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

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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

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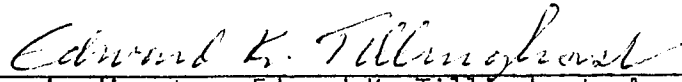
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Graduate School
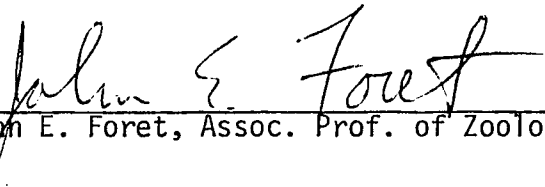
Department of Zoology

September, 1976
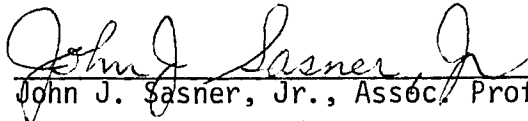
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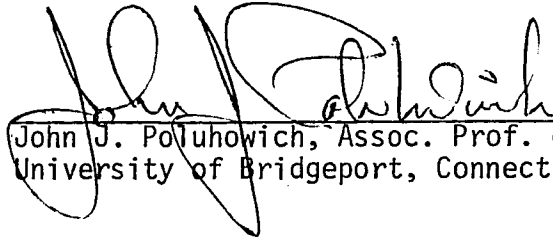
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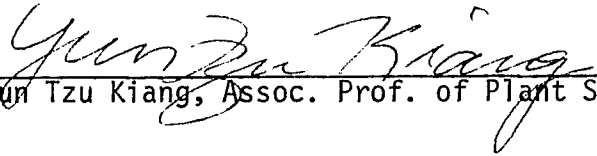
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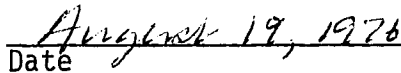
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ABSTRACT

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 (3.4×10^6 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 <u>Lumbricus</u>
and <u>Allolobophora</u> hemoglobins to have identical isoelectric points
(pH 5.38); that of <u>Eisenia</u> 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
$(pH \geq 8.0)$.

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

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

7. The profile of isozymes in <u>Allolobophora</u> changed
dramatically with estivation.

8. With estivation, <u>Allolobophora</u> 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

1

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 foetida_. 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 3.4×10^6 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 foetida: (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 <u>Lumbricus terrestris</u>, and <u>Allolobophora caliginosa</u> were collected locally and maintained at 15°C in soil supplemented with worm bedding (Earlybird Co., Boise, Idaho). <u>Eisenia foetida</u> 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 anaesthezised 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 <u>L</u>. <u>terrestris</u>, 20-30 µl from <u>A</u>. <u>caliginosa</u>, and 5-10 µl from <u>E</u>. <u>foetida</u>. 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 electri-

cally 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 $4^{o}C$ 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 $4^{o}C$ 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 $4^{o}C$ 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 electro-

phoresis, 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-naphthyl acetate by the esterase activity (Markert and Hunter, 1959).

The liberation of beta-naphthol by carbonic anhydrase from beta-naphthyl

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 develop-

ment. 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 corres-
pond 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 hemo-
globins were similar to those of the non-urea treated hemoglobins (Fig. 1).

The hemoglobins from the three species showed an apparent homo-
geneity (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 electro-
phoresis 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 $\pm$ S.E. with the

number of determinations in the parentheses.

	LUMBRICUS	ALLOLOBOPHORA	EISENIA
Hb (g%)	7.0 $\pm$ 0.26 (20)	8.5 $\pm$ 0.66 (20)	11.2 $\pm$ 0.45 (15)
Elution fraction numbers (1 ml)	26-30	26-30	26-30
Absorption spectra			
I. Alpha	576-577 nm	576-577 nm	576-577 nm
II. Beta	540-542 nm	540-542 nm	540-542 nm
III. Soret	416-418 nm	416-418 nm	416-418 nm
Isoelectric point	5.38	5.38	5.48

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.

CANALCO
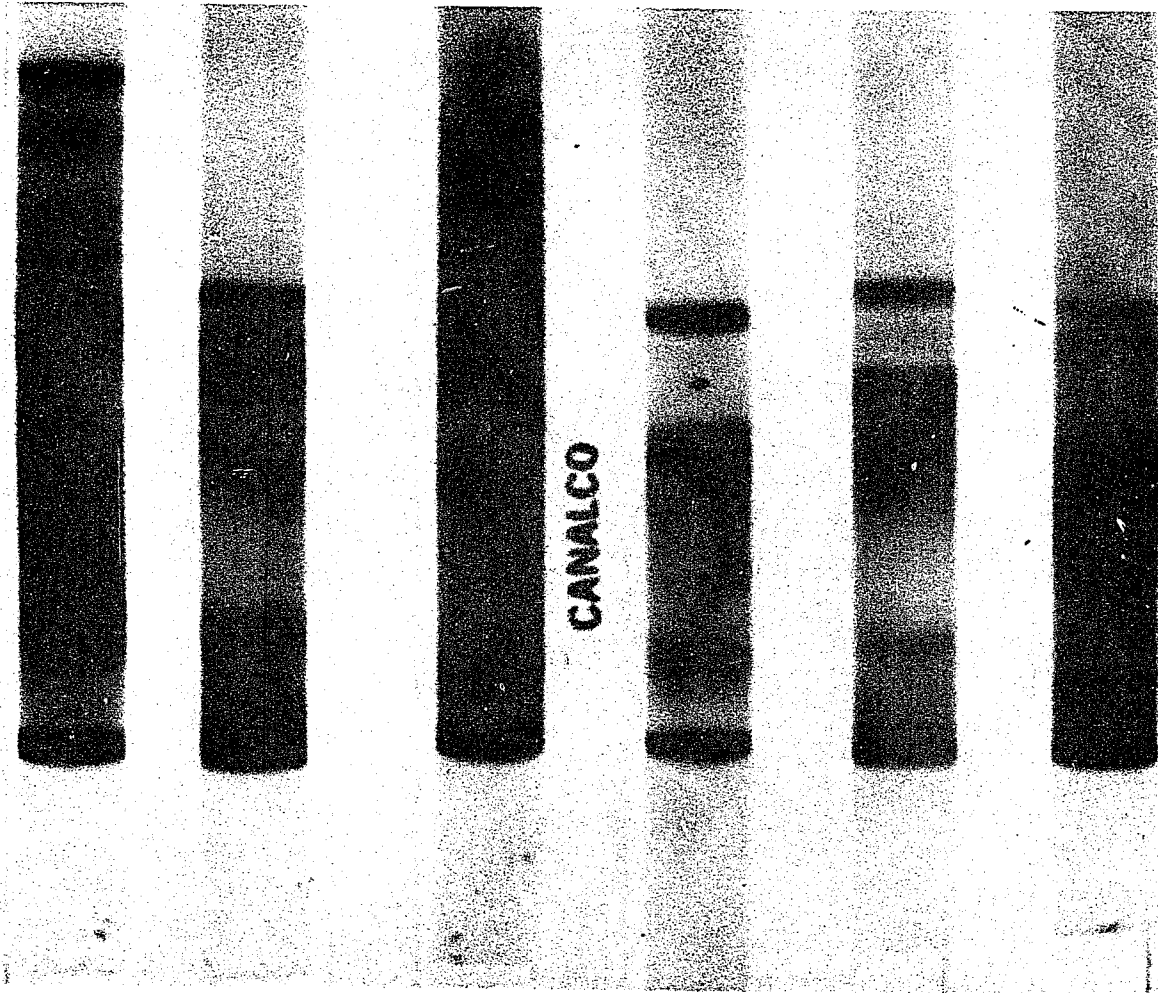
AB   EB   LB   AHb   EHb   LHb

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 = <u>L</u>. <u>terrestris</u>

