutes and the reaction was terminated by the addition of 0.2 ml of cold 10% TCA. After centrifugation a 0.2 ml aliquot of the supernatant was analyzed for inorganic phosphate by the method of Fiske and SubbaRow (1925). Active phosphorylase is defined as the enzyme activity in the absence of 5'-AMP, while total phosphorylase is the enzyme activity in the presence of added 5'-AMP.

**Lysosomal enzymes**

Two acid proteases (cathepsins) were assayed following the procedure of Mycek (1970). General proteolytic activity was measured by the method of Bewley and DeVillez (1968). The three proteinases were differentiated solely by pH. Acid ribonuclease (polynucleotide 2-oligonucleotide transferase
(cyclizing), E.C. 2.7.7.16) was measured by following the increase in absorbance at 260 nm using the procedure of Kalnitsky et al. (1959).

Acid deoxyribonuclease (deoxyribonucleate oligonucleotide hydrolase, E.C. 3.1.4.5) was assayed by the procedure of Kunitz (1950) as described in the Worthington Enzyme Manual (1972). It was necessary to purify partially Lumbricus deoxyribonuclease before enzyme analysis due to a significant precipitation of body wall proteins and, to a lesser extent, gut proteins when incubated with potassium acetate buffer pH 5.0. For this reason homogenates were treated with potassium acetate buffer before the reaction was measured. Equal volumes of the homogenate were incubated with 0.1 M potassium acetate buffer pH 5.0 at 37° for 30 minutes. Acetate-insoluble material was removed by centrifugation. The supernatant was then immediately used for enzyme analysis. Little if any deoxyribonuclease is believed to be lost by prior treatment with acetate buffer inasmuch as the ratio of DNAase activity to RNAase activity in partially purified preparations was the same as in homogenates.

Gluconeogenic enzymes

The assay for fructose-1,6-diphosphatase (D-fructose-1,6-diphosphate 1-phosphohydrolase, E.C. 3.1.3.11) was by the procedure of Pontremoli (1966) as described in the Worthington Enzyme Manual (1972). Freedland and Harper's method (1957) for glucose-6-phosphatase (D-glucose-6-phosphate phosphohydrolase, E.C. 3.1.3.9) was employed. The activities of aspartate transaminase (L-aspartate-alpha-ketoglutarate transaminase,
E.C. 2.6.1.1) and alanine transaminase (L-alanine-alpha-keto-glutarate transaminase, E.C. 2.6.1.2) were measured by the procedure of Chan and Cohen (1964). The mitochondrial enzyme glutamate dehydrogenase (L-glutamate: NAD(P) oxidoreductase (deaminating), E.C. 1.4.1.3) was assayed by the procedure of Fahien and Cohen (1970).

Electrophoresis of gut and body wall proteins

Electrophoresis of the gut and body wall proteins from ammonotelic and ureotelic earthworms was accomplished by the procedure described by the Canal Industrial Corp. (Canalco), Bethesda, Md. 7% acrylamide separating gels pH 8.9 and 2.5% acrylamide stacking and capping gels pH 6.7 were routinely employed. The concentration of proteins in the homogenates was adjusted before electrophoresis to 5.0 mg/ml and 4.4 mg/ml for the gut and body wall homogenates, respectively. After adjustment of the protein concentration equal volumes of 40% sucrose was mixed with the appropriate extract. 100 microliters of gut (containing 250 micrograms protein) and body wall (containing 220 micrograms protein) diluted homogenates were applied to each gel. Electrophoresis was conducted at 4 milliamps/tube using bromphenol blue as the tracking dye. When the tracking dye was within 5 mm of the end of the separating gels electrophoresis was stopped. Each gel was stained in 0.5% aniline blue black in 7% acetic acid for 2 hours. Gels were destained electrophoretically. Qualitative differences in the protein patterns were measured with a Joyce-Loebl Chromoscan Densitometer, Gateshead, England.
Protein concentration was determined by the method of Lowry et al. (1951) using bovine serum albumin, Fraction IV, Sigma Chemical Co., as the standard.
CHAPTER III

RESULTS

Metabolic sources of ammonia nitrogen

In order to determine the possible metabolic sources of ammonia in feeding earthworms, intestinal homogenates were incubated with naturally occurring nitrogenous substrates (amino acids and purines). Table 1 compares the amount of ammonia which was produced when 5 micromoles of each substrate was incubated individually with approximately 0.5 mg of homogenate protein.

Inspection of Table 1 reveals that while all amines were deaminated slightly, L-serine was deaminated approximately six times more than any other amino acid and 5'-AMP was deaminated about 25 times above this.

The enzyme involved in the deamination of L-serine was shown to be L-serine dehydratase (SDH). It was observed to be similar in its catalytic characteristics to mammalian SDH (Jost et al., 1968) i.e., it is a pyridoxal 5'-phosphate requiring enzyme and the products of the reaction, pyruvic acid and ammonia, are produced in a stoichiometric ratio.

Recent work by Jost et al. (1969) has indicated that mammalian SDH is a regulatory enzyme. It is inducible by amino acids and cyclic AMP and repressible by glucose. This observation prompted the following study to determine if the earthworm enzyme exhibited similar behavior.
Table 1. Deamination of nitrogenous substrates by gut homogenates of feeding *Lumbricus*. Each value represents the difference between the control and the experimental. The average value for the control was 6.70 micrograms NH$_3$N/mg protein/hour.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Number of analyses</th>
<th>Mean ug NH$_3$N/mg/hr</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-alanine</td>
<td>2</td>
<td>0.28</td>
</tr>
<tr>
<td>D-arginine</td>
<td>1</td>
<td>1.28</td>
</tr>
<tr>
<td>L-arginine</td>
<td>2</td>
<td>0.61</td>
</tr>
<tr>
<td>L-aspartic acid</td>
<td>2</td>
<td>1.75</td>
</tr>
<tr>
<td>L-citrulline</td>
<td>2</td>
<td>0.36</td>
</tr>
<tr>
<td>L-cysteine</td>
<td>1</td>
<td>0.79</td>
</tr>
<tr>
<td>L-glutamic acid</td>
<td>2</td>
<td>0.16</td>
</tr>
<tr>
<td>glycine</td>
<td>2</td>
<td>1.17</td>
</tr>
<tr>
<td>L-histidine</td>
<td>1</td>
<td>1.88</td>
</tr>
<tr>
<td>DL-isoleucine</td>
<td>2</td>
<td>0.52</td>
</tr>
<tr>
<td>L-leucine</td>
<td>2</td>
<td>0.75</td>
</tr>
<tr>
<td>lombricine</td>
<td>1</td>
<td>2.43</td>
</tr>
<tr>
<td>L-lysine</td>
<td>2</td>
<td>0.68</td>
</tr>
<tr>
<td>DL-methionine</td>
<td>2</td>
<td>1.16</td>
</tr>
<tr>
<td>D-ornithine</td>
<td>1</td>
<td>0.64</td>
</tr>
<tr>
<td>L-ornithine</td>
<td>2</td>
<td>0.05</td>
</tr>
<tr>
<td>DL-phenylalanine</td>
<td>2</td>
<td>-0.31</td>
</tr>
<tr>
<td>D-serine</td>
<td>2</td>
<td>1.42</td>
</tr>
<tr>
<td>L-serine</td>
<td>5</td>
<td>11.35</td>
</tr>
<tr>
<td>DL-threonine</td>
<td>2</td>
<td>0.96</td>
</tr>
<tr>
<td>DL-tryptophan</td>
<td>2</td>
<td>-1.33</td>
</tr>
<tr>
<td>L-tyrosine</td>
<td>2</td>
<td>-0.43</td>
</tr>
<tr>
<td>urea</td>
<td>1</td>
<td>0.20</td>
</tr>
<tr>
<td>DL-valine</td>
<td>2</td>
<td>1.03</td>
</tr>
<tr>
<td>adenosine</td>
<td>4</td>
<td>1.17</td>
</tr>
<tr>
<td>5'-AMP</td>
<td>5</td>
<td>251.00</td>
</tr>
<tr>
<td>ATP</td>
<td>4</td>
<td>2.07</td>
</tr>
<tr>
<td>2'-deoxyadenosine</td>
<td>2</td>
<td>0.82</td>
</tr>
</tbody>
</table>
Cyclic AMP and the phosphodiesterase inhibitor theophylline were added to intestinal homogenates to ascertain the effect of the cyclic nucleotide on SDH activity. While cyclic AMP did not stimulate this enzyme (data not shown) theophylline had a pronounced though variable effect (Fig. 1).

