A COMPARATIVE STUDY ON THE HEMOGLOBINS AND INTESTINAL ISOZYMES OF THREE SPECIES OF EARTHWORMS

KENNETH VASKEN KALOUSTIAN

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A COMPARATIVE STUDY ON THE HEMOGLOBINS AND INTESTINAL ISOZYMES OF THREE SPECIES OF EARTHWORMS

Keywords
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A COMPARATIVE STUDY ON THE
HEMOGLOBINS AND INTESTINAL ISOZYMES
OF THREE SPECIES OF EARTHWORMS

by

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A.B., Boston University, 1969
M.S., University of Bridgeport, 1973

A THESIS

Submitted to the University of New Hampshire
in Partial Fulfillment of
The Requirements for the Degree of

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Graduate School
Department of Zoology
September, 1976
This thesis has been examined and approved.

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ABSTRACT

A COMPARATIVE STUDY ON THE
HEMOGLOBINS AND INTESTINAL ISOZYMES
OF THREE SPECIES OF EARTHWORMS

KENNETH VASKEN KALOUSTIAN

Studies on the physiology of earthworms have contributed much to our fundamental knowledge of biological systems. The now classical study of Ramsay (1949) on the nephridia of *Lumbricus* is but one example of the usefulness of these organisms in biological research. Most research on earthworms, however, has been concentrated on the large and conveniently available *Lumbricus terrestris*. Less attention has been paid to the smaller, but frequently more abundant *Allolobophora* and *Eisenia*. In the present study we have examined the relatedness of the hemoglobins and intestinal isozymes of *Lumbricus terrestris*, *Allolobophora caliginosa*, and *Eisenia foetida*. In addition we have examined changes that occur in these molecules with estivation in *Allolobophora caliginosa*.

1. The hemoglobins of all three species had approximately the same molecular weight (3.4 x 10^6 daltons) and identical absorption spectra.

2. Antibody prepared against *Lumbricus* hemoglobin showed a partial identity reaction with both *Allolobophora* and *Eisenia* hemoglobins.
3. Isoelectric focusing experiments showed Lumbricus and Allolobophora hemoglobins to have identical isoelectric points (pH 5.38); that of Eisenia was 5.48.

4. Polyacrylamide disc gel and agarose electrophoresis showed all three hemoglobins to be intact at neutral pH values, but to dissociate into smaller submultiples in alkaline media (pH ≥ 8.0).

5. The stability of the intact pigment was shown not to be dependent upon divalent cations.

6. Eisenia and Allolobophora showed a greater degree of similarity in their isozyme profile compared to the profile of either with Lumbricus.

7. The profile of isozymes in Allolobophora changed dramatically with estivation.

8. With estivation, Allolobophora changed from an ammonotelic pattern of nitrogen excretion to a ureotelic pattern. The tissue urea level rose ten-fold during estivation.
I. INTRODUCTION

The annelids are segmented worms and represent the most advanced morphological development among worms (Eddy and Hodson, 1961). The phylum is divided into several classes; the most important, in terms of number of living species, are the Polychaeta, Oligochaeta, and Hirudinea.

One of the major differences between oligochaetes and polychaetes is in the structure of their genital organs. The genitalia of the Polychaeta, which has separate sexes, are simple. Sexual cells are produced from the coelomic epithelium and released into the sea by rupturing the body wall. The Oligochaeta, by contrast, are hermaphroditic, with their sexual organs confined to two or three segments, and possess specialized and complex mechanisms of fertilization and dispersal of eggs (Meglitsch, 1972).

Dales (1967) divided the oligochaetes into four orders according to the position of the opening of the vasa deferentia in relation to the testes and to the relative position of the spermatheca. In the order Opisthopora, which includes all the earthworms, the vasa deferentia open further back from the segments in which the testes lie, while in the other orders the vasa deferentia open in the same segment or the segment following that containing the testes.

The majority of earthworms fall into one of four families, of which the Lumbricidae, including Lumbricus, Allolobophora, and Eisenia, is a familiar example. The members of all four families have paired testes in the tenth and/or eleventh segments of the body, with ovaries in the thirteenth segment. Thus, it is the arrangement of the genital segments and gonoducts which form the basis for the classification of
the oligochaetes.

The foregoing system as such is regarded as a convenient working classification but not one that reliably reflects the affinities and relationships between the earthworms (Clarke, 1969). An additional approach is to consider the environments in which the earthworms live and the types of physiological and biochemical adaptations required for life in these various habitats.

Most species of earthworms have a wide habitat distribution occurring all over the world, but rarely in deserts, areas under constant snow and ice, mountain ranges, and areas entirely lacking in vegetation (Edwards and Lofty, 1972). Stephenson (1930) has reported *Eisenia foetida* to be the most common earthworm in the United States, with *Allolobophora caliginosa* the second most abundant. The latter is commonest in Western Europe, India, Australia, and Africa. Although *Lumbricus terrestris* is generally identified as the "common earthworm" it is nevertheless less abundant in numbers in areas where *Allolobophora* and *Eisenia* are found (Stephenson, 1930).

To the extent that the comparative physiology and biochemistry of annelids have been investigated, there appears to be little evidence of phyletic trends (Clarke, 1969). The pattern of nitrogen excretion provides but one example of this. The earthworms *L. terrestris* and *A. caliginosa* are ammonotelic while feeding but become ureotelic with fasting (Cohen and Lewis, 1949; Needham, 1957; Tillinghast et al., 1969) whereas *E. foetida* differs from both by excreting primarily urea in both feeding and fasting states. The total nitrogen output of *Eisenia* however does not rise with fasting as seen with *Lumbricus* and *Allolobophora*. Another important difference between these species is the unique capability of
Allolobophora to estivate during unfavorable habitat conditions (e.g. during dry periods). In contrast, both Lumbricus and Eisenia avoid unfavorable conditions by retreating to moister soils (Grant, 1955).

In spite of these apparent variations, earthworms manifest close similarities with respect to their respiratory pigments (Laverack, 1963). Three iron-containing respiratory pigments, hemoglobin, chlorocruorin, and hemerythrin, have been identified in different annelids, and while the distribution of these pigments is very erratic among the polychaetes, most oligochaetes appear to possess hemoglobin (Florkin, 1969). Studies with Lumbricus hemoglobin (Rossi-Fanelli et al., 1970) have shown it to be a very high molecular weight protein (3.4 x 10^6 daltons) composed of many subunits. All existing data confirm that the prosthetic group of Lumbricus hemoglobin is the same as that in mammalian hemoglobin and that there is one iron atom per 17,250 molecular weight units (Florkin, 1969).

The application of electrophoresis in the study of enzymes when used in conjunction with other physiological and biochemical data, has been of considerable value in describing differences and affinities among many species (Leone, 1962; Markert, 1975; Avise, 1975). Certain enzymes such as the esterases and phosphatases, for example, act on a variety of substrates that originate in the environment and thus the isozyme pattern of these enzymes frequently reflect differences in environments (Gillespie and Kojima, 1968; Kojima et al., 1970; Johnson, 1974). The variability of the isozyme distribution may therefore provide additional knowledge about the type of physiological and biochemical adaptation required for survival. To our knowledge, the following represents the first attempt to compare the affinities and the relationships between
the oligochaetes by isozyme studies.

The purpose of the present research was to ascertain the physiological similarities and differences between three closely related species of earthworms: *Lumbricus terrestris*, *Allolobophora caliginosa*, and *Eisenia fetida*. In the first study, we compare the physicochemical characteristics of the hemoglobins as well as the intestinal isozyme profiles of these three species. In the second study we have recorded the changes that occur in the hemoglobin and the intestinal isozymes with estivation in *Allolobophora caliginosa*. 
II. STUDIES ON THE HEMOGLOBINS AND INTESTINAL ISOZYMES OF SEVERAL EARTHWORMS

INTRODUCTION

The structure of a number of annelid extracellular hemoglobins has been examined in detail (Roche, 1965; Rossi-Fanelli et al., 1970; Chiancone et al., 1972; and Wiechelman and Parkhurst, 1972). These hemoglobins share several common features such as acidic isoelectric points and sedimentation constants in the range of 55-60S, corresponding to a molecular weight of about $3.4 \times 10^6$ daltons (Svedberg, 1933). The intact hemoglobin has been shown to dissociate into submultiples of 10S (Levin, 1963). The dissociation of these subunits in certain polychaetes appears to be dependent on the divalent cations Mg$^{2+}$ or Ca$^{2+}$ (Terwilliger et al., 1975) whereas in Lumbricus (Chiancone et al., 1972) and Arenicola (Waxman, 1971) the integrity of the intact hemoglobin is not dependent on the presence of divalent cations.