Gels 3 and 4 = <u>A</u>. <u>caliginosa</u>

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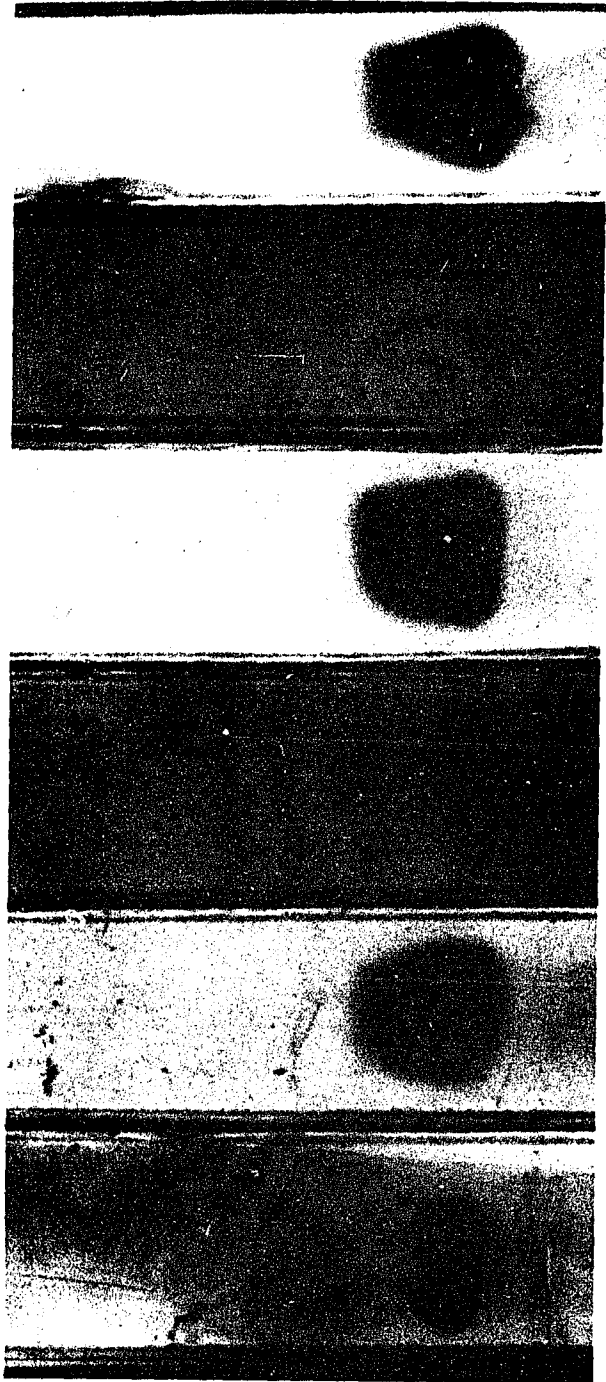
Figure 3. Isoelectric focusing in agarose gel (0.05 M sodium citrate with 10^{-3} M EDTA, pH 6.0) of L. terrestris, A. caliginosa, and E. foetida hemoglobins.

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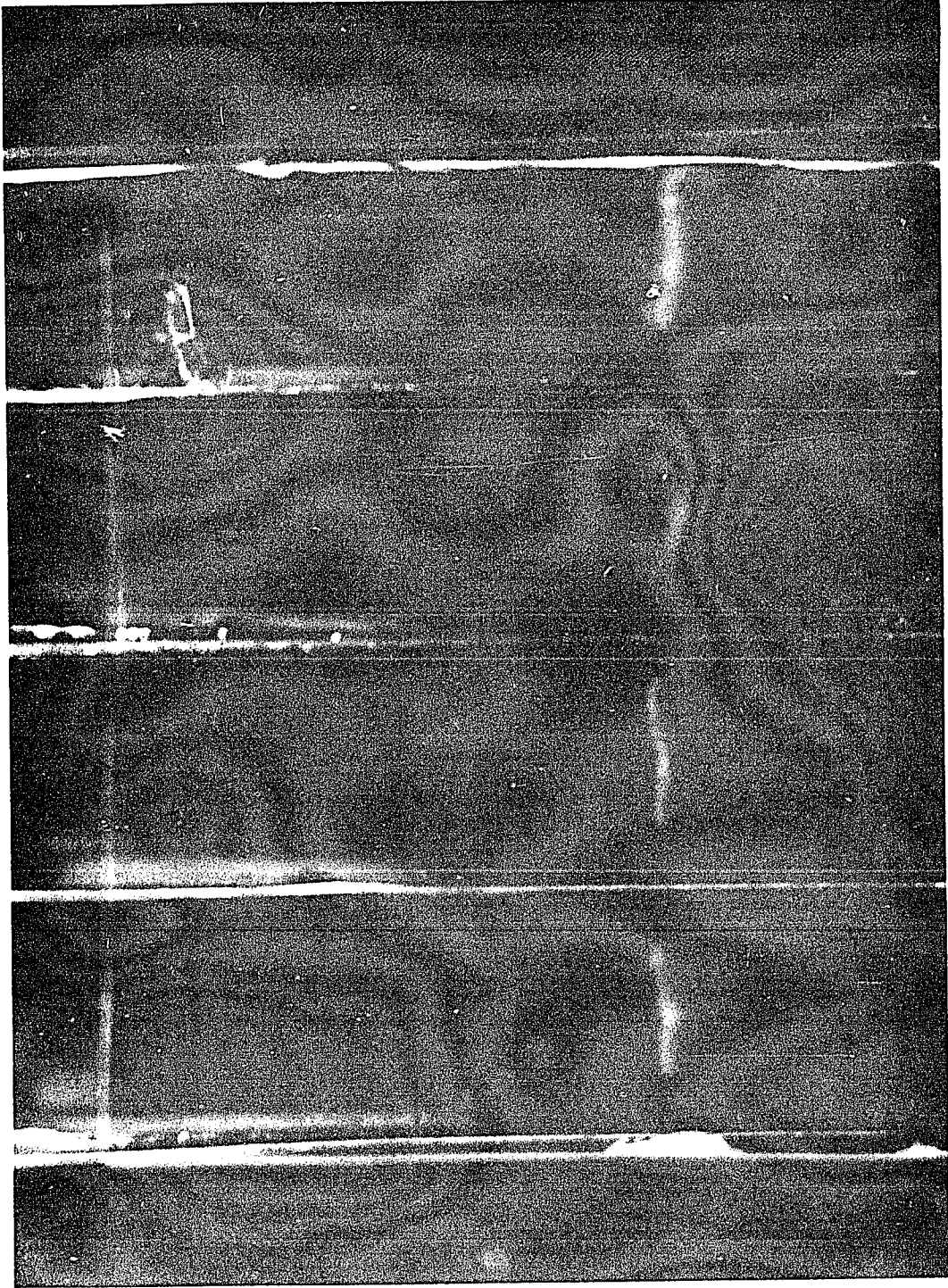
Allolobophora, and 5.48 for Eisenia (Fig. 3).

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 Allolo-bophora and Eisenia hemoglobins were similar to that of the Lumbricus hemoglobin which has a molecular weight of 3.4×10^6 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 hemo-globins 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 <u>L</u>.
terrestris, <u>A</u>. <u>caliginosa</u>, and <u>E</u>. <u>foetida</u> against <u>L</u>. <u>terrestris</u>
hemoglobin antiserum (Ab).

L = <u>Lumbricus</u>, A = <u>Allolobophora</u>, E = <u>Eisenia</u>
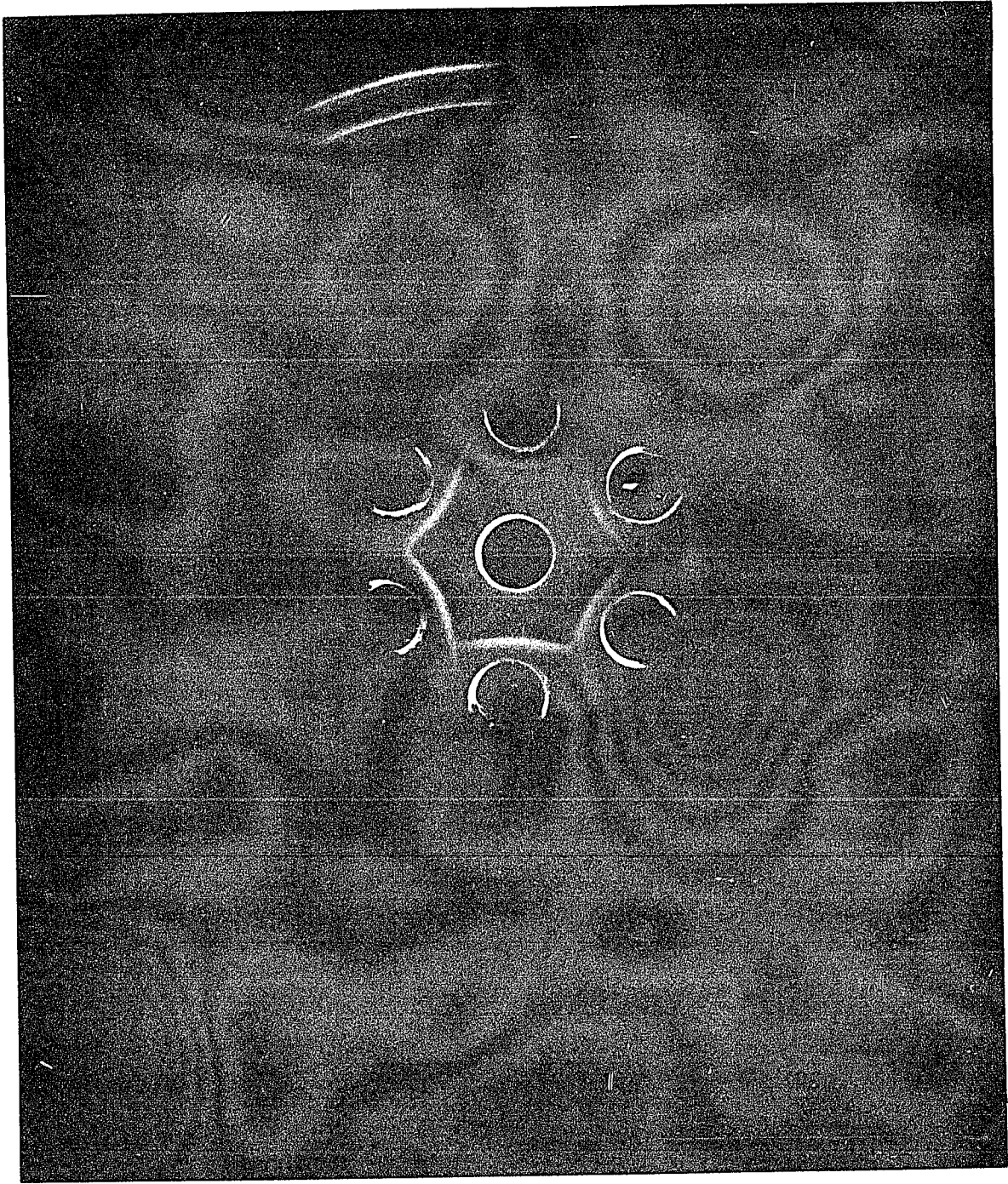
Figure 5. Immunoelectrophoresis of L. terrestris and A. caliginosa