As can be seen from Fig. 1 different concentrations of theophylline either stimulate or inhibit SDH. High concentrations of theophylline depress enzymic activity when the substrate concentration is limiting. As the substrate concentration is increased there appears to be less inhibition. Inhibition is completely abolished when the enzyme is saturated with substrate. In fact, enzymic activity is now stimulated by theophylline.

5'-AMP is also extensively deaminated by earthworm homogenates (Table 1). It was observed that earthworm AMP deaminase was an allosteric enzyme, similar to that of other species (Askari and Rao, 1968). Titration of AMP deaminase with its substrate, 5'-AMP, produces a sigmoidal curve, as seen in Fig. 2. If 2 micromoles of its positive modulator, ATP, are added to the assay system, activity at all concentrations of substrate shows an increase. The greatest increase occurs at low AMP concentrations. ATP causes a shifting of the curve to the left, thus producing an hyperbolic curve.

Simultaneous measurement of both ammonia and phosphate revealed that 5'-AMP is deaminated directly without the prior participation of a dephosphorylating reaction.

The tissue distribution of the two predominant deaminases (SDH and AMP deaminase) along with the other two deaminases involved in purine degradation is presented in
Figure 1. Effect of theophylline on the enzymic activity of serine dehydratase. Each point represents the mean from replicate assays.

- ■■■■ 2 micromoles serine;
- ●●●● 5 micromoles serine;
- ▲▲▲▲ 100 micromoles serine.
\( \mu g \text{ Pyruvic acid/mg protein/hour} \)

\( \mu \text{moles Theophylline} \)
Figure 2. Effect of 2 micromoles ATP on the enzymic activity of 5'-AMP deaminase. Each point represents the mean from replicate assays.
AMP + ATP

AMP
Table 2. AMP deaminase is located predominantly in the gut but some activity is also present in the body wall. SDH, on the other hand, is located exclusively in the gut. Of the other deaminating enzymes, adenosine deaminase appears to possess a higher activity than 2'-deoxyadenosine deaminase.

A comparison of deaminase activity (Table 3) in gut homogenates of feeding and fasting earthworms reveals a significant decrease (p < .05) of adenosine and 2'-deoxyadenosine deaminases while AMP deaminase and SDH remain unchanged in going from the feeding to the fasting state.

**Distribution of enzymes along the intestine**

Inasmuch as ammonia is voided via the gut in *Lumbricus* (Tillinghast, 1967) it was considered of interest to compare the distribution of ammonia-generating enzymes with those enzymes whose function is for digestion or metabolism.

Fig. 3 depicts the distribution of serine dehydratase and AMP deaminase along the linear post-gizzard gut. It is evident that AMP deaminase is situated predominantly in the two anterior sections of the gut. SDH has been observed only in the third region of the post-gizzard gut.

As seen in Figs. 4 and 5 the distribution of protease activity, using either casein (Fig. 4) or the synthetic substrate, TAME (Fig. 5), gives similar results. Greatest activity is observed in sections 1 and 2 with a decline as one progresses posteriorly.

The distribution of acid and alkaline phosphatases (Figs. 6 and 7) differs from that of protease activity with
Table 2. Tissue distribution of deaminases in feeding *Lumbricus*. The body wall and seminal vesicle values represent the activity of pooled homogenates from 5 animals. Gut values were individually assayed from 5 animals.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>micromoles NH₃/N/mg protein/hour ± 1 S.E.M.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>gut</td>
</tr>
<tr>
<td>adenosine deaminase</td>
<td>0.24 ± 0.03</td>
</tr>
<tr>
<td>5'-AMP deaminase</td>
<td>20.73 ± 1.69</td>
</tr>
<tr>
<td>2'-deoxyadenosine deaminase</td>
<td>0.06 ± 0.01</td>
</tr>
<tr>
<td>serine dehydratase</td>
<td>1.79 ± 0.07</td>
</tr>
<tr>
<td></td>
<td>body wall</td>
</tr>
<tr>
<td>adenosine deaminase</td>
<td>0.13</td>
</tr>
<tr>
<td>5'-AMP deaminase</td>
<td>1.20</td>
</tr>
<tr>
<td>2'-deoxyadenosine deaminase</td>
<td>0</td>
</tr>
<tr>
<td>serine dehydratase</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>seminal vesicles</td>
</tr>
<tr>
<td>adenosine deaminase</td>
<td>0.21</td>
</tr>
<tr>
<td>5'-AMP deaminase</td>
<td>0.39</td>
</tr>
<tr>
<td>2'-deoxyadenosine deaminase</td>
<td>0.06</td>
</tr>
<tr>
<td>serine dehydratase</td>
<td>0</td>
</tr>
</tbody>
</table>
Table 3. Comparison of deaminase activities in the gut and body wall of feeding and 2 week fasting *Lumbricus*. Each value represents the mean from 5 animals.

<table>
<thead>
<tr>
<th>Intestinal deaminases</th>
<th>micromoles NH$_2$N/mg protein/hour ± 1 S.E.M.</th>
<th>Feeding</th>
<th>Fasting</th>
<th>p ≤ .05</th>
</tr>
</thead>
<tbody>
<tr>
<td>adenosine deaminase</td>
<td>0.24 ± 0.03</td>
<td>0.10 ± 0.03</td>
<td>&lt; .05</td>
<td></td>
</tr>
<tr>
<td>5'-AMP deaminase</td>
<td>20.73 ± 1.69</td>
<td>20.45 ± 2.43</td>
<td>——</td>
<td></td>
</tr>
<tr>
<td>2'-deoxyadenosine deaminase</td>
<td>0.06 ± 0.01</td>
<td>0.01 ± 0.01</td>
<td>&lt; .05</td>
<td></td>
</tr>
<tr>
<td>serine dehydratase</td>
<td>1.79 ± 0.07</td>
<td>1.60 ± 0.09</td>
<td>——</td>
<td></td>
</tr>
</tbody>
</table>

| Body wall deaminases   |                                                |         |         |         |
| 5'-AMP deaminase       | 1.20 ± 0.03                                    | 1.26 ± 0.11 | —— |
Figure 3. Gut distribution of 5'-AMP deaminase and serine dehydratase. Values in the figure represent the mean from 3 experiments assayed in replicate (+ 1 S.E.M.).