Unlike the situation with the hemoglobins, there are no detailed comparative studies on isozymal distribution in different tissues of the annelids. The occurrence of diverse isozymes in different tissues of organisms is thought to serve metabolic roles that differ somewhat from one tissue to another (Brewer and Sing, 1970). In addition, isozyme studies are currently being used in an attempt to answer questions about relative degree of similarity between different organisms (Avise, 1975).

In this communication we report on two areas of potential differences among the earthworms *Lumbricus terrestris*, *Allolobophora caliginosa* and *Eisenia fetida*: (1) The physical nature of their hemo-
globins, and (2) the distribution of isozymes (phosphatases and esterases) along the length of the digestive tract.

**MATERIALS AND METHODS**

Specimens of mature *Lumbricus terrestris*, and *Allolobophora caliginosa* were collected locally and maintained at 15°C in soil supplemented with worm bedding (Earlybird Co., Boise, Idaho). *Eisenia foetida* were collected locally from compost piles and transferred to jars containing the compost sample and kept at 15°C. All three species were identified using the taxonomic keys prepared by Eddy and Hodson (1961).

**Blood collection**

Individual worms were anaesthetised with 10% ethanol, rinsed with tap water, and dried by blotting. Blood was collected in 40 μl micropipettes by severing the blood vessels and "hearts" in the anterior segments of the worms. About 50 to 70 μl of blood could be obtained from *L. terrestris*, 20-30 μl from *A. caliginosa*, and 5-10 μl from *E. foetida*. Pooled samples of blood (0.3 ml) from each individual species were centrifuged at 13,000 x g for 20 minutes in a Sorvall RC-2B Refrigerated Centrifuge to remove particulate matter. The supernatant fluid was removed and added to 0.1 ml of 0.1 M potassium phosphate buffer, pH 7.0, containing 10^{-3} M EDTA, then centrifuged again at 13,000 x g to assure complete removal of all particulate matter. Blood prepared in this manner was used for hemoglobin isolation.
Hemoglobin isolation

Gel filtration was conducted in a 1.5 x 90 cm column of Bio-Gel A-5m (Bio-Rad Laboratories) and the hemoglobin eluted with 0.1 M potassium phosphate, pH 7.0, containing \(10^{-3}\) M EDTA as described previously (Rossi-Fanelli et al., 1970). A 0.2 ml sample of blood was applied to the column, eluted in 1 ml fractions, and the fractions monitored at 418 nm in Beckman DB-GT spectrophotometer. All column chromatographic separations were performed at 4°C. The fractions of the eluted hemoglobins were pooled and concentrated in an Amicon Ultrafiltration chamber with an X100A filter. Elution profiles and rate of fractionation of the hemoglobins from each species were recorded and used for molecular weight comparisons.

Hemoglobin measurements and absorption spectra

Hemoglobin calculations were carried out by the Hycel cyanmethemoglobin method (Hycel Inc., Houston, Texas). Standard curves were established on both Spectronic 20 (Bausch and Lomb) and Beckman DB-GT spectrophotometers. Twenty microliter samples of blood were assayed for each species. In the case of Eisenia, blood was pooled in order to obtain this volume (20 µl).

Absorption spectra (1000-200 nm) of the hemoglobins from each species were performed in a Beckman DB-GT spectrophotometer at room temperature and recorded on a Beckman 10" recorder.

Subunit dissociations of the hemoglobins

The effects of pH on the dissociation of earthworm hemoglobins into subunits were studied using agarose gel electrophoresis at pH 6.0 and 7.0 (0.05 M sodium citrate with \(10^{-3}\) M EDTA) and pH 8.6 (0.05 M...
sodium barbitol with $10^{-3}$ M EDTA).

**Isoelectric focusing**

Ampholine electrofocusing at a pH range of 3.5-7.0 was carried out in 1% agarose (0.05 M sodium citrate with $10^{-3}$ M EDTA at pH 6.0) to determine the isoelectric points of the earthworm hemoglobins. A steady current of 2mA/slide was applied for 5 hours. Upon completion of the run the hemoglobin bands were fixed in 12% trichloroacetic acid (TCA). The pH gradient of the standards (gels that were not fixed with TCA) were determined by slicing the gels into 5 mm pieces and placing each slice into 2 ml distilled water. After one hour the pH of the solutions was read on a Fisher Accumet Model 120 pH meter.

**Polyacrylamide disc gel electrophoresis**

For disc gel electrophoresis of the purified hemoglobins and blood samples, the 7% acrylamide gels, pH 8.9, of Ornstein (1964) were used as described in Canalco Disc Gel Electrophoresis Instruction Manual. All electrophoretic reagents were purchased from Canalco (Rockville, Maryland). Samples containing 30 µg proteins were mixed with the loading gel and then the reservoir buffer and the tracking dye were layered over the sample. The gels were electrophoresed at 4°C with a current of 4mA/tube until the tracking dye was approximately 0.5 cm from the bottom of the gel. The gels were then treated with 0.5% aniline black in 7% acetic acid for approximately 1.5 hr and destained electrically with 7% acetic acid. Effects of urea on the hemoglobins were studied by incubating the hemoglobins in 8 M urea at room temperature for 2 hours prior to electrophoresis.
Preparation of the antigen and antiserum

*Lumbricus* hemoglobin was dialyzed thoroughly at 4°C against sterilized 0.85% w/v saline (Jaccarini and Harris, 1975) and, using the cyanmethemoglobin data, the necessary dilutions were made to give a final concentration of 375 μg hemoglobin/ml. A 0.4 ml aliquot of this mixture (150 μg hemoglobin) was mixed with Freund's adjuvant and injected into a single rabbit (2.5-3.5 Kg) subcutaneously in each of the hind thighs. This was followed by four equal intravenous injections (150 μg hemoglobin/0.4 ml) at 7 day intervals. A test bleeding was carried out 28 days after the first injection and a ring test performed to estimate the titer of antibody present. Antiserum was prepared from 15 ml of blood drawn from the marginal ear vein. This sample was allowed to clot at room temperature and kept at 4°C for 24 hours. The clot was compressed by centrifugation at 600 x g and the supernatant removed. Antiserum was frozen until required.

Immunodiffusion

Double diffusion was carried out with 1% agarose (Marine Colloids, Rockport, Maine) in 0.85% saline. After heating to boiling, 12.5 ml of agarose solution was layered on a petri dish and allowed to gel for 2 to 3 hrs at room temperature. Wells were made in an hexagonal arrangement, with a center well for the antibody. Purified hemoglobins or whole blood (10 μl) from *L. terrestris*, *A. caliginosa* or *E. foetida* were added to the outer wells and antiserum (10 μl) to *L. terrestris* hemoglobin added to the center well. The petri dish was covered and kept at 4°C and the precipitation reactions were analyzed after 48 hours.
**Immunoelectrophoresis**

Agarose (1%, in 0.1 M sodium barbitol, pH 8.6) was employed for immunoelectrophoresis. The agarose medium was coated on a glass slide (11 x 2.5 cm) and allowed to gel for 2 hours, then a small cut was made at 1.5 cm from the end of the slide. Antigen (10 µl) was pipetted into the cut with a Hamilton microliter syringe. A constant current of 3.5 mA/gel was applied for 3 hours. After electrophoresis, a trough, whose long axis paralleled the axis of the electrophoretic migration, was cut in the gel and antiserum (20 µl) was applied with a micropipette. Precipitation reactions were analyzed after 48 hours at 4°C.

**Gut tissue preparations for isozyme studies**

The gut from the gizzard to the anus was rapidly excised, cut longitudinally and the intestinal contents removed. For the analysis of the distribution of isozymes the post-gizzard gut was divided into four sections. Section 1 (G1) was from the gizzard to the posterior clitellum segments while sections 2 (G2) and 3 (G3) were equal divisions of the remaining chloragogen-containing region. Section 4 (G4) was the terminal chloragogen-free region of the gut.

The sections were weighed and 10% tissue homogenates were prepared in 0.05 M sodium barbitol, pH 8.6, using a Potter-Elvehjem ground glass homogenizer at 4°C. For acid phosphatase, 0.05 M citric acid, pH 6.0, was used. The homogenates were centrifuged for 30 minutes at 27,000 x g. The supernatant was used as the source of enzyme to be assayed electrophoretically.

**Agarose electrophoresis**

A 1% agarose solution was prepared in 0.05 M sodium barbitol,
pH 8.6, and boiled for a few minutes until the solution cleared. Glass slides (11 x 2.5 cm) were coated with 2.5 ml of the warm agar solution and left for 1 hour at room temperature. In order to apply the sample, cuts, about 1 cm in length and perpendicular to the direction of electrophoresis, were made in the gel with a razor blade at 1.5 cm from one end of the slide. Esterase electrophoresis was studied using commercially prepared plates (Agarose Universal Film, ACI, Palo Alto, California).