hemoglobins against L. terrestris hemoglobin antiserum.

In trough, antiserum. Anode on top.

L = Lumbricus, A = Allolobophora

(+)
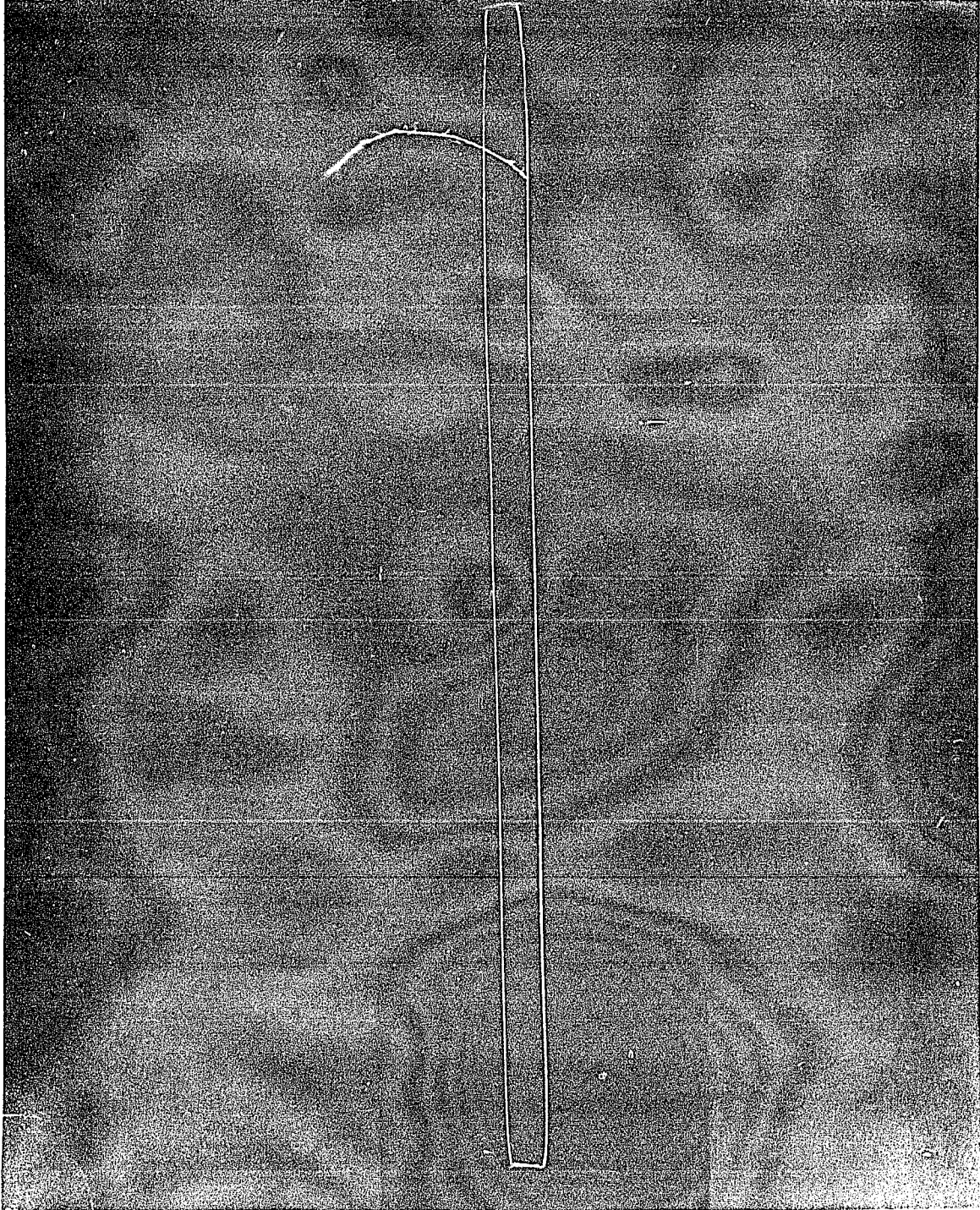
L (−) A

Figure 6.   Immunoelectrophoresis of L. terrestris and E. foetida hemo-

globins against L. terrestris hemoglobin antiserum.   In

trough, antiserum.   Anode on top.