- 5'-AMP deaminase
- Serine dehydratase
Figure 4. Gut distribution of general proteolytic activity (casein as substrate). Values in the figure represent the mean from 3 experiments assayed in replicate (+ 1 S.E.M.).
Figure 5. Gut distribution of trypsin (TAME as substrate).
Values in the figure represent the mean from 3 experiments assayed in replicate (+ 1 S.E.M.).
Figure 6. Gut distribution of acid phosphatase. Values in the figure represent the mean from 3 experiments assayed in replicate (+ 1 S.E.M.).
Gut section

μmoles Nitrophenol/mg protein/20 minutes

0 0.50 1.00 1.50 2.00 2.50 3.00

- I
- II
- III
- IV

30
Figure 7. Gut distribution of alkaline phosphatase. Values in the figure represent the mean from 3 experiments assayed in replicate (+ 1 S.E.M.).
0.20 mols Nitrophenol/mg protein/20 minutes
less activity in section 2 than 1. Again, a decline in activity is observed in the posterior sections.

The distribution of glycosidase activity follows the trend observed for the above enzymes with greater activity in the anterior sections. However, within this group some differences are apparent. Thus, amylase (Fig. 8) and beta-glucuronidase (Fig. 9) are found predominantly in the first two sections while alpha-mannosidase (Fig. 10) and alpha-galactosidase (Fig. 11) activities remain high even in the posterior sections. No alpha-glucosidase (Fig. 12) was detected in the terminal portion of the post-gizzard gut.

In contrast to the foregoing, the distribution of active and total phosphorylase (Fig. 13) was more uniform along the gut. Because the samples were pooled for assay in section 4 the variance of this section could not be ascertained.

Glutamate dehydrogenase (Fig. 14) is most abundant in the third region of the post-gizzard gut. Although some activity is evident in the other regions there is considerably less than in section 3.

It is evident from the above that the distribution of enzymes along the post-gizzard gut of *Lumbricus* is not uniform. While enzymes involved with digestion are concentrated toward the anterior, other enzymes such as SDH and GDH are particularly evident in the hind-gut. Still others such as phosphorylase are rather uniformly distributed.
Figure 8. Gut distribution of amylase. Values in the figure represent the mean from 3 experiments assayed in replicate (+ 1 S.E.M.). Enzyme activity is expressed as the reciprocal of the time of the starch disappearance x 10^2/mg protein.
Figure 9. Gut distribution of beta-glucuronidase. Values in the figure represent the mean from 3 experiments assayed in replicate (+ 1 S.E.M.).
Gut section

\[ \text{\(\mu g\) Phenolphthalein/mg protein/30 minutes} \]

<table>
<thead>
<tr>
<th>Gut section</th>
<th>0</th>
<th>10</th>
<th>20</th>
<th>30</th>
<th>40</th>
<th>50</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>II</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>III</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IV</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Figure 10. Gut distribution of alpha-mannosidase. Values in the figure represent the mean from 3 experiments assayed in replicate (+ 1 S.E.M.).
μmoles Nitrophenol/mg protein/30 minutes

Gut section

I
II
III
IV
Figure 11. Gut distribution of alpha-galactosidase. Values in the figure represent the mean from 3 experiments assayed in replicate (± 1 S.E.M.).
μmoles Nitrophenol/mg protein/30 minutes

Gut section

I

II

III

IV
Figure 12. Gut distribution of alpha-glucosidase. Values in the figure represent the mean from 3 experiments assayed in replicate (+ 1 S.E.M.).
Gut section

$\mu\text{moles Nitrophenol/mg protein/30 minutes}$

Graph showing the activity of different gut sections (I, II, III, IV) with $\mu\text{moles Nitrophenol/mg protein/30 minutes}$ on the y-axis and a scale from 0 to 0.120 on the x-axis.
Figure 13. Gut distribution of active and total phosphorylase. Values in the figure represent the mean from 3 experiments assayed in replicate (+ 1 S.E.M.) except that the value from gut section IV is from pooled tissue samples.

- active phosphorylase
- total phosphorylase
$\mu$moles Phosphate/g wet wt. tissue/30 minutes

Gut section

- I
- II
- III
- IV
Figure 14. Gut distribution of glutamate dehydrogenase. Values in the figure represent the mean from 3 experiments assayed in replicate (+ 1 S.E.M.).
Metabolic changes in the transition to ureotelism

A) Protein electrophoresis

During starvation *Lumbricus* displays a marked negative nitrogen balance, the urea nitrogen output far exceeding the feeding ammonia output. We therefore attempted to determine whether any of the tissue proteins were selectively lost during the transition to the ureotelic state.

Acrylamide gel disc electrophoresis was employed to observe if tissue proteins changed with starvation. Duplicate samples from the gut and body wall of four feeding as well as four fasting animals were analyzed. Protein bands were stained and gels were subsequently scanned with a densitometer. However, these proteins could not be individually quantitated because of the overlapping of peaks.

It should be noted that there was some variation in banding patterns within both feeding and fasting animals but this was less than the variation between the gut and body wall samples. It would not be feasible to include all of the densitometer tracings, therefore only the most representative of the samples are included in the present studies.

Fig. 15 depicts two gut protein densitometer tracings: one from an ammonotelic and the other from a ureotelic animal. While differences are apparent the overlap in bands precluded precise analysis. For purposes of discussion the patterns were arbitrarily divided into 8 groups. Groups G and H did not vary under either regime, i.e. no conspicuous differences were noted in any densitometer tracings including those which are not presented here. Sections A through F tended to be
Figure 15. Comparison of gut tissue proteins from a representative feeding and 2 week fasting *Lumbricus*. Absorbance of the destained gels was measured with a Joyce-Loebl Densitometer using a red filter (620 nm). 250 micrograms of the gut tissue protein from each individual were applied to the respective gel.
Absorbance

| A | B | C | D | E | F | G | H |

Ammonotelic
Ureotelic

Origin ——— Direction of migration
more variable in fasting animals than they were in feeding individuals. Ammonotelic animals consistently possessed well developed peaks in groups A through E while ureotelic animals either had a lack of or a great reduction in these groups.

Protein groups in the body wall (Fig. 16) were divided into 5 groups. While changes during fasting were not as extreme in the body wall as compared to the gut, notable changes did occur in groups B and C. These protein complexes were reduced in fasting animals but the relative ratio of the two groups varied; sometimes B appeared to be greater than C and vice versa.

Even with a relatively crude separation of tissue proteins from ammonotelic and ureotelic animals it appears that marked changes in the relative ratio of various protein groups occurs during starvation. In all cases, the most notable changes were evident in the gut but some differences were also observed in the body wall.

B) Lysosomal and gluconeogenic enzymes

Lysosomal enzymes were released by osmotic shock. No attempt was made to differentiate between free and particulate-bound enzymes. Five to ten animals were routinely used for each analysis. For fasting conditions, earthworms were starved for at least two and no more than three weeks, at which time they were completely ureotelic. Six lysosomal enzymes were analyzed; general proteolytic activity measured at pH 8.0 was included to indicate the extent of total proteolysis.