The methods of Tashian and Shaw (1962) and Tashian (1969) were used to identify esterase and carbonic anhydrase isozymes. The sites of the esterase isozymes were observed by the coupling of alpha-naphthol with a diazonium salt (Blue RR) after the alpha-naphthol was liberated from alpha-napthyl acetate by the esterase activity (Markert and Hunter, 1959). The liberation of beta-naphthol by carbonic anhydrase from beta-napthyl acetate and its complex with Blue RR salt was used for identification of carbonic anhydrase isozymes.

Acid and alkaline phosphatases were revealed by the technique of Brewer and Sing (1970). The liberation of naphthol by phosphatase from alpha-naphthyl phosphate and its complex with Blue RR salt indicated the presence of alkaline phosphatase. The sites of acid phosphatases were indicated by the splitting of phosphate of phenolphthalein diphosphate at an acid pH (6.0). In this reaction the liberated phenolphthalein turns red under the alkaline conditions upon the addition of ammonium hydroxide to the staining mixture (Hopkinson et al., 1964).

Due to the transitory nature of both acid phosphatase and carbonic anhydrase bands, diagrams were made immediately following color development. All electrophoretic runs were performed at 4°C for 2.5 hours at
3.5 mA/slide.

**Esterase identification**

Esterases were identified by means of inhibitors as reported by Holmes and Masters (1967). After electrophoresis, the gels were allowed to stand in 0.05 M Tris buffer, pH 7.4, containing the inhibitor. After 20-30 minutes they were removed, rinsed with distilled water followed by a single rinse with the Tris buffer and stained as reported above. A variety of inhibitors were used for classification purposes and these are listed in Table 3.

**RESULTS**

Blood hemoglobin concentrations were the highest in *Eisenia* while *Allolobophora* and *Lumbricus* showed values that were only 76% and 62% respectively that of *Eisenia* (Table 1). Polyacrylamide disc gel electrophoresis revealed that the hemoglobins of all three species were heterogeneous, with *Allolobophora* and *Eisenia* having 6 bands and *Lumbricus* 5 bands (Fig. 1). *Allolobophora* and *Lumbricus* showed the presence of one major band (band 3) and three minor bands (1, 2, and 6) that correspond to each other in electrophoretic mobility. *Eisenia*, on the other hand, had two bands in common with both *Allolobophora* and *Lumbricus*. The electrophoretic (polyacrylamide) patterns of the urea-treated hemoglobins were similar to those of the non-urea treated hemoglobins (Fig. 1).

The hemoglobins from the three species showed an apparent homogeneity (one band) on agarose electrophoresis at pH 6.9. At pH 8.6, however, they dissociated into three bands (Fig. 2).

Isoelectric focusing of the intact hemoglobins by agarose electrophoresis revealed isoelectric points of 5.38 for both *Lumbricus* and
Table 1. Summary of the hemoglobin concentrations, elution profiles (Bio-Gel A 5m column, 0.1 M potassium phosphate, with $10^{-3}$ M EDTA, pH 7.0), absorption spectra, and isoelectric points from *L. terrestris*, *A. caliginosa*, and *E. foetida*. Values for the Hb concentrations are the means ± S.E. with the number of determinations in the parentheses.

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<th>ALLOLOBOPHORA</th>
<th>EISENIA</th>
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<td><strong>Hb (g%)</strong></td>
<td>7.0 ± 0.26</td>
<td>8.5 ± 0.66</td>
<td>11.2 ± 0.45</td>
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<td><strong>Absorption spectra</strong></td>
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<td>I. Alpha</td>
<td>576-577 nm</td>
<td>576-577 nm</td>
<td>576-577 nm</td>
</tr>
<tr>
<td>II. Beta</td>
<td>540-542 nm</td>
<td>540-542 nm</td>
<td>540-542 nm</td>
</tr>
<tr>
<td>III. Soret</td>
<td>416-418 nm</td>
<td>416-418 nm</td>
<td>416-418 nm</td>
</tr>
<tr>
<td>Isoelectric point</td>
<td>5.38</td>
<td>5.38</td>
<td>5.48</td>
</tr>
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</table>
Figure 1. Polyacrylamide (7%) disc gel zymograms of *L. terrestris*,
*A. caliginosa*, and *E. foetida* hemoglobins.

$L =$ Lumbricus, $A =$ Allolobophora, $E =$ Eisenia, $B =$ blood,
$Hb =$ hemoglobin.
Figure 2. The effect of pH on the dissociation of earthworm hemoglobins. Gels 2, 4, and 6 are at pH 8.6 (0.05 M sodium barbitol with $10^{-3}$ M EDTA) and gels 1, 3, and 5 are at pH 6.9 (0.05 M sodium citrate with $10^{-3}$ M EDTA).

Gels 1 and 2 = \textit{L. terrestris}

Gels 3 and 4 = \textit{A. caliginosa}

Gels 5 and 6 = \textit{E. foetida}
Figure 3. Isoelectric focusing in agarose gel (0.05 M sodium citrate with $10^{-3}$ M EDTA, pH 6.0) of \textit{L. terrestris}, \textit{A. caliginosa}, and \textit{E. foetida} hemoglobins.

\textit{L} = \textit{Lumbricus}, \textit{A} = \textit{Allolobophora}, \textit{E} = \textit{Eisenia}, \textit{LAE} = combined hemoglobins of the earthworms.
Allolobophora, and 5.48 for Eisenia (Fig. 3).

Absorption spectra of the three species were identical. The maxima of absorption for the alpha band of the oxygenated hemoglobins was between 540-542 nm and the beta band was between 576-577 nm. The Soret absorption region was between 416-418 nm (Table 1).

Column chromatographic elution profiles (Table 1) for both Allolobophora and Eisenia hemoglobins were similar to that of the Lumbricus hemoglobin which has a molecular weight of $3.4 \times 10^6$ daltons (Rossi-Fanelli et al., 1970). When the hemoglobins from the three species were mixed and allowed to fractionate all three were recovered in the same fraction (fractions 26-30).

Double diffusion of Lumbricus, Allolobophora, and Eisenia hemoglobins and of the antiserum produced against Lumbricus hemoglobin revealed an apparent partial identity of the hemoglobins between the three species (Fig. 4). Single precipitation arcs were observed for Lumbricus and Allolobophora whereas double precipitation arcs were obtained with Eisenia. Similar results were obtained with the whole blood of the earthworms using the antiserum produced against the Lumbricus hemoglobin. Partial identities were indicated by the presence of spurs. The second precipitation arc produced by the reaction between Lumbricus antiserum and Eisenia antigen was more diffuse than the first arc (Fig. 4).

Eisenia and Allolobophora hemoglobins migrated anodally during immunoelectrophoresis at pH 8.6 and gave a single precipitation arc when reacted against Lumbricus antiserum. The mobilities of the hemoglobins from both Eisenia and Allolobophora appear to be similar to that of the Lumbricus hemoglobin (Figs. 5 and 6). In contrast to the results obtained
Figure 4. Double diffusion in agarose gel of the hemoglobins from *L. terrestris*, *A. caliginosa*, and *E. foetida* against *L. terrestris* hemoglobin antiserum (Ab).

*L = Lumbricus, A = Alolobophora, E = Eisenia*
Figure 5. Immunoelectrophoresis of *L. terrestris* and *A. caliginosa* hemoglobins against *L. terrestris* hemoglobin antiserum.

In trough, antiserum. Anode on top.

*L* = *Lumbricus*, *A* = *Allolobophora*
Figure 6. Immunoelectrophoresis of *L. terrestris* and *E. foetida* hemoglobins against *L. terrestris* hemoglobin antiserum. In trough, antiserum. Anode on top.

*L* = *Lumbricus*, *E* = *Eisenia*
by the double diffusion technique, *Eisenia* hemoglobin did not show a double precipitation arc against the *Lumbricus* antiserum.

Alkaline phosphatase isozymes were observed in the G1 and G2 sections of *Lumbricus*, *Allolobophora*, and *Eisenia* but not in the G3 and G4 sections (Fig. 7). Of the combined total of three electrophoretically different isozymes observed, *Lumbricus* had two isozymes (1 and 3), *Eisenia* two (isozymes 2 and 3), and *Allolobophora* three (isozymes 1, 2, and 3). The zymogram revealed the isozymic pattern to be similar in both G1 and G2 sections from all three species although the staining intensity suggests the isozymes to be more active in the G1 sections. All three isozymes from G1 and G2 of *Allolobophora* showed the same staining intensity suggesting no decline in the activity of these isozymes in the G2 sections.