L = Lumbricus, E = Eisenia

(+)
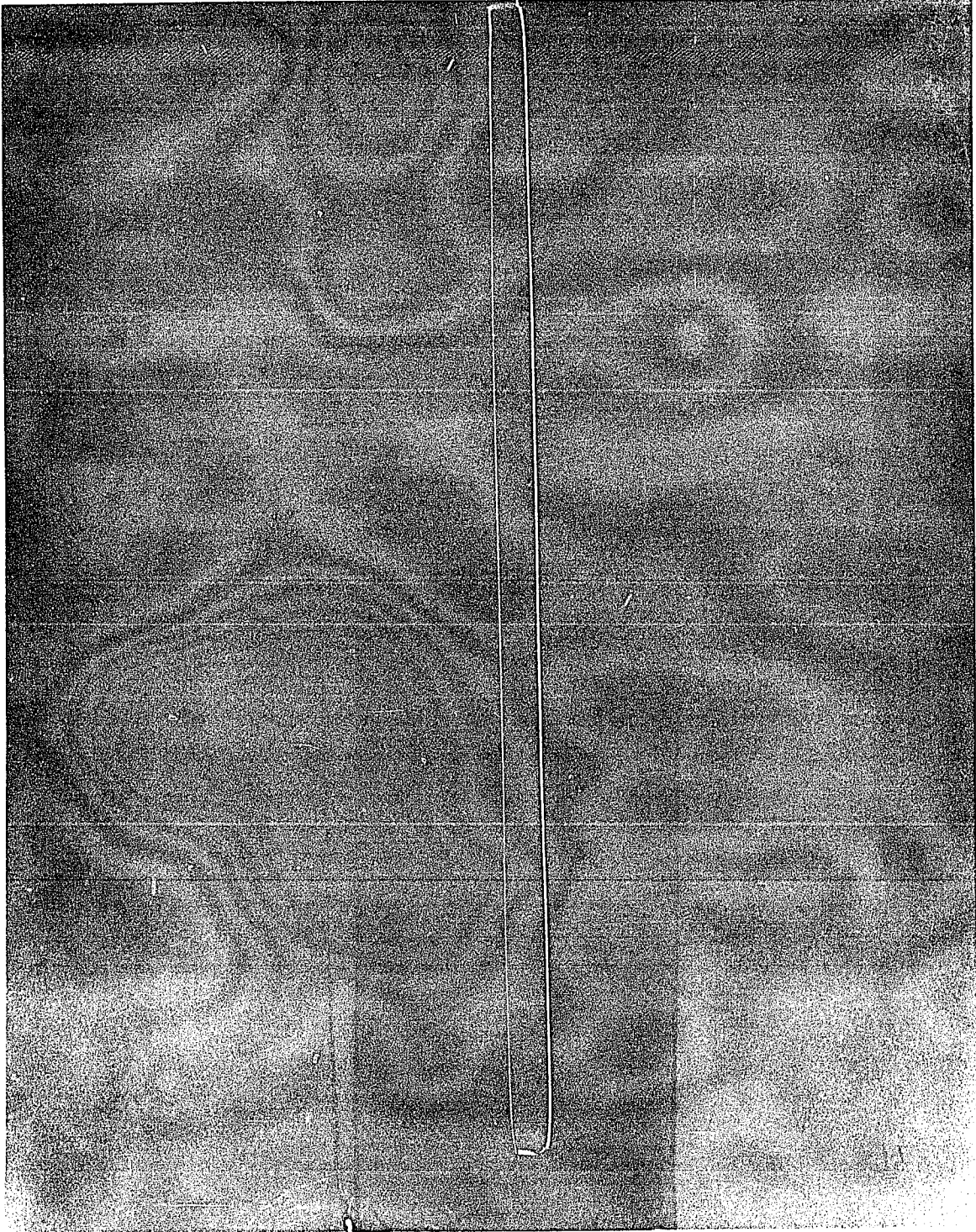
L (−) E

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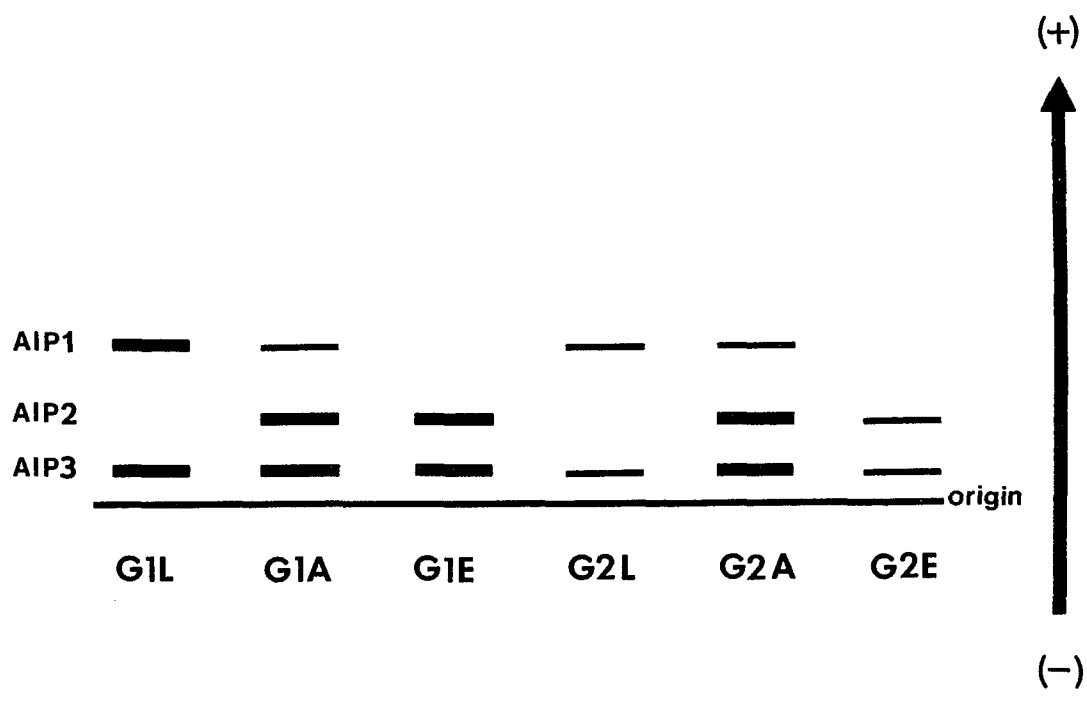
Figure 8. Zymogram illustrating the distribution of acid phosphatase
in the gut sections of L. terrestris, A. caliginosa, and E.
foetida. Staining intensities are indicated by the width
of the bands.

L = Lumbricus, A = Allolobophora, E = Eisenia, G = gut
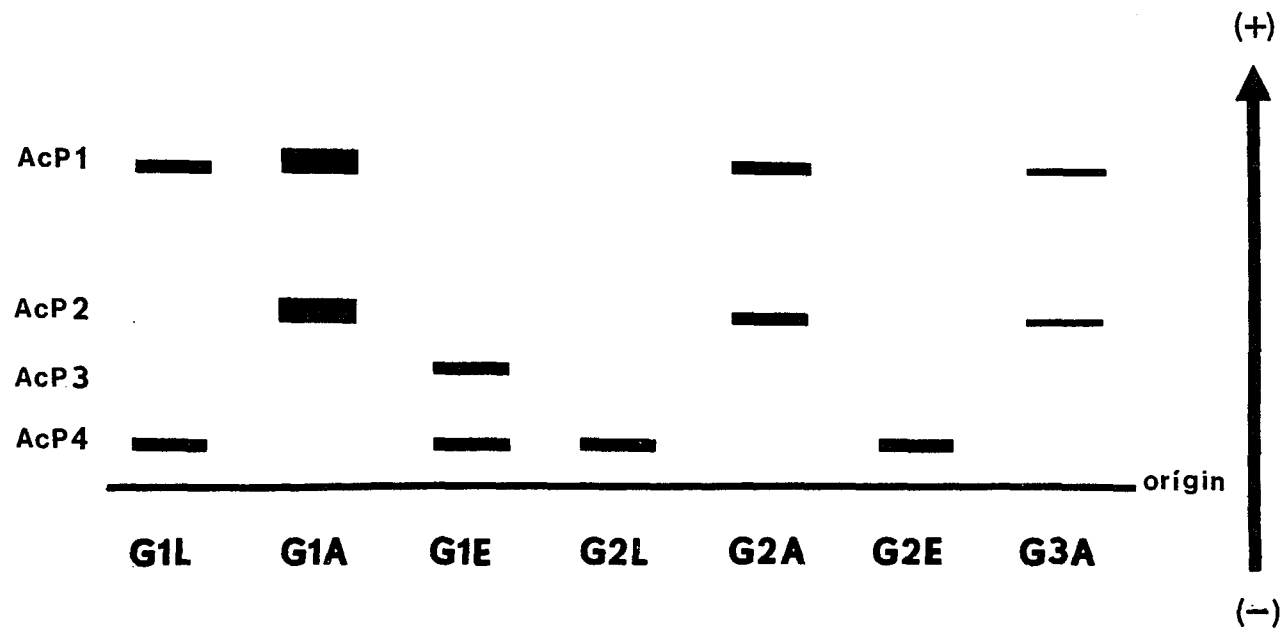
gut sections of Lumbricus, Allolobophora, and Eisenia, with isozyme six

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 class-

ification 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.
L = Lumbricus, A = Allolobophora, E = Eisenia, G = gut

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

L = Lumbricus, A = Allolobophora, E = Eisenia, G = gut

		G1			G2			G3			G4		
		L	A	E	L	A	E	L	A	E	L	A	E
Alkaline Phosphatase	1	+	+	-	+	+	-	-	-	-	-	-	-
	2	-	+	+	-	+	+	-	-	-	-	-	-
	3	+	+	+	+	+	+	-	-	-	-	-	-
Acid Phosphatase	1	+	+	-	-	+	-	-	+	-	-	-	-
	2	-	+	-	-	+	-	-	+	-	-	-	-
	3	-	-	+	-	-	-	-	-	-	-	-	-
	4	+	-	+	+	-	+	-	-	-	-	-	-
Carbonic Anhydrase	1	-	-	+	-	-	+	-	-	-	-	-	-
	2	+	+	-	+	+	-	+	-	-	-	-	-
	3	+	+	+	+	+	+	+	-	+	+	-	+
	4	-	-	-	+	-	-	+	-	-	+	-	-
	5	-	-	-	-	-	-	-	+	-	-	+	-
	6	-	-	-	+	-	-	-	-	+	-	-	+
Esterase	1	-	+	+	-	+	+	=	+	+	+	+	+
	2	-	+	+	-	+	+	-	+	+	+	+	+
	3	+	+	+	+	+	+	+	+	+	+	+	+
	4	+	+	+	+	+	+	+	+	+	+	+	+
	5	-	+	-	-	+	-	-	+	-	-	+	-

Table 3. Classification of esterases by inhibitors (According to Holmes and Masters, 1967). Inhibition is denoted by +.

	Arylesterase	Cholinesterase	Acetylcholinesterase
Eserine (10^{-3} M)	-	+	+
PCMB* (10^{-2} M)	+	-	-
Acetylcholine Iodide (10^{-3} M)	-	-	+
Urea 10 M	-	+	+
Heat 47°C	-	-	-
Heat 55°C	-	+	+

*PCMB, p-chloromercuribenzoate

Table 4. Classification of the esterases in the gut sections of $\underline{L}$.

terrestris, $\underline{A}$. caliginosa, and $\underline{E}$. foetida by specific inhi-

bitors. Numbers indicate esterase isozymes.