The results, shown in Tables 4 and 5, reveal that in the gut three enzymes increase in activity, cathepsin 5.2,
Figure 16. Comparison of body wall proteins from a representative feeding and 2 week fasting *Lumbricus*. Same procedure as in Fig. 15. 220 micrograms of the body wall protein from each individual were applied to the respective gel.
Table 4. Lysosomal enzyme activities in the gut of feeding and 2 week fasting *Lumbricus*. Each value represents the mean from 5 animals.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Feeding Activity ± S.E.M./mg protein</th>
<th>Fasting Activity ± S.E.M./mg protein</th>
<th>p ≤ .10</th>
</tr>
</thead>
<tbody>
<tr>
<td>acid phosphatase</td>
<td>4.4 ± 0.5</td>
<td>5.4 ± 0.6</td>
<td>—</td>
</tr>
<tr>
<td>beta-glucuronidase</td>
<td>26.3 ± 5.2</td>
<td>30.2 ± 3.8</td>
<td>—</td>
</tr>
<tr>
<td>cathepsin (2.8)</td>
<td>31.7 ± 1.8</td>
<td>32.0 ± 4.9</td>
<td>—</td>
</tr>
<tr>
<td>cathepsin (5.2)</td>
<td>33.1 ± 2.4</td>
<td>60.7 ± 10.5</td>
<td>&lt; .10</td>
</tr>
<tr>
<td>neutral protease</td>
<td>111.8 ± 14.2</td>
<td>199.0 ± 35.5</td>
<td>&lt; .10</td>
</tr>
<tr>
<td>deoxyribonuclease</td>
<td>23.1 ± 3.6</td>
<td>19.0 ± 2.1</td>
<td>—</td>
</tr>
<tr>
<td>ribonuclease</td>
<td>6.2 ± 0.6</td>
<td>15.8 ± 0.8</td>
<td>&lt; .01</td>
</tr>
</tbody>
</table>

* The activity of acid phosphatase is expressed as micromoles nitrophenol/mg protein/hour; beta-glucuronidase as micrograms phenolphthalein/mg protein/hour; cathepsin 2.8, cathepsin 5.2, and neutral protease activity as the change in absorbance at 280 nm/mg protein/10 minutes x 10²; deoxyribonuclease as the change in absorbance at 260 nm/mg protein/minute x 10²; and ribonuclease as the change in absorbance at 260 nm/mg protein/20 minutes.
Table 5. Lysosomal enzyme activities in the body wall of feeding and 2 week fasting Lumbricus. Each value represents the mean from 5 (feeding) or 10 (fasting) animals. Activities of the enzymes are expressed as in Table 4.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Feeding</th>
<th>Fasting</th>
<th>p ≤ .10</th>
</tr>
</thead>
<tbody>
<tr>
<td>acid phosphatase</td>
<td>0.6 ± 0.1</td>
<td>0.9 ± 0.1</td>
<td>&lt; .05</td>
</tr>
<tr>
<td>beta-glucuronidase</td>
<td>0.7 ± 0.1</td>
<td>0.8 ± 0.1</td>
<td>—</td>
</tr>
<tr>
<td>cathepsin (2.8)</td>
<td>6.6 ± 0.9</td>
<td>3.2 ± 0.7</td>
<td>&lt; .05</td>
</tr>
<tr>
<td>cathepsin (5.2)</td>
<td>20.2 ± 0.8</td>
<td>10.3 ± 1.2</td>
<td>&lt; .01</td>
</tr>
<tr>
<td>neutral protease</td>
<td>40.5 ± 1.7</td>
<td>30.1 ± 2.2</td>
<td>&lt; .01</td>
</tr>
<tr>
<td>deoxyribonuclease</td>
<td>31.4 ± 7.9</td>
<td>34.7 ± 12.1</td>
<td>—</td>
</tr>
<tr>
<td>ribonuclease</td>
<td>4.9 ± 0.8</td>
<td>3.4 ± 0.8</td>
<td>—</td>
</tr>
</tbody>
</table>
neutral protease activity, and ribonuclease, while the others remain relatively constant. In the body wall, on the other hand, three enzymes, cathepsin 2.8, cathepsin 5.2, and neutral protease activity, decrease in activity during prolonged starvation. The data presented in Table 6 indicate that the activity of proteolytic enzymes are higher in the gut than in the body wall. The extent of the differences in enzymic activity between these two tissues varies as much as 38 fold.

Gluconeogenic and transaminase enzymes exhibited three notable features during fasting (Tables 7 and 8). First, the ratio of aspartate/alanine transaminase in the body wall remained relatively constant in feeding and fasting individuals (7.2 to 6.9, respectively). This ratio increased in the gut during fasting (9.2 to 15.2). Second, glutamate dehydrogenase in the gut increased during fasting more than 12 fold; no such change was evident in the body wall. Finally, fructose diphosphatase activity declined while glucose-6-phosphatase activity remained relatively constant following the transition to ureotelism.

The control of the ammonia-urea transition

When the antibiotic actinomycin D (Sigma Chemical Co.) was injected intracoelomically a dramatic inhibition of urea output was observed. A dose-response curve, employing increasing quantities of actinomycin D, was therefore performed in order to determine the quantitative effect of the antibiotic on the inhibition of urea production.
Table 6. Comparison of lysosomal enzyme activities in the body wall and gut of feeding *Lumbricus*. Data derived from Tables 4 and 5. Activities of the enzymes are expressed as in Table 4.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Activity ± 1 S.E.M./mg protein</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Body wall</td>
</tr>
<tr>
<td>acid phosphatase</td>
<td>0.6 ± 0.1</td>
</tr>
<tr>
<td>beta-glucuronidase</td>
<td>0.7 ± 0.1</td>
</tr>
<tr>
<td>cathepsin (2.3)</td>
<td>6.6 ± 0.9</td>
</tr>
<tr>
<td>cathepsin (5.2)</td>
<td>20.2 ± 0.8</td>
</tr>
<tr>
<td>neutral protease</td>
<td>40.5 ± 1.7</td>
</tr>
<tr>
<td>deoxyribonuclease</td>
<td>31.4 ± 7.9</td>
</tr>
<tr>
<td>ribonuclease</td>
<td>4.9 ± 0.8</td>
</tr>
</tbody>
</table>
Table 7. Gluconeogenic enzyme activities in the gut of feeding and 2 week fasting *Lumbricus*. Each value represents the mean from 5 animals.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Activity* ± 1 S.E.M./mg protein</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Feeding</td>
</tr>
<tr>
<td>aspartate transaminase</td>
<td>0.43 ± 0.04</td>
</tr>
<tr>
<td>alanine transaminase</td>
<td>0.05 ± 0.01</td>
</tr>
<tr>
<td>fructose diphosphatase</td>
<td>1.88 ± 0.04</td>
</tr>
<tr>
<td>glucose-6-phosphatase</td>
<td>0.24 ± 0.03</td>
</tr>
<tr>
<td>glutamate dehydrogenase</td>
<td>0.02 ± 0.01</td>
</tr>
</tbody>
</table>

* The activities of aspartate transaminase, alanine transaminase, and glutamate dehydrogenase are expressed as the change in absorbance at 340 nm/mg protein/minute; fructose diphosphatase and glucose-6-phosphatase as micromoles inorganic phosphate/mg protein/hour.
Table 8. Gluconeogenic enzyme activities in the body wall of feeding and 2 week fasting Lumbricus. Each value represents the mean from 5 animals. Activities of the enzymes are expressed as in Table 7.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Feeding</th>
<th>Fasting</th>
<th>p ≤ .10</th>
</tr>
</thead>
<tbody>
<tr>
<td>aspartate transaminase</td>
<td>0.29 ± 0.02</td>
<td>0.31 ± 0.01</td>
<td>—</td>
</tr>
<tr>
<td>alanine transaminase</td>
<td>0.04 ± 0.01</td>
<td>0.05 ± 0.01</td>
<td>—</td>
</tr>
<tr>
<td>fructose diphosphatase</td>
<td>5.20 ± 0.17</td>
<td>4.14 ± 0.09</td>
<td>&lt; .01</td>
</tr>
<tr>
<td>glutamate dehydrogenase</td>
<td>0.04 ± 0.01</td>
<td>0.05 ± 0.01</td>
<td>—</td>
</tr>
</tbody>
</table>
Twenty fresh earthworms were divided into four groups of five individuals. On days 1 and 2 each group was injected intracoelomically with 0.2 ml of solution containing either 0, 5, 10, or 15 micrograms of actinomycin D, corresponding to 0, 1, 2, or 3 micrograms/g body weight, respectively. The 24 hour urea output was then monitored periodically for one week.