Four different acid phosphatase isozymes were observed from the gut sections of *Lumbricus*, *Allolobophora*, and *Eisenia* (Fig. 8). The zymogram revealed *Lumbricus* to have two (isozymes 1 and 4) in the G1 and one (isozyme 4) in the G2, *Allolobophora* two (isozymes 1 and 2) in the G1, G2, and G3, and *Eisenia* two (isozymes 3 and 4) in the G1 and one (isozyme 4) in the G2. No acid phosphatase isozymes were observed in the G4 section from any of the species. Isozyme 1 of *Lumbricus* and 3 of *Eisenia* were not seen in their G2 sections whereas isozyme 4 from both species seen in G1 and G2 sections, stain equally in both sections. There was no decrease in the number of acid phosphatase isozymes in *Allolobophora* although the staining intensity decreased from anterior to posterior.

Six different carbonic anhydrase isozymes were observed from the
Figure 7. Zymogram illustrating the distribution of alkaline phosphatase in the gut sections of *L. terrestris*, *A. caliginosa*, and *E. foetida*. Staining intensities are indicated by the width of the bands.

*L = Lumbricus, A = Allolobophora, E = Eisenia, G = gut*
Figure 8. Zymogram illustrating the distribution of acid phosphatase in the gut sections of *L. terrestris*, *A. caliginosa*, and *E. foetida*. Staining intensities are indicated by the width of the bands.

*L* = *Lumbricus*, *A* = *Allolobophora*, *E* = *Eisenia*, *G* = gut
gut sections of *Lumbricus*, *Allolobophora*, and *Eisenia*, with isozyme six showing cathodal migration (Fig. 9). Gut 1 and 4 of *Lumbricus* had isozymes 2 and 3 while G2 revealed two additional isozymes (4 and 6) and G3 one (isozyme 3). Like *Lumbricus*, isozymes 2 and 3 were also found in the G1 and G2 sections of *Allolobophora*; however, these isozymes were replaced in G3 and G4 by isozyme 5. *Eisenia* had isozymes 1 and 3 in the G1 and G2 section. In the G3 and G4 section isozyme 6 replaced 1. In general, the staining intensity decreased from anterior to posterior.

Zymograms of the esterases revealed the pattern of these isozymes to be similar throughout the gut sections of both *Allolobophora* and *Eisenia* (Fig. 10). *Allolobophora* had all five isozymes present in its gut sections while *Eisenia* had isozymes 1 through 4. Except for isozyme 4 in G3 and G4 and isozyme 5 found only in *Allolobophora* no noticeable changes in staining intensity were observed from all three species. *Lumbricus* showed isozyme 3 and 4 to be present in G1, G2, and G3. In the G4 section, in addition to isozymes 3 and 4, isozymes 1 and 2 also appeared. A summary of the intestinal isozyme distribution is presented in Table 2.

A comparison of *Lumbricus*, *Allolobophora*, and *Eisenia* zymograms for esterases from the gut sections revealed a strong similarity in banding patterns. This similarity is further strengthened by the classification based on inhibitor sensitivity (Tables 3 and 4). Isozyme 3, which is found in all the gut sections of the earthworms, is sensitive to PCMB indicating it to be arylesterase. Isozymes 1, 2, and 4 found in every section of both *Allolobophora* and *Eisenia* but only in G4 of *Lumbricus* was inhibited by eserine, an inhibitor of both cholinesterase and acetylcholinesterase. Further inhibition tests with acetylcholine
Figure 9. Zymogram illustrating the distribution of carbonic anhydrase in the gut sections of *L. terrestris, A. caliginosa*, and *E. foetida*. Staining intensities are indicated by the width of the bands.

*L = Lumbricus, A = Allolobophora, E = Eisenia, G = gut*
Figure 10. Zymogram illustrating the distribution of esterases in the gut sections of *L. terrestris*, *A. caliginosa*, and *E. foetida*. Staining intensities are indicated by the width of the bands. 

Table 2. Summary of the distribution of alkaline and acid phosphatase, carbonic anhydrase, and esterases in the gut tissues of \( L \). terrestis, \( A \). caliginosa, and \( E \). foetida. + indicates the presence and - the absence of the isozymes.

\( L = \) Lumbricus, \( A = \) Allolobophora, \( E = \) Eisenia, \( G = \) gut
<table>
<thead>
<tr>
<th></th>
<th>G1</th>
<th>G2</th>
<th>G3</th>
<th>G4</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Alkaline Phosphatase</strong></td>
<td>L A E</td>
<td>L A E</td>
<td>L A E</td>
<td>L A E</td>
</tr>
<tr>
<td>1</td>
<td>+ + -</td>
<td>+ + -</td>
<td>- - -</td>
<td>- - -</td>
</tr>
<tr>
<td>2</td>
<td>- + +</td>
<td>- + +</td>
<td>- - -</td>
<td>- - -</td>
</tr>
<tr>
<td>3</td>
<td>+ + +</td>
<td>+ + +</td>
<td>- - -</td>
<td>- - -</td>
</tr>
<tr>
<td><strong>Acid Phosphatase</strong></td>
<td>L A E</td>
<td>L A E</td>
<td>L A E</td>
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<tr>
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<td>4</td>
<td>+ - +</td>
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</tr>
<tr>
<td><strong>Carbonic Anhydrase</strong></td>
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<td>L A E</td>
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<td>6</td>
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<td>+ - -</td>
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<tr>
<td><strong>Esterase</strong></td>
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<td>L A E</td>
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<td>5</td>
<td>- + -</td>
<td>- + -</td>
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Table 3. Classification of esterases by inhibitors (According to Holmes and Masters, 1967). Inhibition is denoted by +.

<table>
<thead>
<tr>
<th></th>
<th>Arylesterase</th>
<th>Cholinesterase</th>
<th>Acetylcholinesterase</th>
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<tr>
<td>Eserine (10^{-3} M)</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>PCMB* (10^{-2} M)</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Acetylcholine Iodide (10^{-3} M)</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Urea 10 M</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Heat 47°C</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Heat 55°C</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

*PCMB, p-chloromercuribenzoate
Table 4. Classification of the esterases in the gut sections of *L.* terrestris, *A.* caliginosa, and *E.* foetida by specific inhibitors. Numbers indicate esterase isozymes.

L = Lumbricus, A = Allolobophora, E = Eisenia, G = gut
<table>
<thead>
<tr>
<th></th>
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<th>G4</th>
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<tr>
<td>PCMB</td>
<td>L A E 3</td>
<td>L A E 3</td>
<td>L A E 3</td>
<td>L A E 3</td>
</tr>
<tr>
<td>Eserine</td>
<td>4 1,2,4</td>
<td>1,2,4</td>
<td>4 1,2,4</td>
<td>1,2,4</td>
</tr>
<tr>
<td>Acetylcholine iodide</td>
<td>4 1,2,4</td>
<td>1,2,4</td>
<td>4 1,2,4</td>
<td>1,2,4</td>
</tr>
<tr>
<td>Urea 10 M</td>
<td>3 1,2,3,5</td>
<td>1,2,3</td>
<td>3 1,2,3,5</td>
<td>1,2,3</td>
</tr>
<tr>
<td>Heat 47°C</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Heat 55°C</td>
<td>3 1,2,3</td>
<td>1,2,3</td>
<td>3 1,2,3</td>
<td>1,2,3</td>
</tr>
</tbody>
</table>
iodide, specific inhibitor of acetylcholinesterase, revealed these isozymes to be acetylcholinesterase. 10 M urea had a marked inhibitory effect on all the isozymes except isozyme 4. Gels incubated at 47°C did not show any inhibition. On the contrary, the staining intensity increased at this temperature compared to gels incubated at room temperature. At 55°C isozymes 1, 2, and 3 were inhibited.