L = Lumbricus, A = Allolobophora, E = Eisenia, G = gut

	G1			G2			G3			G4		
	L	A	E	L	A	E	L	A	E	L	A	E
PCMB	3	3	3	3	3	3	3	3	3	3	3	3
Eserine	4	1,2,4	1,2,4	4	1,2,4	1,2,4	4	1,2,4	1,2,4	1,2,4	1,2,4	1,2,4
Acetylcholine iodide	4	1,2,4	1,2,4	4	1,2,4	1,2,4	4	1,2,4	1,2,4	1,2,4	1,2,4	1,2,4
Urea 10 M	3	1,2,3,5	1,2,3	3	1,2,3,5	1,2,3	3	1,2,3,5	1,2,3	1,2,3	1,2,3,5	1,2,3
Heat 47°C	-	-	-	-	-	-	-	-	-	-	-	-
Heat 55°C	3	1,2,3	1,2,3	3	1,2,3	1,2,3	3	1,2,3	1,2,3	1,2,3	1,2,3	1,2,3

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 phos-
phatases, 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

	L A	L E	A E
G1	53.8	38.5	46.7
G2	42.9	38.5	50.0
G3	18.2	37.5	40.0
G4	50.0	71.4	50.0
X̄	41.2	46.5	46.9

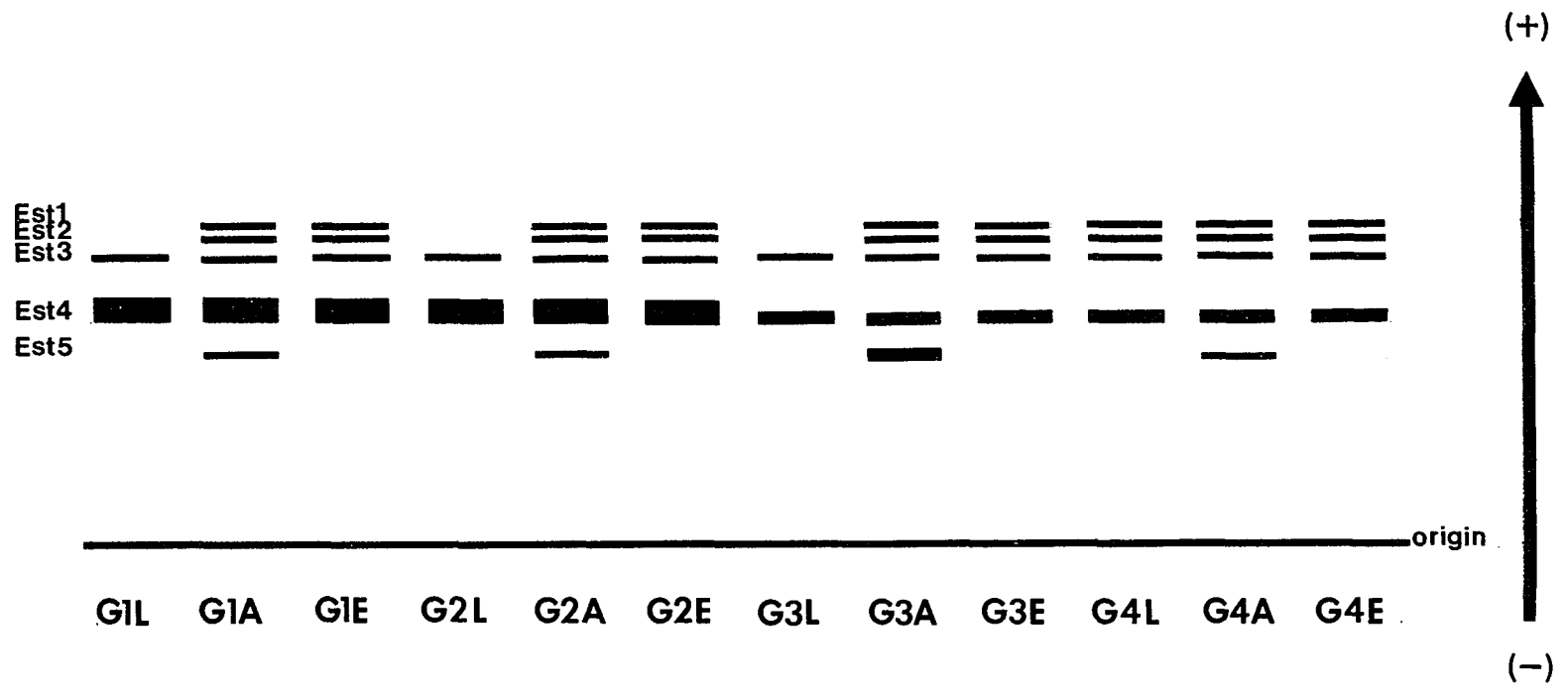Table 6. Percent similarity of the individual isozymes of alkaline and
acid phosphatase, carbonic anhydrase, and esterases in the gut
sections of L. terrestris, A. caliginosa, and E. foetida.
L = Lumbricus, A = Allolobophora, E = Eisenia

	L A	L E	A E
AlP	66.7	33.3	66.7
AcP	12.5	50.0	00.0
C.A.	30.8	28.6	16.7
Est	50.0	62.5	80.0

DISCUSSION

The apparent molecular weight and the dissociation of the hemo-globins 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 x 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 P. pacifica hemoglobin under alkaline conditions (Terwilliger et al., 1975).

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

The immunoelectrophoretic reactions showed Allolobophora and Eisenia hemoglobins to give a single precipitation arc when reacted against the antibody produced against 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 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 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 at 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 architec-
ture of Lumbricus, Allolobophora, and Eisenia hemoglobins suggests that
their pigments have similar physiological functions.

The percent similarity of the isozyme distributions in the diges-
tive 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 meta-
bolic 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 (AlP) and acid phosphatase (AcP) to be present throughout the gut of Lumbricus. AlP 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 chlorogogen 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 AlP) 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 acetylesterases
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 acetylesterases
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 recep-
tacles (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, <u>ALLOLOBOPHORA</u> <u>CALIGINOSA</u> (SAVIGNY)

INTRODUCTION

During the summer months, when the soil becomes too dry, <u>Allolobophora</u> <u>caliginosa</u> 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, <u>A</u>. <u>caliginosa</u> 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 <u>Allolobophora</u>. 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 oliogochaetes.

During normal feeding activity <u>Allolobophora</u> 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 esti-vating 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 $15^{O}C$ 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 $15^{O}C$ 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 K_2CO_3 (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_2CO_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 Allolobophora 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 $\pm$ 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 ($P \leq 0.05$).

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 <u>Allolobophora</u>. Values are
the weighted means $\pm$ S.E., with the number of determinations
in parentheses. N = nitrogen

Condition	Ammonia (N)	Urea (N)	Total % nitrogen excreted as urea
Estivating			
Tissue	0.08 $\pm$ 0.046 (4)	3.16 $\pm$ 0.358 (4)	97.53
Excreta	1.60 $\pm$ 0.70 (14)	22.11 $\pm$ 8.6 (14)	93.25
Non-Estivating			
Tissue	0.438 $\pm$ 0.0559 (4)	0.045 $\pm$ 0.024 (4)	9.32
Excreta	14.99 $\pm$ 2.96 (14)	1.657 $\pm$ 0.886 (14)	9.95

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 Allolo-
bophora. Staining intensities are indicated by the width of
the bands.