Fig. 17 presents the urea output as a function of time using variable amounts of actinomycin D. Only a trace amount of urea was detected on day 3 in all groups. A rapid rise in urea output was observed in the control group after day 3. Depending upon the dose of actinomycin D which was injected, day 5 values indicate that the antibiotic depressed urea output (73 to 90% inhibition observed). As fasting progressed to day 7 the control group continued to excrete increased amounts of urea while the two experimental groups receiving the higher dosage of actinomycin D exhibit the opposite behavior. Low amounts of actinomycin D (1 microgram/g body weight) produces a 72% reduction in urea output, a proportion which remains unchanged from day 5. When 2 or 3 micrograms of the antibiotic/g body weight were injected the animals at day 7 show a complete suppression of urea excretion.

The inability to become ureotelic may in part be a reflection of the toxicity of the antibiotic. Animals injected with actinomycin D began to die on days 5 and 6 with the majority of deaths occurring on days 7 to 9. Animals which were injected with water only did not exhibit any mortality. No relationship between the dose of the antibiotic and mortality rate nor time of mortality was evident.
Figure 17. Effect of various concentrations of actinomycin D on urea nitrogen output (± 1 S.E.M.). Actinomycin D was injected on days 1 and 2.

- control;
- 1 microgram actinomycin D/g body weight;
- 2 micrograms/g body weight;
- 3 micrograms/g body weight.
μg Urea N/g body weight/day

Time (days)
The above results would suggest that DNA transcription is essential to the onset of ureotelism. In order to evaluate the effect of the antibiotic on the post-transcriptional ureotelic state the following experiment was performed.

Twenty-one fresh animals were divided into three groups: 5 animals served as controls, 8 animals were injected with 3 micrograms actinomycin D/g body weight on days 1 and 2, and 8 completely ureotelic worms were injected with the antibiotic twice (3 micrograms/g body weight), 8 hours apart, on day 6. The 24 hour output of urea was assayed at intervals throughout the experiment.

Fig. 18 demonstrates the relationship between time of injection of actinomycin D (early, days 1 and 2 and late, day 6) and the amount of excreted urea. As in the previous experiment injection of the antibiotic into ammonotelic animals almost completely inhibited urea excretion (70 to 100% inhibition, depending upon the day assayed). Controls exhibited the identical type of ureotelic pattern to that described above: noticeable amount of urea being excreted by day 4 and increasing linearly to at least day 10. Ureotelic worms injected with actinomycin D on day 6, in contrast, continued to excrete normal amounts of urea. From day 6 to day 10 urea output parallels the control value. Animals in both experimental groups survived for approximately 6 to 8 days post-injection. No mortality was noticed in the control group.

Inasmuch as actinomycin D did not block urea production once the animals were ureotelic but did prevent the onset of ureotelism in ammonotelic worms an attempt was made to ascertain the critical period in this nitrogen transition.
Figure 18. Effect of time of injection of actinomycin D (3 micrograms/g body weight) on urea nitrogen output (± 1 S.E.M.).

- control;
- actinomycin D injected on day 6;
- actinomycin D injected on days 1 and 2.
Sixteen fresh ammonotelic worms were divided into three groups: 6 animals served as controls, 5 animals were injected with 1 microgram actinomycin D/g body weight on days 1 and 4, while 5 animals were injected only on day 4. The urea output was determined at intervals for 10 days.

Fig. 19 depicts, as in previous studies, that actinomycin D administered to ammonotelic animals inhibits urea output by 85 to 92%. Urea excretion in the controls did not vary from the two previous experiments. Animals injected with the antibiotic on day 4 became ureotelic, as did the controls, but they exhibited at least a 50% reduction in the output of urea (p < .05). In this experiment, in contrast to the previous two, no mortality was observed in any of the groups, even up to day 10.

Puromycin and cycloheximide, inhibitors of protein synthesis at the translational level, were investigated in order to study the possibility of protein synthesis being necessary for either the development of ureotelism or the maintenance of the ureotelic state. A dose-response curve employing puromycin was constructed for purposes of determining the amount of puromycin needed to inhibit urea output.

Sixteen freshly collected earthworms were divided equally into four groups. Animals were injected on days 1 and 2 with 0.2 ml of solution containing 0, 25, 100, or 200 micrograms puromycin, corresponding to 0, 5, 20, and 40 micrograms puromycin/g body weight. The 24 hour output of urea was then measured on days 3, 5, and 7.
Figure 19. Effect of time of injection of actinomycin D (1 microgram/g body weight) on urea nitrogen output (± 1 S.E.M.).

- control;
- actinomycin D injected on day 4;
- actinomycin D injected on days 1 and 4.
The data presented in Fig. 20 is plotted using bar graphs instead of the usual line graphs because of the smaller degree of inhibition which was observed during this study. Animals in the puromycin injected groups were not significantly different (p > .05) with respect to urea output from the controls. The data suggests the possibility of the involvement of protein synthesis during the ureotelic state. Due to the lack of adequate response the experiment was not pursued after day 7.

The effect of cycloheximide on urea production in ureotelic animals was investigated in order to determine if continued protein synthesis was necessary for the prolongation of the ureotelic state. Ten ureotelic worms, having fasted for 10 days at the end of which time they were excreting approximately 50 micrograms urea nitrogen/g body weight/day were divided into two groups of 5 individuals each. On days 11, 12, and 13, 40 micrograms cycloheximide/g body weight were injected intracoelomically posterior to the clitellum. Urea output was then measured for the following 24 hours.

Fig. 21 demonstrates the possible involvement of continued protein synthesis for maintaining the ureotelic state. Controls show a gradual increase in urea output until day 13 after which a slight decline was noted. Cycloheximide injected animals initially showed an increase in urea output to day 12. After day 12 a dramatic decline in urea excretion occurs. Within a two day period of time (day 12 to 14) and 85% inhibition of urea excretion occurs in cycloheximide injected animals.
Figure 20. Effect of various concentrations of puromycin on urea nitrogen output (+ 1 S.E.M.). Puromycin was injected on days 1 and 2.
Figure 21. Effect of cycloheximide (40 micrograms/g body weight) on urea nitrogen output (± 1 S.E.M.). Cycloheximide was injected on days 11, 12, and 13.

●—● control;
■—■ cycloheximide.
All animals injected with cycloheximide died one day after the termination of the experiment.
CHAPTER IV

DISCUSSION

Metabolic sources of ammonia nitrogen

There are a variety of enzymes which are known to produce ammonia in animals. These include oxidative and non-oxidative deaminases, amidases, nucleodeaminases, and hexosamine deaminases (Cohen and Brown, 1960). To this one may add urease as an important source of ammonia in mammals and one which is apparently of microbial origin (Visek, 1972). While the ammonia-generating enzymes have been shown to play an important role in nitrogen metabolism in vertebrates their role in the metabolic economy of the invertebrates is less well understood. The present investigation was undertaken in an attempt to determine the metabolic source of ammonia in the earthworm and to try to correlate its production with the activity of some of the above enzymes.