A percent similarity test of all the isozymes studied showed *Allolobophora* and *Eisenia* to have the most isozymes in common (46.8%) and *Lumbricus* and *Eisenia* to have the least (39.9%). Individual gut sections, except for G1 and G4, also revealed *Allolobophora* and *Eisenia* to have the most isozymes in common (Table 5) whereas *Lumbricus* and *Eisenia* showed the least similarity in G1 and G2. The greatest similarity of any isozyme recorded was between *Allolobophora* and *Eisenia* (esterase, 80%) and the least recorded was also between *Allolobophora* and *Eisenia* (acid phosphatase, 0%) (Table 6).
Table 5. Percent similarity of the isozymes of acid and alkaline phosphatases, carbonic anhydrase, and esterases from the individual gut sections of *L. terrestris*, *A. caliginosa*, and *E. foetida*.  
*L = Lumbricus, A = Allolobophora, E = Eisenia, G = gut*

<table>
<thead>
<tr>
<th></th>
<th>L</th>
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<th>L</th>
<th>E</th>
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<td></td>
<td>46.5</td>
<td></td>
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Table 6. Percent similarity of the individual isozymes of alkaline and acid phosphatase, carbonic anhydrase, and esterases in the gut sections of *L. terrestris*, *A. caliginosa*, and *E. foetida*. 
*L* = *Lumbricus*, *A* = *Allolobophora*, *E* = *Eisenia*

<table>
<thead>
<tr>
<th></th>
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<td>AIP</td>
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<td>33.3</td>
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<tr>
<td>AcP</td>
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<tr>
<td>C.A.</td>
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<td>28.6</td>
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<tr>
<td>Est</td>
<td>50.0</td>
<td>62.5</td>
<td>80.0</td>
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DISCUSSION

The apparent molecular weight and the dissociation of the hemoglobins of both Allolobophora and Eisenia (Table 1; Figs. 1 and 2) are similar to that of Lumbricus hemoglobin which has a molecular weight of $3.4 \times 10^6$ daltons and can be dissociated into submultiples (Rossi-Fanelli et al., 1970; Wiechelman and Parkhurst, 1972; Chiancone et al., 1972). It is likely that the structural model of the hemoglobin that has been proposed for Lumbricus and other annelid hemoglobins (Levin, 1963; Roche, 1965) may also be characteristic of Allolobophora and Eisenia hemoglobins.

Most high molecular weight annelid hemoglobins dissociate into smaller submultiples at alkaline pH (Svedberg and Eriksson-Quensel, 1933). Lumbricus hemoglobin begins to dissociate from the intact duodecamer (12 subunits, 60S) into 10S components near pH 8.0. As the pH is increased to 9.7 the 60S material disappears and the 10S and the 3.5S submultiples increase in concentration (Chiancone et al., 1972). The present study with polyacrylamide disc gel electrophoresis has demonstrated that all three species of earthworm hemoglobins are heterogeneous. At pH 9.6 (electrophoretic pH) Eisenia and Allolobophora hemoglobins dissociate into 6 subunits while Lumbricus hemoglobin dissociates into 5 (Fig. 1).

Shlom and Vinogradov (1973) have reported Lumbricus hemoglobin to consist of at least 6 polypeptide chains possessing molecular weights of 13,000, 14,000, 16,000, 19,000, 31,000, and 36,000 daltons. Weichelman and Parkhurst (1972) have suggested that at pH 9.0 Lumbricus hemoglobin exists primarily in dissociated forms, one-fourth to one-sixth of the intact duodecamer. In our study the electrophoretic separations suggest
that at alkaline pH *Allolobophora* and *Eisenia* hemoglobins also exist in the dissociated form. Furthermore, some of the dissociated subunits from the three species share similar electrophoretic mobilities.

The hemoglobins of the three species, when incubated at room temperature for two hours in 8 M urea prior to electrophoresis, did not show any change in the electrophoretic patterns from that of the controls. Weichelman and Parkhurst (1972) have reported similar findings with cellulose acetate electrophoretic patterns for the *Lumbricus* hemoglobin. In their study both urea and non-urea runs gave four bands, however, the best resolution was obtained when urea was present in the presoak, the running buffer, and in the globin solution. They calculated the molecular weight of each subunit to be 19,000 daltons.

Unlike certain polychaete hemoglobins (Terwilliger et al., 1975) and hemocyanins (van Holde and van Bruggen, 1971) the dissociation-association equilibria of earthworm hemoglobins are not dependent on the presence of Mg$^{2+}$ or other divalent cations which in general stabilize the larger aggregated states. Our study showed that in the presence of $10^{-3}$ M EDTA and at a pH of between 6.0 and 7.0 earthworm hemoglobins did not dissociate and thus showed only one band on agarose gel electrophoresis (Fig. 2). In contrast, hemoglobin from the polychaete *Pista pacifica* exists almost exclusively in the dissociated form over the same pH range, in the presence of EDTA (Terwilliger et al., 1975). At pH values above 8.0, in the presence or absence of EDTA, earthworm hemoglobins dissociated into subunits (3 bands at pH 8.6 in agarose gel electrophoresis and 5 to 6 bands at pH 9.6 in disc gel polyacrylamide electrophoresis). In earthworms the dissociation of hemoglobin appears to be pH dependent. Similar
results have been obtained from \textit{P. pacifica} hemoglobin under alkaline conditions (Terwilliger \textit{et al}., 1975).

Double diffusion of the hemoglobins from all three earthworms and the antiserum produced against \textit{Lumbricus} hemoglobin revealed an apparent partial identity of their hemoglobins. While the appearance of two precipitation arcs from \textit{Eisenia} hemoglobin cannot be explained at this time, it is possible that \textit{Eisenia} hemoglobin may have dissociated into two subunits, each of which showed a partial identity reaction against the antibody.

The immunoelectrophoretic reactions showed \textit{Allolobophora} and \textit{Eisenia} hemoglobins to give a single precipitation arc when reacted against the antibody produced against \textit{Lumbricus} hemoglobin. The mobilities of all three hemoglobins appear to be similar, further suggesting some similarities in the amino acid composition of these molecules. It is interesting that only a single precipitation arc was obtained from all three species since at the electrophoretic pH (8.6) the hemoglobins dissociated into subunits (Fig. 2). It is unlikely that the hemoglobins dissociated into the smallest possible subunits under these conditions since our study with the three different hemoglobins and that of Wiechelman and Parkhurst (1972) with \textit{Lumbricus} hemoglobin have shown the pigments to dissociate into different electrophoretic components. An alternate possibility is that the antiserum is directed solely against a single electrophoretic species.

The absorption spectra of the oxyhemoglobin from all three species showed the presence of absorption peaks at 576-577, 540-542, and 416-418 nm (Table 1). Prosser (1973) has reported the absorption spectra of several annelids and the report on \textit{Lumbricus} is similar to our results. It appears
that the alpha band in the annelids ranges from 574-578 nm and the beta ranges from 538-544 nm. The Soret peak of *Lumbricus* has been reported to be 417 nm (Rossi-Fanelli *et al.*, 1970), corresponding to the value we obtained for *Lumbricus* as well as the other two species. The identical spectra of the earthworm hemoglobins would indicate that the prosthetic group of the pigments are the same.

The hemoglobins of vertebrates have isoelectric points between 6.8-7.2 (Svedberg, 1933). In contrast, the hemoglobins of annelids, as well as their chlorocruorins, have isoelectric points between 4.4 and 5.6 (Florkin, 1969). In our study the isoelectric focusing of the earthworm hemoglobins gave evidence of apparent homogeneity. The isoelectric points of *Lumbricus* and *Allolobophora* hemoglobins were identical (5.38) while *Eisenia* was slightly higher (5.48). Using more sophisticated methods, Rossi-Fanelli *et al.* (1970) had previously reported a value of 5.1-5.2 for the isoelectric point of *Lumbricus* hemoglobin.

The concentrations of the hemoglobins in the blood were more closely related from *Lumbricus* and *Allolobophora* than *Eisenia* (Table 1). Aside from this difference, the hemoglobins of all three species have a close degree of similarity in their biochemical structure. Earthworms are usually exposed to oxygen tensions near that of air and their hemoglobins show high affinity, heme-heme interactions, and a normal Bohr effect (Laverack, 1963). Manwell (1959) has suggested that these characteristics in *Lumbricus* appear to be a specialization for oxygen transport under conditions of low internal oxygen tensions as a result of an inefficient mechanism for external respiration. Apparently gas exchange through the epidermis is relatively inefficient in *Lumbricus*, but this is compensated
for by the properties of the hemoglobins in the blood system. Edwards and Lofty (1972) reported that hemoglobins of earthworms can be saturated with oxygen at pressures as low as 19 mm Hg. Tembe and Dubash (1961) have suggested that the oxygen tensions of coelomic fluids of earthworms can be as low as 14 mm Hg; oxygen can thus reach the tissues even when small amounts are available. The similar biochemical architecture of *Lumbricus*, *Allolobophora*, and *Eisenia* hemoglobins suggests that their pigments have similar physiological functions.

The percent similarity of the isozyme distributions in the digestive tracts of the earthworms was less than 50% (Table 5). *Allolobophora* and *Eisenia* had slightly more isozymes in common than *Lumbricus* and *Allolobophora* or *Lumbricus* and *Eisenia*. Our results agree with those of previous studies with invertebrate isozymes in which levels of genic similarity between different species of invertebrates were shown to be between 25-50% (for a review, see Avise, 1975).