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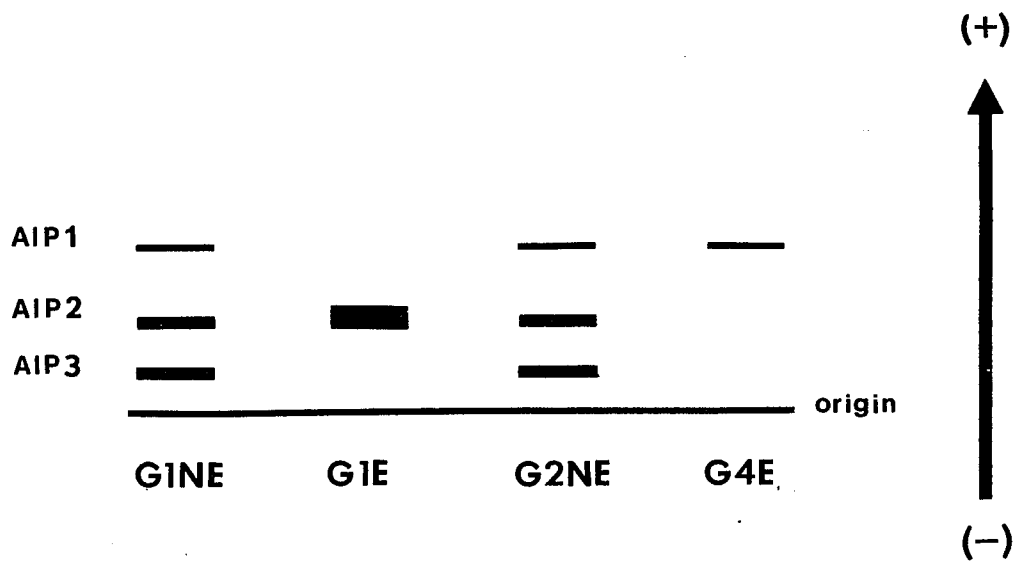
Figure 12. Zymogram illustrating the distribution of acid phosphatase in the gut sections of estivating and non-estivating <u>Allolobophora</u>. Staining intensities are indicated by the width of the bands. E = estivating, NE = non-estivating, G = gut
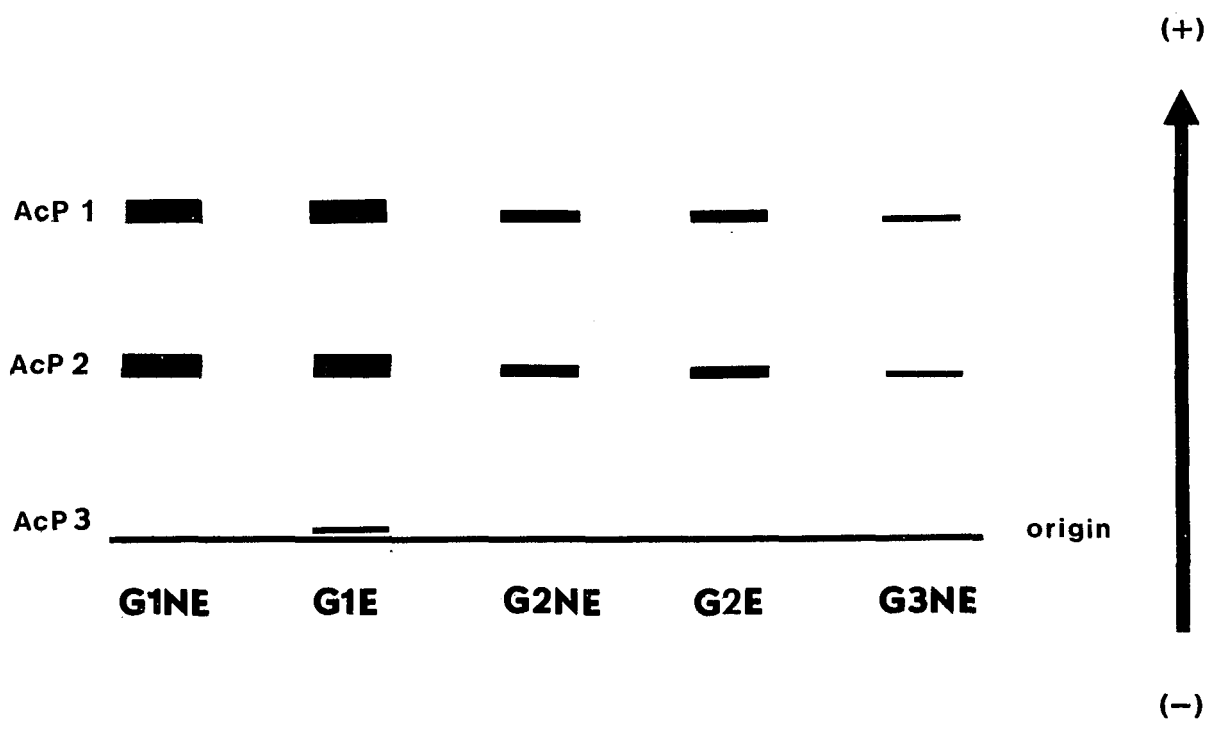
Figure 13. Zymogram illustrating the distribution of carbonic anhydrase

in the gut sections of estivating and non-estivating <u>Allolo-</u>

<u>bophora</u>. Staining intensities are indicated by the width of

the bands.

E = estivating, NE = non-estivating, G = gut
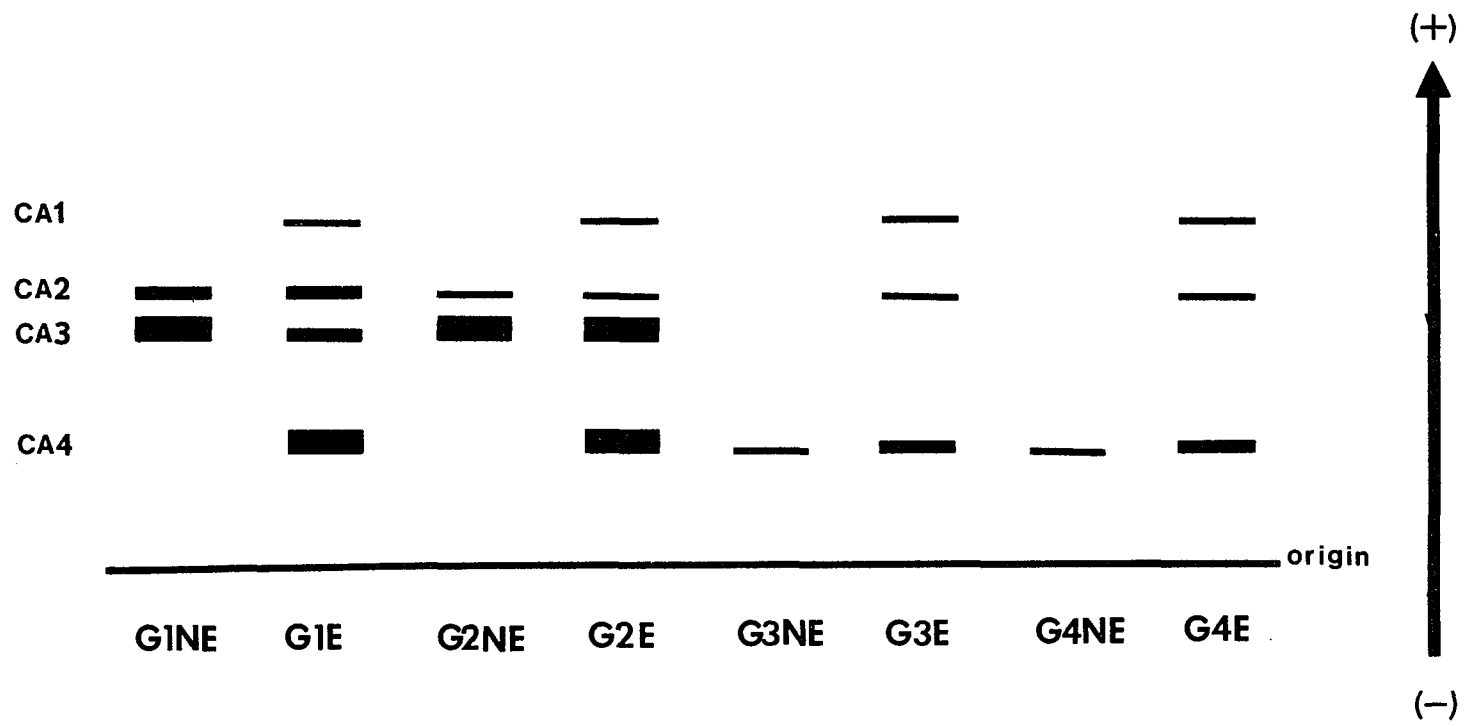
Figure 14. Zymogram illustrating the distribution of esterases in the gut

sections of estivating and non-estivating <u>Allolobophora</u>.

Staining intensities are indicated by the width of the bands.