When individual amino acids were incubated with gut homogenates from feeding earthworms there was a consistently higher production of ammonia from serine (Table 1). While it was later shown that the deamination of serine was due to serine dehydratase (SDH), a pyridoxal 5'-phosphate requiring enzyme, the data in Table 1 was obtained without added cofactor. Apparently, sufficient cofactor is present in crude homogenates for SDH activity. Further analysis demonstrated that SDH activity in homogenates without added cofactor was approximately two thirds its maximal activity when cofactor was present.
Subsequent experiments therefore, were performed with the addition of exogenous pyridoxal 5'-phosphate.

In comparison to serine, other amino acids were deaminated only slightly. The minimal deamination of aspartate, histidine, and glutamate appears unusual inasmuch as other investigators have demonstrated that these amino acids are extensively deaminated in various ammonotelic species. For example, teleost liver homogenates (Janicki and Lingis, 1970) are capable of deaminating aspartate and glutamate even in the absence of exogenously added cofactor, NAD^+. And in a related study teleost and elasmobranch homogenates (liver, kidney, brain) were able to deaminate aspartate as well as histidine (Salvatore et al., 1965). It appears that Lumbricus may be unique among ammonotelic species in that only serine is able to serve as a deaminating substrate, other amino acids being deaminated only to a slight extent. Alternatively, enzyme cofactors may be lacking in our homogenates which might prevent the deamination of certain amino acids.

In earlier studies in vivo (Cohen and Lewis, 1949) it was found that arginine and citrulline slightly increased the ammonia output of intact Lumbricus. In a more recent dietary study earthworms fed ad libitum with protein dramatically increased their ammonia output (Tillinghast and Janson, 1971). Apparently a relationship exists between dietary nitrogen and ammonia production. No similar relationship would appear to exist between protein intake and urea synthesis (Cohen and Lewis, 1949; Tillinghast and Janson, 1971).
During the course of the present studies it was observed that ATP was occasionally deaminated by gut homogenates with the amount of ammonia liberated from ATP being dependent upon the relative age of the ATP preparation. It was therefore assumed that ATP underwent spontaneous dephosphorylation to produce a variety of purine intermediates. Additionally, a recent report demonstrated the production of ammonia from 5'-AMP in marine polychaetes (Bishop and Barnes, 1971). For these reasons a number of purine derivatives of adenine were investigated to observe if they could possibly serve as substrates for ammonia liberation. Table 1 reveals that of the four purines assayed the mononucleotide 5'-AMP was deaminated to the greatest extent, surpassing the deamination of serine by more than an order of magnitude.

AMP deaminase, the enzyme involved in the deamination of 5'-AMP, is a widely distributed enzyme, having been found in vertebrates from elasmobranchs (Makarewicz, 1968) to mammals (Cunningham and Lowenstein, 1966; Askari and Rao, 1968). While information on its distribution in invertebrates is incomplete, AMP deaminase has been found in polychaetes (Bishop and Barnes, 1971) and in the earthworm Lumbricus (Umiastowski, 1964). On the other hand its activity in crayfish and lobster (Roush and Betz, 1956), oysters (Ishida et al., 1969), scallops (Kitagawa and Tonomura, 1957) and the cockroach (Cochran, 1961) is very low or absent.

AMP deaminase in Lumbricus is located predominantly in the gut, with approximately 20 times greater activity than that of the adjacent body wall (Table 2). A similar though
less accentuated distribution has been recorded in the marine polychaete *Abarenicola pacifica* (Bishop and Barnes, 1971). Previous results with the earthworm (Umiastowski, 1964) were concerned only with the body wall and therefore no comparisons can be made with the present work.

AMP deaminase in vertebrates has repeatedly been shown to be an allosteric enzyme (Cunningham and Lowenstein, 1966; Lee and Wang, 1968). ATP functions as an allosteric activator (Setlow and Lowenstein, 1968) while 2,3-diphosphoglyceric acid (Askari and Rao, 1968) and GTP (Setlow et al., 1966) appear to be allosteric inhibitors. The present studies suggest a similar allosteric nature for *Lumbricus* AMP deaminase.

AMP deaminase exhibits a typical sigmoidal, substrate-dependent concentration curve in the absence of exogenous ATP (Fig. 2). The addition of a constant amount of ATP (2 micromoles) shifts the curve, producing an hyperbolic curve. This effect has also been observed by a number of investigators (Cunningham and Lowenstein, 1966; Setlow and Lowenstein, 1967; Askari and Rao, 1968; Lee and Wang, 1968) with AMP deaminase from other species.

ATP activation of AMP deaminase is dependent upon the concentration of substrate employed. Maximum activation occurs at low substrate concentrations with little or no effect observed when the enzyme is saturated with its substrate (Fig. 2).

The apparent Km for AMP deaminase in a number of vertebrates has been calculated by Ronca-Testoni and his co-workers (1970) to be slightly less than 1 millimolar AMP while
others have shown that the Km lies between 1 to 6 millimolar AMP (Dingle and Hines, 1967; Makarewicz, 1969; Currie and Webster, 1962). The present work with *Lumbricus* indicates that the Km is 1.1 millimolar AMP. The addition of 2 micro­moles ATP decreases the Km to 0.6 millimolar AMP. ATP in this system appears to decrease the Km about two-fold while having no effect on the maximum velocity of the reaction. Cunningham and Lowenstein (1966) have shown that ATP increases the apparent affinity of calf brain AMP deaminase for its substrate about six fold.

It presently appears that 5'-AMP may be degraded by two major pathways depending upon whether the amine or phosphate group is removed first. In either case inosine, ammonia, and phosphate are the eventual end products. The difference lies in whether adenosine or 5'-IMP is the intermediate. Thus, the two pathways can be summarized as follows:

Scheme A: 5'-AMP $\xrightarrow{-\text{NH}_2}$ 5'-IMP $\xrightarrow{-\text{PO}_4}$ inosine

Scheme B: 5'-AMP $\xrightarrow{-\text{PO}_4}$ adenosine $\xrightarrow{-\text{NH}_2}$ inosine

The present evidence suggests that scheme A may occur in *Lumbricus*. First, when 5'-AMP is added to homogenates inorganic phosphate was not liberated into the reaction medium but ammonia was rapidly produced in large amounts. Second, ammonia liberation from adenosine is two orders of magnitude less than from 5'-AMP (Tables 1 and 2). Although the appearance of 5'-IMP was not investigated it appears reasonable to suggest that 5'-AMP in *Lumbricus* is degraded by an initial
deamination reaction (scheme A) and not by a dephosphorylation reaction as illustrated in scheme B.

Scheme A (deamination) is present and believed to be operational for the production of ammonia in marine polychaetes (Bishop and Barnes, 1971). Not only is ammonia liberated from 5'-AMP but $^{14}$C-labeled 5'-AMP is converted to $^{14}$C-labeled 5'-IMP by polychaete gut homogenates.