Of the four enzymes studied, the esterases generally showed the greatest heterogeneity in *Allolobophora*, *Eisenia*, and the G4 section of *Lumbricus*. Gillespie and Kojima (1968), Kojima et al. (1970), and more recently Johnson (1974) reasoned that most enzymes in known metabolic pathways (e.g. glucose metabolism) have a single substrate in a relatively constant concentration. Other enzymes, such as the phosphatases and esterases, act on a variety of substrates which may vary greatly in concentrations and many of these substrates originate outside the organism and thus reflect the characteristic of the environment. In this regard the higher interspecific tissue similarity in esterase multiplicity between *Allolobophora* and *Eisenia* compared to *Lumbricus* and *Allolobophora* is
interesting since the latter two have been suggested to be associated with one another as a result of some characteristic of their habitat (Edwards and Lofty, 1972). This inconsistency of the esterase data suggests that the diet may differ in some particulars between these two species. Species living in the uppermost part of the soil, such as *Allolobophora* and *Eisenia*, feed mainly on the dead vegetation of the litter while species which burrow to deeper layers, such as *Lumbricus*, ingest considerable amounts of soil (Jeunieux, 1969). The similarity observed in the esterases as well as the higher percent similarity of the total isozymes studied between *Allolobophora* and *Eisenia* may reflect a somewhat similar type of food utilization by these two species.

Of all the enzymes studied, carbonic anhydrase (CA) isozymes showed the most tissue specific variation while alkaline phosphatase showed the least. The presence of CA isozymes with characteristic tissue distributions may suggest a physiological role for their existence. CA isozymes in the alimentary canal of some mammals have been extensively studied (for a review, see Carter, 1972), and it appears that HACA (high activity CA) is present in the gastric mucosa whereas LACA (low activity CA) is absent. Other regions of the gut show considerable variation in the concentrations of HACA and LACA. At present the HACA in the gastric mucosa is thought to be essential for the splitting of water into hydrogen and hydroxyl ions, the latter reacting with carbon dioxide to form bicarbonate ions. Similar functions have been attributed to the earthworm CA (Laverack, 1963). The multiplicity and tissue specific distribution of CA in the intestine as well as its absence from the blood system of earthworms (Laverack, 1963) suggest that the hydration of CO$_2$ may occur in the intestinal tract.
The distribution of acid and alkaline phosphatase isozymes indicates that their localization is primarily towards the anterior sections of the gut (except for acid phosphatase isozymes in the G3 section of *Allolobophora*). Esterase and carbonic anhydrase isozymes were present in all gut sections, however, the staining intensities generally indicated higher concentrations in the anterior regions. In view of van Gansen's (1963) report that anterior portions of the gut are richly provided with glandular cells, secreting mucus and enzymes, Jeunieux (1969) has reasoned that the fore- and middle-intestine are chiefly concerned with enzyme secretion and with absorption, while the hind intestine is exclusively devoted to absorption. Both alkaline and acid phosphatases have been shown to be present in the fore- and mid-gut of earthworms (Laverack, 1963; Tillinghast and MacDonnell, 1973). The former which is present in the intestinal wall and the latter, found in the chloragogen cells, function in the active transport and subsequent utilization of absorbed substances (van Gansen, 1956).

Tillinghast and MacDonnell (1973) have shown both alkaline phosphatase (A1P) and acid phosphatase (AcP) to be present throughout the gut of *Lumbricus*. A1P declined in activity from anterior to posterior whereas AcP did not show this trend since the G3 sections had slightly higher AcP activity than the G2 sections. The presence of AcP in the G4 section would indicate that the source of this enzyme is not from the chloragogen cells, since this region of the gut is free of these cells. We cannot explain at this time why we could not detect phosphatases in the posterior sections of the gut. One possibility may be that since the activity of the phosphatases (AcP and A1P) are weak in the posterior sections the individual isozymes that share this already weak activity could not be detected by our method.
Studies of the esterases with specific inhibitors revealed *Allolobophora* and *Eisenia* to have three acetylcholinesterase isozymes and one arylesterase isozyme while *Lumbricus* G1, G2, and G3 had one acetylcholinesterase and one arylesterase; G4, however, had an isozyme distribution similar to that of *Allolobophora* and *Eisenia*. One of the acetylcholinesterase isozymes (isozyme 4) showed extreme stability to heat and urea while all other bands were inhibited by urea. Isozyme 5 in *Allolobophora*, which could not be classified by our inhibition studies, was also heat stable. Holmes and Masters (1967) have shown slow migrating carboxyesterases and acetyl esterases in the guinea pig tissues to be very stable at high temperatures with no visible loss in activity occurring over 20 minute incubation period at 55°C. The results of urea treatment showed that acetyl esterases were also stable to 10 M urea. In regard to the multiformity of the esterases, the present study revealed a somewhat extensive heterogeneity of these enzymes in *Eisenia* and *Allolobophora*. The distribution of these multiple forms in different sections of intestinal tissues does not seem to be tissue specific. Comparison of the gut tissue with other earthworm tissues, such as the seminal vesicles and seminal receptacles (unpublished data), have however shown a characteristic tissue specificity for these isozymes. The absence of cholinesterase in the intestinal tissues of these earthworms may be significant. While Holmes and Masters (1967) have shown cholinesterase to be a major contributor to the intestinal esterase patterns of the guinea pig, Augustinsson (1961), Holmes *et al.* (1968), Holmes and Whitt (1970), and Metcalf *et al.* (1972) have reported cholinesterase to be absent from lower vertebrates. It would be interesting to see if a similar pattern is generally true of other invertebrates.
III. CHANGES IN INTESTINAL ISOZYMES AND NITROGEN EXCRETION
OF THE ESTIVATING EARTHWORM, ALLOLOBOPHORA CALIGINOSA (SAVIGNY)

INTRODUCTION

During the summer months, when the soil becomes too dry, Allolobophora caliginosa and certain other species of earthworms enter a condition of estivation. At this time the animals isolate themselves, expel their gut contents, construct an oval chamber lined with mucus, and roll into a tight sphere (Gerard, 1967). This coiled shape and mucus-lined chamber reduces water loss to a minimum (Edwards and Lofty, 1972). In addition, A. caliginosa loses its secondary sexual characteristics, including a regression of the clitellum (Satchell, 1967). Once estivating, a certain length of time must elapse before the worms again become active and it thus appears that internal factors are responsible for the regulation of this adaptation (Michon, 1954).

In the course of a comparative study of isozyme patterns of several species of earthworms, we observed rather dramatic differences between estivating and active Allolobophora. Changes in specific tissue isozyme patterns during development (for an extensive review see Lindsay, 1963; Markert, 1963; Holmes and Masters, 1967; Holmes, 1972; Markert, 1975) and environmental adaptation (Hochachka, 1965; Hochachka and Somers, 1973; Markert, 1975) are now well documented in certain animals but this is the first observation of such a phenomenon in the oligochaetes.

During normal feeding activity Allolobophora has been shown to
be ammonotelic (Needham, 1957). However, many ammonotelic animals such as Xenopus (Balinsky et al., 1967) and snails (Horne, 1971) assume a ureotelic pattern of nitrogen metabolism when estivating. In our study we have examined the pattern of nitrogen excretion in estivating Allolobophora.

MATERIALS AND METHODS

Experimental animals

Specimens of mature, non-estivating Allolobophora were collected locally during the months of May and June and identified as A. caliginosa. They were maintained at 15°C in soil supplemented with Worm-Bedding (Earlybird Co., Boise, Idaho). Allolobophora were found estivating at soil depths of 15 to 40 cm in late August and early September. In order to keep the worms in their natural estivating condition, they were carefully collected and transferred into jars containing the same soil sample in which they were found, then kept at 15°C until analyzed.

Measurement of excretory and tissue ammonia and urea

For the measurement of excretory nitrogenous compounds, earthworms were placed individually in 50 ml Erlenmeyer flasks containing 0.5 ml of tap water. This fluid was later assayed for the 24 hour ammonia and urea output by the phenate-hypochlorite method as described previously (Tillinghast, 1968).

Tissue ammonia and urea determinations were carried out using Conway diffusion chambers. Whole body tissue extract (0.5 ml) and saturated K₂CO₃ (0.5 ml) were placed separately in the outer well of the chambers. For urea determinations, 1 ml of 0.4 units urease/ml was also
added. Following the addition of 1 ml of 0.1 N HCl to the center well, the chambers were covered and sealed with saturated K$_2$CO$_3$. The outer well solutions were mixed by a slight tilting of the closed chambers and, after adequate time for diffusion, ammonia and urea determinations were performed on aliquots of the center well by the phenate-hypochlorite method.