E = estivating, NE = non-estivating, G = gut.
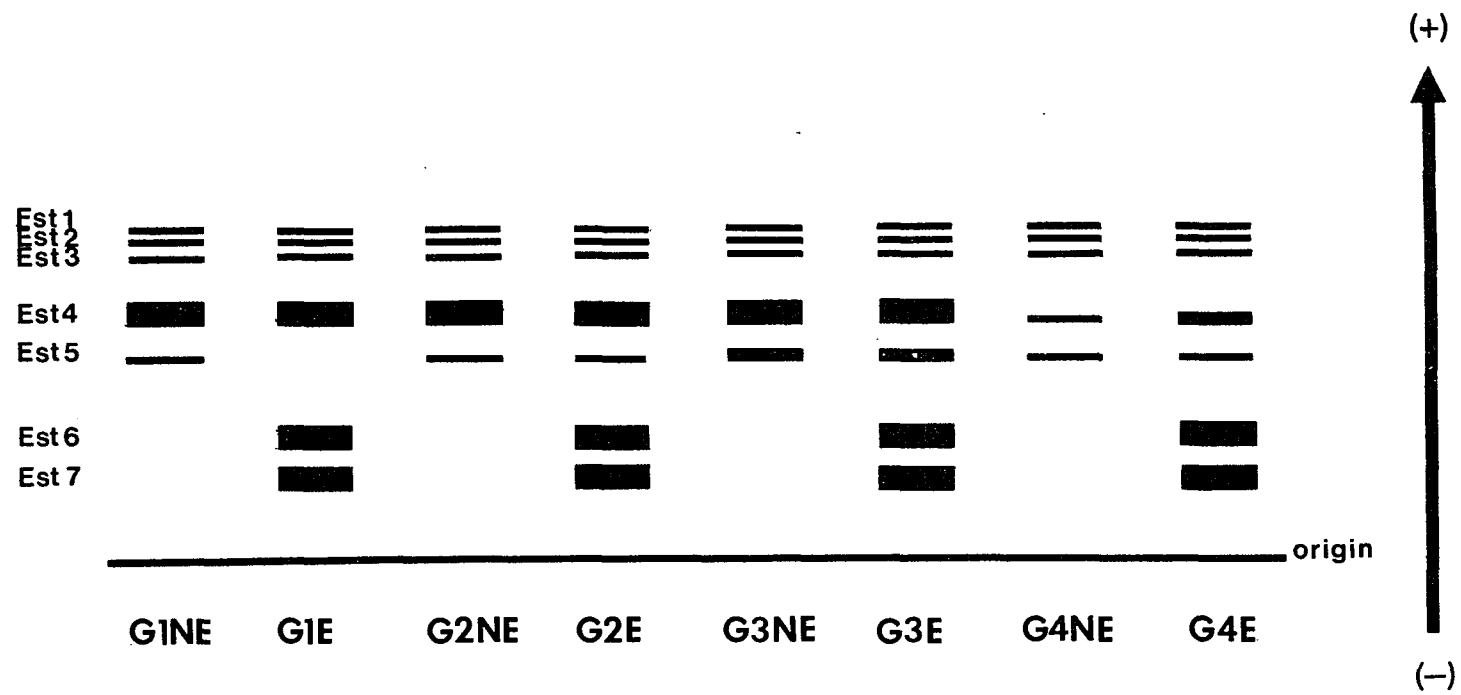
and non-estivating animals are essentially identical. Isozymes 6 and 7 were also found in the seminal vesicles of the estivating worms (unpublished data). Inhibition studies showed these two isozymes to be sensitive to p-chloromercuribenzoate (PCMB), an inhibitor of arylesterase, and eserine, an inhibitor 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 <u>Allolobophora</u>. + indicates the presence and - the absence of the isozymes.

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

		G1		G2		G3		G4	
		E	NE	E	NE	E	NE	E	NE
Alkaline Phosphatase	1	−	+	−	+	−	−	+	−
	2	+	+	−	+	−	−	−	−
	3	−	+	−	+	−	−	−	−
Acid Phosphatase	1	+	+	+	+	−	+	−	−
	2	+	+	+	+	−	+	−	−
	3	+	−	−	−	−	−	−	−
Carbonic Anhydrase	1	+	−	+	−	+	−	+	−
	2	+	+	+	+	+	−	+	−
	3	+	+	+	+	−	−	−	−
	4	+	−	+	−	+	+	+	+
Esterase	1	+	+	+	+	+	+	+	+
	2	+	+	+	+	+	+	+	+
	3	+	+	+	+	+	+	+	+
	4	+	+	+	+	+	+	+	+
	5	−	+	−	+	+	+	+	+
	6	+	−	+	−	+	−	+	−
	7	+	−	+	−	+	−	+	−

Table 9. Percent similarity of isozymal (alkaline and acid phosphatase,
carbonic anhydrase, and esterases) banding pattern between
estivating and non-estivating <u>Allolobophora</u>.

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

		E			
		G1	G2	G3	G4
NE	G1	52.9	-	-	-
	G2		53	-	-
	G3			60	-
	G4				54.5

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 concen-
tration 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-

ciation 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

Augustinsson K.B. (1961) Multiple forms of esterase in vertebrate blood plasma. Ann. N.Y. Acad. Sci. 94, 844-860.

Avise J.C. (1975) Systematic value of electrophoretic data. Syst. Zool. 23, 465-481.

Baldwin J. & Hochachka P.W. (1970) Functional significance of isoenzymes in thermal acclimation: acetylcholinesterase from trout brain. Biochem. J. 116, 883-887.

Balinsky J.B., Choritz E.L., Coe C.G.L. & van der Schans G.S. (1967) Amino acid metabolism and urea synthesis in naturally aestivating Xenopus laevis. Comp. Bioch. Physiol. 22, 59-68.

Bevelander G. & Nakahara H. (1959) A histochemical and cytological study of the calciferous glands of Lumbricus terrestris. Physiol. Zool. 32, 40-46.

Boyer S.H. (1961) Alkaline phosphatase in human sera and placenta. Science 134, 1002-1004.

Brewer G.J. & Sing C.F. (1970) An Introduction to Isozyme Techniques. Academic Press, New York.

Carter M.J. (1972) Carbonic anhydrase: isoenzymes, properties, distribution, and functional significance. Biol. Rev. 47, 465-513.

Chiancone E., Veccini P., Rossi-Fanelli M.R. & Antonini E. (1972) Studies on erythrocruorin-II. Dissociation of earthworm erythrocruorin. J. molec. Biol. 70, 73-84.

Clark A.M. (1957) The distribution of carbonic anhydrase in the earthworm and snail. Aust. J. Sci. 19, 205-207.

Clarke R.B. (1969) Systematics and phylogeny: Annelida, Echiura, Sipuncula. In Chemical Zoology (Edited by Florkin M. and Scheer B.T.) Vol. IV, pp. 1-62. Academic Press, New York.

Cohen S. & Lewis H.B. (1949) The nitrogenous metabolism of the earthworm (Lumbricus terrestris). J. biol. Chem. 180, 79-91.

Cooper E.L. & Baculi B.S. (1968) Degenerative changes in the annelid, Lumbricus terrestris. J. Geront. 23, 375-381.

Dales R.P. (1967) Annelids. Hutchinson University Library, London.

Delaney R.G., Lahiri S. & Fishman A.P. (1974) Aestivation of the African lungfish Protopterus aethiopicus: cardiovascular and respiratory functions. J. exp. Biol. 61, 111-128.

Dietz T. (1974) Active chloride transport across the skin of the earthworm, Lumbricus terrestris L. Comp. Biochem. Physiol. 49A, 251-258.

Eddy S. & Hodson A.C. (1961) Taxonomic Keys To The Common Animals of The North Central States. Burgess Publishing Company, Minnesota.

Edwards C.A. & Lofty J.R. (1972) Biology of Earthworms. Chapman and Hall, London.

Florkin M. (1969) Respiratory proteins and oxygen transport. In Chemical Zoology (Edited by Florkin M. and Scheer B.T.), Vol. IV, pp. 111-132. Academic Press, New York.

Gerard B.M. (1967) Factors affecting earthworms in pastures. J. Anim. Ecol. 36, 235-252.

Gillespie J.H. & Kojima K. (1968) The degree of polymorphism in enzymes involved in energy production compared to that in nonspecific enzymes in two Drosophila ananassae populations. Proc. Natl. Acad. Sci. 61, 582-585.

Grant W.C. (1955) Studies in moisture relationships in earthworms. Ecology 36, 400-407.

Heran H. (1956) Ein Beitrag zur Verdauungsphysiologie von Lumbricus terrestris L. Z. Vergleich Physiol. 39, 44-62 (cited in Laverack M.S., 1963).

Hochachka P.W. (1965) Isoenzymes in metabolic adaptation of a poikilotherm: subunit relationships in lactic dehydrogenase of goldfish. Arch. Biochim. Biophys. 111, 96-103.

Hochachka P.W. & Somero G.N. (1973) Strategies of Biochemical Adaptation W.B. Saunders Company, Philadelphia.

Holmes R.S. & Masters C.J. (1967) The developmental multiplicity and isoenzyme status of cavian esterases. Biochim. Biophys. Acta 132, 379-399.

Holmes R.S., Masters C.J. & Webb E.C. (1968) A comparative study of vertebrate esterases multiplicity. Comp. Biochem. Physiol. 26, 837-852.

Holmes R.S. & Whitt G.S. (1970) Developmental genetics of the esterase isozymes of Fundulus heteroclitus. Biochem. Genet. 4, 471-480.

Hopkinson D.A., Spencer N. & Harris H. (1964) Genetical studies on human red cell acid phosphatase. Am. J. Human Genet. 16, 141-155.