Some invertebrates have been shown to possess scheme B (dephosphorylation). Cochran (1961), studying the time course of ammonia and phosphate release from 5'-AMP in the thoracic muscle of the cockroach Periplaneta americana, has shown that phosphate is released prior to ammonia; ammonia liberation from adenosine takes place at a faster rate than from 5'-AMP; and adenosine and inosine, but not 5'-IMP, accumulated when 5'-AMP served as substrate. He concluded that dephosphorylation followed by deamination of adenosine occurred in the cockroach. Likewise, the midgut of the oyster possesses scheme B inasmuch as 5'-AMP is dephosphorylated and the product, adenosine, is then deaminated to form inosine (Kitagawa and Tonomura, 1957).

There are two alternative metabolic pathways by which ammonia may be generated in vivo. One of these is via the transaminase-glutamate dehydrogenase series of reactions in which the amine group is shuttled through a series of intermediate reactions until split from glutamic acid by glutamate dehydrogenase. This series of reactions may be depicted as follows:
(a) amino acid + alpha-ketoglutarate $\xrightarrow{\text{transaminases}}$ glutamate + alpha-keto acid

GDH

(b) glutamate $\rightarrow$ alpha-ketoglutarate + NH$_3$

In tissues where glutamate dehydrogenase is very low or absent (e.g. skeletal muscle) there appears to be a second mechanism for ammonia production, a purine nucleotide-transaminase cycle. By means of spectrophotometric analysis, Lowenstein and his colleagues have demonstrated the complete cycle of reactions in this metabolic scheme (Lowenstein and Tornheim, 1971; Tornheim and Lowenstein, 1972; Lowenstein, 1972). This may be outlined as follows:

(a) 5'-AMP + H$_2$O $\xrightarrow{\text{deaminase}}$ 5'-IMP + NH$_3$

(b) 5'-IMP + GTP + aspartate $\xrightarrow{\text{synthetase}}$ adenylosuccinate + GDP + Pi

(c) adenylosuccinate $\rightarrow$ 5'-AMP + fumarate

Bishop and Barnes (1971), noting a low GDH and a high AMP deaminase activity in polychaetes, have suggested the participation of the purine cycle in the production of ammonia. Earlier, Makarewicz (1963) came to the conclusion that some form of nucleotide cycle must exist in certain teleosts and lampreys inasmuch as a low level of GDH and a high activity of AMP deaminase in the gills of these animals was observed.

Lumbricus is similar to the polychaetes and some teleosts in that it possesses a high AMP deaminase activity.
(Table 2 and 3) and a very low GDH level (Tables 7 and 8).

It should be noted at this point that GDH dramatically increases in *Lumbricus* during fasting. We believe that the increase in GDH activity is involved in urea, but not ammonia production. This point will be considered in detail later.

Hence, although it is not possible at this time to state unequivocally that the purine nucleotide-transaminase cycle is functional in *Lumbricus* the preliminary results discussed above suggest this possibility. Further studies are necessary to establish this critical point.

**Enzyme distribution along the intestine**

It has been demonstrated that although urea is voided via the nephridia of *Lumbricus* ammonia nitrogen is discharged with the gut contents (Tillinghast, 1967). In order to locate the site of origin of ammonia we have analyzed the gut along its length for a variety of ammonia-generating enzymes and have used both digestive enzymes as well as selected metabolic enzymes (e.g. phosphorylase) as a frame of reference.

The distribution of digestive enzymes observed in the present study (Figs. 4 to 12) in general supports observations made earlier on both oligochaetes and polychaetes. Thus, Heren (1956) and Van Gansen (1963) have recorded high levels of both proteolytic and amylolytic enzymes in the anterior intestine of *Lumbricus* as well as *Eisenia* with declining values in the posterior intestine. While Li and Shetlar (1965) did not extend their work on glycosidases in *Lumbricus* to the linear distribution, the present studies (Figs. 10 to 12) show a parallel with other digestive enzymes. This linear
specialization of the gut is also suggested by the work on polychaetes by Dales (1955) as well as Marsden (1963). The present study supports the general description of the intestine by Jeuniaux (1969) that the "stomach and foreintestine are mainly the regions of enzyme secretion and food degradation, while the function of the mid-intestine and hind-intestine are merely restricted to absorption and feces formation".

Of necessity, the analysis of the linear distribution of gut enzymes, both here and in all earlier studies (Heran, 1956) has utilized homogenates containing both gut luminal epithelium and chloragogen tissue as well. This procedure is dictated by the close apposition of these tissues and the lack of simple and suitable methods for their separation. Thus, one can only make conjectures as to the location of particular enzymes.

It would seem reasonable to postulate that digestive enzymes are located in the luminal epithelium. On the other hand, because glycogen is located in the chloragogen tissue of Lumbricus (Tillinghast, unpublished observations) one would expect glycogen phosphorylase to be so located. Acid and alkaline phosphates, however, have different distributions, the latter in the luminal epithelium and the former in the chloragogen tissue as demonstrated histochemically (Van Gansen, 1963). We suggest that the ammonia-generating enzymes are located in the luminal epithelium.

The post-gizzard gut of Lumbricus is highly specialized both in its gross and cellular morphology (Arthur, 1963). By contrast, the chloragogen tissue shows no such regional
specialization. It is easier to imagine that the unique location of ammonia-generating enzymes (AMP deaminase, serine dehydratase, and glutamate dehydrogenase) is correlated with the observed anatomical specialization of the luminal epithelium. Moreover, this location would allow ammonia excretion directly into the gut, rather than indirectly via the coelomic fluid and blood.

Needham (1957) has observed that feeding worms void greater quantities of titratable acid than fasting worms and has suggested that ammonia may serve a buffer function in feeding individuals. In view of the near neutral pH values recorded in the intestinal lumen, it is conceivable that ammonia serves to regulate the pH for optimal digestive enzyme action. Alternatively, ammonia may serve in a base conservation mechanism such as exists in the mammalian kidneys (reviewed by Lotspeich, 1967). That two centers of ammonia production were observed in this study indicate that these two functions are spatially separated. Thus, it is of interest that while the pH of the anterior gut is near neutrality (pH 6.5 to 7.5), the pH of the hind-gut is slightly more alkaline (pH 7.6 to 8.0) (Heran, 1954).

**Metabolic changes in the transition to ureotelism**

Since *Lumbricus* enters a strong negative nitrogen balance with fasting it seemed appropriate to investigate the possible tissue origin of urea nitrogen. Cohen and Lewis (1949) suggested that urea nitrogen in *Lumbricus* might be derived from the catabolism of tissue proteins and, in a later study,
Cooper and Baculi (1968) observed histological changes in gut and body wall tissues during a protracted period of inadequate nutrition. The present study was therefore initiated in an attempt to observe not only changes in gross tissue proteins but also to correlate these changes with the activity of lysosomal and gluconeogenic enzymes.

That proteins are mobilized in a selective manner during starvation is clearly indicated in Figs. 15 and 16. Thus, while some proteins appear unchanged others are particularly labile. The changes in tissue proteins were more evident in the gut tissue than in the body wall. It is of considerable interest that proteolytic activity rises in the gut tissue during fasting and is considerably higher than in the body wall. These observations would seem to suggest that selected gut tissue proteins are used as a source of urea nitrogen, at least during the first two weeks of fasting.

The rise in proteolytic enzymes in the gut poses something of a dilemma in that it has previously been thought to indicate that with fasting, digestive proteases may be accumulating. That this rise is most evident in the anterior gut (Heran, 1956) would lend support to this view. Alternatively, as we have suggested, this rise may be related to proteolytic activity in support of urea biosynthesis. A comparison of the changes in activity of other digestive enzymes with fasting would be of value.