**Preparation of gut tissue**

Preparation of the gut tissue was as previously described (this report, Part II). The post-gizzard gut was divided into four sections: section 1 (G1) was from the post gizzard to the posterior clitellum segment while sections 2 and 3 (G2 and G3) were equal divisions of the chloragogen containing region. Section 4 (G4) was the terminal chloragogen-free region of the gut.

**Hemoglobin isolation**

The hemoglobins of both estivating and non-estivating Alolobophora were prepared by gel filtration in a 1.5 x 90 cm column of Bio-Gel A-5m (Bio-Rad Laboratories) and eluted with 0.1 M potassium phosphate, pH 7.0, containing 10$^{-3}$ M EDTA as described previously (Rossi-Fanelli et al., 1970).

**Gel electrophoresis**

For disc gel electrophoresis of the hemoglobins and blood samples, the 7% acrylamide gels, pH 8.9, of Ornstein (1964) were used as described in the Canalco Disc Gel Electrophoresis Instruction Manual. The gels were run at a current of 4mA/tube and stained with 0.5% aniline black in 7% acetic acid.

**Preparation of 1% agarose for the electrophoretic separations**
of isozymes was as previously described (this report, Part II). Esterase determinations were carried out on commercially prepared plates (Agarose Universal Film, ACI, Palo Alto, California). All agarose gel electro-phoretic separations were carried out at 4°C for 2.5 hours at 3.5 mAmps per slide.

Gel Stains

(1) Esterases and carbonic anhydrase. The staining mixture was essentially the same as that described by Markert and Hunter (1959). The substrates used to detect these enzymes were alpha-naphthyl acetate for the esterase and beta-naphthyl acetate for carbonic anhydrase (Tashian and Shaw, 1962; Tashian, 1969). Since the stained bands of carbonic anhydrase (CA) were very transitory, diagrams were made within 10-15 minutes of color development (Brewer and Sing, 1970).

(2) Acid phosphatase. The staining method of Hopkinson et al. (1964) with phenolphthalein diphosphate as the substrate was employed. Due to the transitory nature of the stained bands, diagrams were made immediately following color development (Brewer and Sing, 1970).

(3) Alkaline phosphatase. The staining mixture was that of Boyer (1961) as modified by Brewer and Sing (1970); the substrate used was alpha-naphthyl phosphate.

Preparation of the antiserum and immunodiffusion

Antiserum was prepared against Lumbricus hemoglobin as described previously (this report, Part II). Double diffusion of estivating and non-estivating Allolobophora hemoglobins and blood was carried out in 1% agarose gel (Marine Colloids, Rockport, Maine) in 0.85% saline.

Statistical procedures used were from Sokal and Rohlf (1969). Values are reported as mean ± 1 S.E. Student's t-test was used to determine
the significance of the difference between means at the 5% or 1% level. The percent similarity test was that of Sokal and Sneath (1963).

RESULTS

As seen in Table 7, *A. caliginosa* exhibits a transition to ureotelism with estivation. Although there is a wide variation in the rate of urea excretion between specimens, urea is, nevertheless, the major nitrogenous waste excreted. Active *Allolobophora*, collected after the estivation period, show an ammonotelic pattern of nitrogen excretion. The percentage of the sum of urea plus ammonia represented by urea in the excreta of estivating animals was 93.25 compared with 9.95 for the non-estivating worms (Table 7). The difference between the two groups was statistically significant ($P < 0.05$).

Whole body concentrations of urea and ammonia in estivating and non-estivating worms were determined. The values in Table 7 show that tissue urea was considerably higher in the estivating worms. Although urea was observed in the non-estivating worms it represented only a small percentage of the total nitrogen excreted. The percentage of the sum of urea plus ammonia represented by urea in the tissues of the estivating worms was 97.53 compared with 9.32 for the non-estivating. This difference was significant ($P \leq 0.05$).

Immunodiffusion and electrophoresis

There were no observed differences in either the electrophoretic or the immunodiffusion profiles between estivating and non-estivating *Allolobophora* hemoglobins.

Alkaline phosphatase isozymes

Three similar isozymes could be resolved in the G1 and G2 sections
Table 7. Comparison of the ammonia and urea levels in the tissues (μg N/g wet wt.) and excreta (μg N/g wet wt/24 hrs.) of estivating and non-estivating Allolobophora. Values are the weighted means ± S.E., with the number of determinations in parentheses. N = nitrogen

<table>
<thead>
<tr>
<th>Condition</th>
<th>Ammonia (N)</th>
<th>Urea (N)</th>
<th>Total % nitrogen excreted as urea</th>
</tr>
</thead>
<tbody>
<tr>
<td>Estivating</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tissue</td>
<td>0.08 ± 0.046 (4)</td>
<td>3.16 ± 0.358 (4)</td>
<td>97.53</td>
</tr>
<tr>
<td>Excreta</td>
<td>1.60 ± 0.70 (14)</td>
<td>22.11 ± 8.6 (14)</td>
<td>93.25</td>
</tr>
<tr>
<td>Non-Estivating</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tissue</td>
<td>0.438 ± 0.0559 (4)</td>
<td>0.045 ± 0.024 (4)</td>
<td>9.32</td>
</tr>
<tr>
<td>Excreta</td>
<td>14.99 ± 2.96 (14)</td>
<td>1.657 ± 0.886 (14)</td>
<td>9.95</td>
</tr>
</tbody>
</table>
of the non-estivating Allolobophora (Fig. 11). Gut 1 of the estivating Allolobophora showed only isozyme 2; however, the intensity of the staining suggests a higher activity of this enzyme. No isozymes could be seen in G3 of either estivating or non-estivating worms, or in G4 of the non-estivating Allolobophora. A single faint band (isozyme 1) was present in the G4 section of the estivating worms.

**Acid phosphatase**

Estivating and non-estivating Allolobophora showed two similar isozymes (1 and 2) in G1 and G2 sections (Fig. 12). An additional isozyme (isozyme 3) could be resolved in the G1 section of the estivating Allolobophora, very close to the origin. Isozymes 1 and 2 were also present in the G3 section of the non-estivating worms but absent in the estivating animals. No isozymes were observed in the G4 sections of either estivating or non-estivating Allolobophora.

**Carbonic anhydrase**

As can be seen in Figure 13 there are four carbonic anhydrase isozymes in G1 and G2 sections and three isozymes in G3 and G4 sections of the estivating Allolobophora. The staining intensities of the four isozymes decrease in intensity from anterior to posterior. Isozyme 4 was the predominant one in each of the gut sections of estivating Allolobophora, while in the non-estivating worms it was only present as a weak band in sections 3 and 4. Isozymes 2 and 3 were present in G1 and G2 sections but not in G3 and G4 of the non-estivating worms. Isozyme 1 was absent from the gut sections of the non-estivating worms.

**Esterases**

Figure 14 illustrates the results of the esterase isozyme analyses. Except for bands 6 and 7 the patterns of the isozymes for both estivating
Figure 11. Zymogram illustrating the distribution of alkaline phosphatase in the gut sections of estivating and non-estivating *Allolobophora*. Staining intensities are indicated by the width of the bands.

E = estivating, NE = non-estivating, G = gut
Figure 12. Zymogram illustrating the distribution of acid phosphatase in the gut sections of estivating and non-estivating Alolobophora. Staining intensities are indicated by the width of the bands. E = estivating, NE = non-estivating, G = gut
Figure 13. Zymogram illustrating the distribution of carbonic anhydrase in the gut sections of estivating and non-estivating Allolobophora. Staining intensities are indicated by the width of the bands.

E = estivating, NE = non-estivating, G = gut
Figure 14. Zymogram illustrating the distribution of esterases in the gut sections of estivating and non-estivating *Allolobophora*. Staining intensities are indicated by the width of the bands. E = estivating, NE = non-estivating, G = gut.
and non-estivating animals are essentially identical. Isozymes 6 and 7 were also found in the seminal vesicles of the estivating worms (unpublished data). Inhibition studies showed these two isozymes to be sensitive to p-chloromercuribenzoate (PCMB), an inhibitor of arylesterase, and eserine, an inhibitor of acetylcholinesterase (Holmes and Masters, 1967).

The percent similarity of the gut isozymes between estivating and non-estivating Allolobophora was 55% (Table 9); individual gut sections ranged from 52.9% in the G1 to 60% in G3.
Table 8. Summary of the distribution of alkaline and acid phosphatase, carbonic anhydrase, and esterases in the gut tissues of estivating and non-estivating Allolobophora. + indicates the presence and - the absence of the isozymes.