Horne F.R. (1971) Accumulation of urea by a pulmonate snail during aestivation. Comp. Bioch. Physiol. 38A, 565-570.

Huang K.C. & Chen T.S.T. (1971) Ion transport across the intestinal mucosa of winter flounders, Pseudopleuronectus americanus. Am. J. Physiol. 220, 1734-1738.

Jaccarini V. & Harris R.R. (1975) An immunological comparison of haemocyanins from geographically isolated populations of some Brachyura (Decapods). Comp. Bioch. Physiol. 50A, 729-731.

Janssens P.A. & Cohen P.P. (1968) Biosynthesis of urea in the aestivating African lungfish and Xenopus laevis under conditions of water shortage. Comp. Biochem. Physiol. 24, 887-898.

Jeuniaux C. (1969) Nutrition and digestion. In Chemical Zoology (Edited by Florkin M. and Scheer B.T.), Vol. IV, pp. 69-91. Academic Press, New York.

Johnson G.B. (1974) Enzyme polymorphism and metabolism. Science 184, 28-37.

Kinnery V.R. & Code C.F. (1964) Canine ileal chloride absorption: effect of carbonic anhydrase inhibitor on transport. Am. J. Physiol. 207, 998-1004.

Kojima K., Gillespie J. & Tobari Y.N. (1970) A profile of Drosophila species enzymes assayed by electrophoresis I. Number of alleles, heterozygosities, and linkage disequilibrium in glucose-metabolizing systems and some other enzymes. Biochem. Genet. 4, 627-637.

Lahiri S., Szidon J.P. & Fishman A.P. (1970) Potential respiratory and circulatory adjustments to hypoxia in the African lungfish. Fedn. Proc. 29, 1141-1148.

Laverack M.S. (1963) The Physiology of Earthworms. Pergamon Press, Oxford.

Leone C.A. (1962) Taxonomic Biochemistry and Serology. The Ronald Press Company, New York.

Levin O. (1963) Electron microscope observations on some 60S erythrocruorins and their split products. J. molec. Biol. 6, 95-101.

Lindsay D.T. (1963) Isozymic patterns and properties of lactate dehydrogenase from developing tissues of the chicken. J. exp. Zool. 152, 75-89.

Maetz J. (1973) Na^+/NH_4^+, Na^+/H^+ exchanges and NH_3 movement across the gill of Carassius auratus. J. exp. Biol. 58, 255-275.

Manwell C. (1959) Alkaline denaturation and oxygen equilibrium of annelid hemoglobins. J. cell and comp. Physiol. 53, 61-74.

Markert C.L. & Hunter R.L. (1959) The distribution of esterases in mouse tissues. J. Histochem. Cytochem. 7, 42-50.

Markert C.L. (1963) Dissociation and recombination of lactate dehydrogenase subunits. Science 140, 1329-1330.

Markert C.L. & Holmes R.S. (1972) Isoenzymes and ontogeny. Biol. Rev. 47, 309-361.

Markert C.L. (1975) Isozymes. Physiological Function, Vol. II Academic Press, New York.

Metcalf R.A., Whitt G.S., Childers W.F. & Metcalf R.L. (1972) A comparative analysis of the tissue esterases of the white crappie (Pomoxis annularis Rafinesque) and black crappie (Pomoxis nigromamaculatus Lesueur) by electrophoresis and selective inhibitors. Comp. Biochem. Physiol. 41B, 27-38.

Meglitsch P.A. (1972) Invertebrate Zoology, 2nd edn. Oxford University Press, New York.

Michon J. (1954) Influence de l'isolement à partir de la maturité sexuelle sur la biologie des lumbricidae. C.R. Acad. Sci., Paris, 238, 2457-2458.

Moon T.W. & Hochachka P.W. (1971) Temperature and enzyme activity in poikilotherms: isocitrate dehydrogenase in rainbow-trout liver. Biochem. J. 123, 695-705.

Needham A.E. (1957) Components of nitrogenous excreta in the earthworms Lumbricus terrestris L. and Eisenia foetida (Savigny). J. exp. Biol. 34, 425-446.

Ornstein L. (1964) Disc electrophoresis-I. Background and Theory. Ann. N.Y. Acad. Sci. 121, 321.

Prosser, C.L. (1973) Comparative Animal Physiology, 3rd edn. W.B. Saunders Company, Philadelphia.

Ramsay, J.A. (1949) The site of formation of hypotonic urine in the nephridium of Lumbricus. J. exp. Biol. 26, 65-75.

Roche J. (1965) Electron microscope studies on high molecular weight erythrocruorins of annelids. In Studies in Comparative Biochemistry. (Edited by Munday K.A.), Vol. 23, pp. 62-80, Pergamon Press, Oxford.

Roots B.I. (1956) The water relations of earthworms. 2. Resistance to dessication, immersion and behaviour when submerged and when allowed a choice of environment. J. exp. Biol. 33, 29-44.

Rossi-Fanelli M.R., Chiancone E., Vecchini P. & Antonini E. (1970) Studies in erythrocruorin 1. Physiocochemical properties of earthworm erythrocruorin. Archs. Biochem. Biophys. 141, 278-283.

Satchell J.E. (1967) Lumbricidae. In Soil Biology (Edited by Burgess A. and Raw F.), pp. 259-322, Academic Press, New York.

Shlom J. & Vinogradov S. (1973) A study of the subunit structure of the extracellular hemoglobin of Lumbricus terrestris. J. biol. Chem. 248, 7904-7912.

Smith H.W. (1930) Metabolism of the lungfish Protopterus aethiopicus. J. biol. Chem. 88, 97-130.

Sokal R.R. & Sneath P.H.A. (1963) Principles of Numerical Taxonomy. W.H. Freeman and Company, San Francisco.

Sokal R.R. & Rohlf F.J. (1969) Biometry. W.H. Freeman and Company, San Francisco.

Svedberg T. (1933) The sedimentation constants of the respiratory proteins. J. biol. Chem. 103, 311-325.

Svedberg T. & Eriksson-Quensel I.B. (1933) The molecular weight of erythrocruorins. J. Am. Chem. Soc. 55, 2834-2841.

Stephenson J. (1930) The Oligochaeta. Oxford at the Clarendon Press, London.

Tashian R.E. & Shaw M.W. (1962) Inheritance of an erythrocyte acety-lesterase variant. Am. J. Human Genet. 14, 295-300.

Tashian R.E. (1969) from In Biochemical Methods In Red Cell Genetics (Edited by Yunis Y.), pp. 307-334, Academic Press, New York.

Tembe V.B. & Dubash P.J. (1961) The earthworms: a review. J. Bombay Nat. Hist. Soc. 58, 171-201. (cited in Edwards and Lofty, 1972).

Terwilliger R.C., Terwilliger N.B. & Roxby R. (1975) Quarternary structure of Pista pacifica vascular hemoglobin. Comp. Biochem. Physiol. 50B, 225-232.

Tillinghast E.K. (1968) Variations in blood and coelomic fluid ammonia and urea levels in the earthworm Lumbricus terrestris. Comp. Biochem. Physiol. 24, 621-623.

Tillinghast E.K., McInnes D.C. & Duffill R.A. Jr. (1969) The effect of temperature and water availability on the output of ammonia and urea by the earthworm Lumbricus terrestris L. Comp. Biochem. Physiol. 29, 1087-1092.

Tillinghast E.K. & Huffman D.G. (1973) The pattern of nitrogen excretion during fasting of two fresh-water oligochaetes. Comp. Biochem. Physiol. 45A, 555-557.

Tillinghast E.K. & MacDonnell P.C. (1973) The distribution of ammonia-generating enzymes along the intestine of the earthworm Lumbricus terrestris L. J. exp. Zool. 185, 153-158.

van Gansen P. (1956) Les cellules chloragogènes des Lombriciens. Bull. Biol. 90, 335-356.

van Gansen P. (1963) Structure et fonctions due tube digestif du Lombricien Eisenia foetida Savigny. Ann. Soc. Roy. Zool. Belg. 93, 1-20.

van Holde K.E. & van Bruggen E.F.J. (1971) The hemocyanins. In Subunits in Biological Systems (Edited by Timasheff S.N. and Fasman G.E.), pp. 1-53, Marcel Dekker, New York.

Varela J.M. (1975) Potentials in exploring the physiological role of acetylcholinesterase isozymes. In Isozymes. Physiological Functions (Edited by Markert C.L.), Vol, II, pp. 315-343. Academic Press, New York.

Waxman L. (1971) The hemoglobin of Arenicola cristata. J. biol. Chem. 246, 7318-7327.

Wiechelman K.J. & Parkhurst L.J. (1972) Kinetics of ligand binding in the hemoglobin of Lumbricus terrestris. Biochemistry 11, 4515-4520.