The changes observed in the activity of the lysosomal enzymes in Lumbricus were not as marked as those in the dissolution of the tail of the amphibian Xenopus (Weber, 1967).
This would be expected in view of the fact that tissue histolysis is not as extensive in Lumbricus as in Xenopus.

In the present studies the ratio of aspartate/alanine transaminase increased slightly (9.2 to 15.2) in the gut but not in the body wall during ureotelism. In other species exhibiting a similar ammonotelic-ureotelic transition these two transaminases have also been studied. In the African lungfish aspartate transaminase does not change in activity either during starvation or during estivation but alanine transaminase decreases approximately 90% in estivating animals (Janssens, 1964a). However, during metamorphosis of Rana catesbeiana there is a 4 fold increase in aspartate transaminase and a slight decline in alanine transaminase (Chan and Cohen, 1964). In Xenopus, a similar phenomenon is observed, the ratio of aspartate transaminase to alanine transaminase showing an increase at the period of maximum urea synthesis (Janssens, 1964b). It appears that there may be two mechanisms for varying the transaminase ratio: increasing aspartate and maintaining alanine transaminase at a constant level or maintaining aspartate constant and decreasing alanine transaminase. Therefore, gluconeogenesis may be occurring to a slight extent in Lumbricus inasmuch as these changes in the transaminases were in the same direction as in amphibians and teleosts.

Glutamate dehydrogenase, although not primarily considered as a gluconeogenic enzyme, exhibits a marked increase in activity in gut tissue but not body wall. Of all the enzymes investigated in the present study, GDH exhibits the
most noticeable change, increasing by more than 12 fold during ureotelism. The increased level of GDH has also been reported by Cohen's group (DeGroot and Cohen, 1962; Wiggert and Cohen, 1966) in metamorphosing amphibians.

The pivotal role of the GDH reaction has been reported in other systems (Hochachka, personal communication). It appears at the present time that $\text{NH}_4^+$ can be either channeled into ammonia or urea production, ammonia production by the glutamine synthetase reaction and urea production by the carbamyl phosphate synthetase and N-acetyl glutamate systems. Whether or not this actually occurs in *Lumbricus* is open to question, but it may be possible since the present work raises the possibility that GDH and the transaminases may be closely regulated during ureotelism.

Studies on the control of the ammonia-urea transition

The thyroid hormone has been shown to initiate ureotelism in the metamorphosing tadpole by stimulating both transcription and translation (Nakagawa and Cohen, 1967; Unsworth and Cohen, 1968). While no hormone has yet been demonstrated to initiate the onset of ureotelism in *Lumbricus*, the present experiments suggest that if such a hormone exists it most likely functions at the level of the genome. The results of experiments with actinomycin D (Fig. 17) demonstrate that urea excretion was markedly effected by the antibiotic, the amount of voided urea being inversely related to the dose of the antibiotic. Little effect is observed, however, if actinomycin D is administered to animals which are ureotelic.
(6 days starvation, Fig. 18). It appears that once the urea-generating apparatus is firmly established the antibiotic is without effect.

If actinomycin D is injected when earthworms are just becoming ureotelic (excreting about 1 microgram urea nitrogen/g body weight/day) the pattern of urea excretion is entirely different (Fig. 19). Thus, earthworms injected with actinomycin D after only four days of fasting excrete approximately half the normal amount of urea for the remaining six days.

The effects of puromycin and cycloheximide, inhibitors of protein synthesis at the translational level, were studied to observe if continued protein synthesis was necessary for ureotelism. Because puromycin and cycloheximide are rapidly metabolized it is frequently necessary to employ repeated doses to achieve the most effective result. This was not possible in the present studies inasmuch as multiple injections were repeatedly observed to cause tissue autolysis near the site of injection.

While injections of puromycin administered to ammonotelic animals did exert a slight inhibitory effect on subsequent urea output, more frequent doses may be required to observe changes as recorded for actinomycin D. By contrast, cycloheximide clearly inhibited urea production (Fig. 21). It would therefore appear that urea synthesis is dependent upon continued protein synthesis.

While the foregoing results clearly indicate the essential role of RNA synthesis to the onset of ureotelism in *Lumbricus*, the fact cannot be dismissed that the antibiotics
employed have other metabolic effects. These side effects could lead to observations similar to those recorded here.

(1) Actinomycin D, being a highly toxic antibiotic, causes complete mortality within three to four days in tadpoles using as little as 1 microgram/g body weight (Nakagawa et al., 1967). Hence, its effect on urea excretion in Lumbricus may be a reflection of its general toxicity. Although actinomycin D causes mortality within 6 to 9 days in Lumbricus it appears that the antibiotic has no side effect once ureotelism is established (Fig. 18).

(2) Two of the intermediate reactions of the urea cycle require ATP. That ATP production may be inhibited by actinomycin D has been shown by the work of Laszlo et al., (1966). They noted that actinomycin D (50 micrograms/ml) depressed ATP production approximately 90% in cultured human leukemic lymphocytes. In our work with Lumbricus actinomycin D was employed at much lower concentrations (1 to 3 micrograms/g body weight). If ATP production in Lumbricus is effected by actinomycin D it is highly unlikely that ATP is the limiting factor inasmuch as urea synthesis continues unabated in ureotelic animals injected with this compound (Fig. 18).

Respiration and glycolysis in cultured human lymphocytes (Laszlo et al., 1966) and oxygen consumption in aggregating embryonic sea urchin cells (Giudice, 1965) have been shown to be depressed in the presence of actinomycin D.

It has long been known that the urea cycle enzymes rise in activity during the transition to ureotelism in
amphibians. (Dolphin and Frieden, 1955; Brown et al., 1959; Domm and Janssens, 1971). Similar data have been obtained for the transition in *Lumbricus* (Bishop and Campbell, 1965). Evidently, the rate-limiting reaction differs among these species; for *Rana catesbeiana* it is argininosuccinate synthetase (Brown et al., 1959), while it is the arginine synthetase system for the corroboree frog *Pseudophryne corroboree* (Domm and Janssens, 1971), and the citrulline synthesis system in *Lumbricus terrestris* (Bishop and Campbell, 1965).

Domm and Janssens (1971) were unable to detect two of the urea cycle enzymes in tadpoles of *Pseudophryne*, namely argininosuccinate synthetase and argininosuccinate lyase. No such deficiencies were noted in feeding *Lumbricus* in the studies of Bishop and Campbell (1965); all enzymes are evident, albeit at lower activity than in the fasting state.

For two reasons we have some uncertainty whether the observations of Bishop and Campbell (1965) are correct. Firstly, we have observed that commercially obtained worms frequently void small amounts of urea in contrast to fresh, locally obtained specimens which void no urea during the first 24 hours of analysis. Bishop and Campbell (1965) used a commercial source of worms. Secondly, according to the data of Bishop and Campbell (1965) their animals were already partially ureotelic. Evidence presented in Fig. 19 indicates that partially ureotelic animals injected with actinomycin D become ureotelic. Apparently, the urea cycle apparatus is already established at this time.
Even though discrepancies exist in the literature regarding the ammonotelic-ureotelic transition in *Lumbricus* it is possible to conclude from the present studies that for ureotelism to develop, prior RNA synthesis is required. Within the first four days of fasting synthesis of RNA occurs to support partial ureotelism. An additional two days of fasting will allow the complete development of ureotelism. It appears, therefore, that the stability of RNA in our system is moderately long-lived in the sense that it is present for at least six days post-injection during which time ureogenesis continues to occur.
REFERENCES CITED


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