E = estivating, NE = non-estivating, G = gut
<table>
<thead>
<tr>
<th></th>
<th>G1</th>
<th>G2</th>
<th>G3</th>
<th>G4</th>
</tr>
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<tbody>
<tr>
<td>E</td>
<td>NE</td>
<td>E</td>
<td>NE</td>
<td>E</td>
</tr>
<tr>
<td>Alkaline Phosphatase</td>
<td>1</td>
<td>-</td>
<td>+</td>
<td>-</td>
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<tr>
<td></td>
<td>2</td>
<td>+</td>
<td>+</td>
<td>-</td>
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<tr>
<td></td>
<td>3</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Acid Phosphatase</td>
<td>1</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Carbonic Anhydrase</td>
<td>1</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<td></td>
<td>3</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td></td>
<td>4</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Esterase</td>
<td>1</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<td>3</td>
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<td></td>
<td>6</td>
<td>+</td>
<td>-</td>
<td>+</td>
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<tr>
<td></td>
<td>7</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>
Table 9. Percent similarity of isozymal (alkaline and acid phosphatase, carbonic anhydrase, and esterases) banding pattern between estivating and non-estivating *Allolobophora*. 

E = estivating, NE = non-estivating, G = gut

<table>
<thead>
<tr>
<th></th>
<th>G1</th>
<th>G2</th>
<th>G3</th>
<th>G4</th>
</tr>
</thead>
<tbody>
<tr>
<td>G1</td>
<td>52.9</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>G2</td>
<td>53</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>G3</td>
<td>60</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>G4</td>
<td></td>
<td></td>
<td></td>
<td>54.5</td>
</tr>
</tbody>
</table>

Percent similarity of the combined gut sections = 55%
DISCUSSION

Allolobophora are ammonotelic when active but become ureotelic when estivating (Table 7). Our results show that whereas there is very little urea present in the tissues of active Allolobophora, the concentration increases more than ten-fold in tissues of the estivating worms. The metabolic significance of the transition from ammonotelism to ureotelism may be an important adaptation in the prevention of body water loss (Horne, 1971; Tillinghast and Huffman, 1973). Thus, as the urea concentration increases, the enhanced osmotic pressure may reduce evaporative water loss during prolonged drought. The African lungfish Protopterus (Smith, 1930) and the toad Xenopus laevis (Janssens and Cohen, 1968) also assume a ureotelic pattern of nitrogen metabolism as an adaptation to water conservation during estivation. The transition to ureotelism might be particularly important for earthworms since they have been shown to possess little capability in preventing desiccation through reduced permeability to water (Roots, 1956).

The stability of the hemoglobin from estivating earthworms, as observed by electrophoretic and immunological methods, suggests that the hemoglobin retains physical characteristics identical to non-estivating organisms. In view of the radical changes in isozyme patterns with estivation, this stability is rather surprising. Lahiri et al. (1970) have shown the oxygen consumption of estivating African lungfish to be less than 50% that of non-estivating animals and Delaney et al. (1974) have reported a decrease in arterial pH in the same organism during estivation. One would expect similar metabolic changes to occur with estivation in Allolobophora, and corresponding changes in oxygen disso-
cation characteristics of their hemoglobin to parallel the former. Our attempts to measure oxygen consumption during estivation, however, were hampered by the agitation accompanying the respirometry. Similar difficulties have been reported by Delaney et al. (1974).

Carbonic anhydrase activity has been observed in the esophagus, crop, and gizzard of Lumbricus, but was low or absent in the intestine and blood (Clark, 1957). Apparently carbonic anhydrase does not play a role in blood CO$_2$ transport in Lumbricus (Laverack, 1963) as it does in higher organisms. In our study we observed a gradient of activity of carbonic anhydrase, with the highest levels recorded in the anterior gut and lower ones in the posterior. There is a dramatic increase in both numbers of isozymes as well as staining intensity in all gut sections during estivation in Allolobophora (Fig. 13). Carbonic anhydrase isozymes resembling those in the gut sections were also seen in the corresponding body wall sections.

The presence of carbonic anhydrase in the gut sections of Allolobophora may be related to electrolyte transport. Such a relationship between electrolyte transport and intestinal carbonic anhydrase has been demonstrated in the winter flounder (Huang and Chen, 1971) as well as in dogs (Kinney and Code, 1964). Indirect evidence has been presented for a Cl$^-$/HCO$_3^-$ exchange system in earthworms, with chloride transport being blocked by acetazolamide, a carbonic anhydrase inhibitor (Dietz, 1974). Maetz (1973) has linked this exchange system with the requirements for maintaining hydro-mineral and acid-base balance. Moreover, Dietz (1974) has observed a significant correlation between HCO$_3^-$ production and the influx of chloride in worms.
It is difficult for us to explain both the increase in numbers of carbonic anhydrase isozymes and their increased activity with estivation, but current interpretations of the role of this enzyme would imply an involvement in a buffer system.

The presence of alkaline phosphatase has been demonstrated by histochemical methods in the calciferous glands of the earthworm *Lumbricus terrestris* (Bevelander and Nakaris, 1959). The amount present increases as cellular activity rises, and decreases as the cell passes into a quiescent phase. Our study has shown a decrease in the number of intestinal alkaline phosphatase isozymes during estivation in *Allolobophora*. Only isozyme 2 appeared in the G1 and none were present in the G2 of the estivating organisms. Three alkaline phosphatase isozymes were observed in both G1 and G2 of the non-estivating worms. In feeding *Allolobophora* this enzyme has been shown to occur in high concentrations in the distal ends of the intestinal cells, lying alongside the chloragocytes, indicating the active transport of absorbed substances across the cell boundaries. When worms were starved for one month acid phosphatase replaces the alkaline phosphatase (van Gansen, 1956). In our study the increase in the acid phosphatase isozymes (appearance of isozyme 1 in the G1 of the estivating worms) and the apparent decrease observed in the number of alkaline phosphatase isozymes agrees with van Gansen's observation that intestinal acid phosphatase replaces intestinal alkaline phosphatase under conditions of starvation.

The increase in acid phosphatase isozymes with estivation in *Allolobophora* may be related to the regression of tissues such as the clitellum and seminal vesicles. Tillinghast and MacDonnell (1973)
have shown an increase in acid phosphatase, cathepsins, and neutral proteases in the gut and body wall tissue of fasting *Lumbricus* and Cooper and Baculi (1968) have recorded histological changes which support the view that catabolism of tissue proteins provides the source of nitrogen for urea synthesis during starvation (Cohen and Lewis, 1949).

It is of particular interest that the acid phosphatases increase in only the G1 section of estivating worms and they disappear entirely in the G3 and G4 (posterior) sections. Moreover, whereas there is a decrease in the number of alkaline phosphatase isozymes in the G1 section, the single isozyme that remains during estivation is quite active. Heran (1956) has demonstrated that protease activity is highest in the anterior gut sections of *Lumbricus* and this increases differentially with starvation. These observations, taken together, strongly suggest that the anterior gut of fasting and estivating worms plays some critical role during these metabolic states. The elevated acid phosphatase and protease may function in this region for catabolic purposes; the alkaline phosphatase may aid in the transport of catabolic metabolites.

The change in the esterase pattern between estivating and non-estivating worms implies that esterases have an important role in the organisms' economy. All the esterases, except isozyme 6 and 7, appear to be homologous between the estivating and non-estivating worms in terms of intensity and general electrophoretic mobility (Fig. 14). The appearance of the two new bands, similar in electrophoretic mobility in all the gut sections of the estivating worms, suggests that a specific metabolic role should be associated with these isozymes. Since isozymes 6 and 7 also appear in the seminal vesicles which, in the estivating
condition, greatly regress in size, one might speculate that at least one of these isozymes functions in tissue catabolism. Indeed, all estivating *Allolobophora* collected showed a regression of the tail end as well as of the secondary sexual characteristics. The presence of acetylcholinesterase in the estivating *Allolobophora* may suggest a role in cell function unconnected with synaptic activity. This enzyme, for example, has been shown to control the level of cytosolic acetyl groups through regulation of the level of the intermediates in the Krebs cycle (Varela, 1975). Its presence suggests a possible importance in the metabolic adjustment of the earthworm to estivating conditions.

The percent similarity test (Table 9) shows a 45% change in total isozymes with estivation. In the foregoing discussion we have attempted to explain these changes in accordance with the currently accepted metabolic roles assigned to these enzymes. Even so, more work is necessary to explain the exact mechanisms underlying these changes.
REFERENCES


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