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CELL AND COELOMIC FLUID VOLUME REGULATION IN THE EARTHWORM *LUMBRICUS TERRESTRIS*

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Abstract—1. The earthworm *Lumbricus terrestris* is capable of volume regulation in dilute media.
2. Total body water content remains constant over a wide range of salinities, but a greater percentage of body water is extracellular in more dilute media.
3. Worms in dilute media have lower integumental water exchange, which reduces osmotic influx of water.
4. Free amino acids in the coelomic fluid increase in both hypotonic and hypertonic media, providing a source of solutes for volume regulation.
5. Intracellular free amino acids vary as a function of salinity, suggesting iso-osmotic cell volume regulation.

INTRODUCTION

Since early in the nineteenth century (Darwin, 1896), earthworms have been known to thrive in aqueous environments. Roots (1956) has shown that several species of terrestrial oligochaetes, although preferring moist soil, will live indefinitely in fresh water. Physiological studies of water regulation have thus treated *Lumbricus terrestris* and other megadriles as freshwater animals. Much of this early literature, reviewed by Oglesby (1978) and summarized by Carley (1982), deals primarily with descriptions of the parameters of water balance. It is now well established that *Lumbricus* regulates the concentration of its coelomic fluid in media of less than 100 mOsm (Dietz & Alvarado, 1970). Less clear is whether *Lumbricus* can also regulate its volume when transferred from one salinity to another (Oglesby, 1978). Recently, several workers have studied the mechanisms of water balance. Dietz & Alvarado (1970) and Carley (1975, 1978a) have measured ion and water fluxes across the integument of *Lumbricus* to study osmoregulation, and the role of free amino acids in volume regulation of *Eisenia foetida* has been examined by Takeuchi (1980c).

Many questions about the mechanisms of volume and ionoregulation, however, remain unanswered. We have examined several aspects of water balance in *Lumbricus*, placing special emphasis on the regulation of volume in external media of different concentrations. Of particular interest are the role of integumental water fluxes and the significance of small organic ions in regulating volume. We have also made preliminary observations on cell volume regulation and its contribution to overall volume regulation in this freshwater animal.

Although most reports on hydromineral metabolism in earthworms, including the experiments described here, were carried out in controlled aqueous media, the natural habitat of *Lumbricus* is moist soil. Carley (1978a) and Oglesby (1978) have pointed out that certain traits associated with freshwater existence (e.g. production of copius, dilute urine) are not consonant with survival in terrestrial environments. Therefore, while the earthworm serves as a useful model for understanding hydromineral metabolism, results of experiments in water must be interpreted in light of the ecological relationships of these animals.

MATERIALS AND METHODS

Mature *Lumbricus terrestris* weighing 2–7 g were obtained from local suppliers and maintained in artificial pond water (APW, Dietz & Alvarado, 1970, 2.7 mOsm total) at 12°C. Test salinities were prepared by adding 25, 50, 75, or 100 mM/l NaCl to APW. All experiments were performed on animals adapted to the test salinity for at least 48 hr.

Total body water was determined by drying animals to constant weight at 100°C. Extracellular fluid volume was estimated by dilution of a radioactive tracer. 100 μl of a 10 μCi/ml solution of [^{3}H]inulin was injected into the coelom of unanesthetized animals and allowed to equilibrate for 2 hr in a measured volume of the test salinity. Following equilibration worms were weighed and duplicate 10 μl samples of coelomic fluid were withdrawn in tapered Pasteur pipettes. Radioactivity of samples was measured in a Beckman 100LS liquid scintillation system using New England Nuclear Biofluor as a scintillation cocktail. Analysis of aliquots of the medium allowed correction for possible loss of inulin through the nephridia or dorsal pores.

Determination of integumental water exchange followed previously described methods (Welsh *et al.*, 1964; Carley, 1975, 1978a) except that tritiated water replaced deuterium oxide. Coelomic incorporation of ^{3}H from a 0.1 μCi/100 ml solution was measured during 30 min exposures. The rate constant for water exchange, K, expresses the fractional water exchange per hour.

Both total osmotic pressure and concentrations of organic solutes in the coelomic fluid were tested in several

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salinities. Osmolality was measured using a Wescor 5100B vapor pressure osmometer. Analysis of the free amino nitrogen included separate determinations of total ninhydrin-positive-substances (NPS), urea nitrogen, and ammonia nitrogen. NPS was measured by the methods of Clark (1976). Pooled coelomic fluid samples from five animals were treated with five volumes of ice-cold 95% ethanol for 15 min and centrifuged to remove proteins and particulates. 100 μl aliquots of this extract were reacted with ninhydrin and ascorbic acid (reducing agent) for 40 min in a boiling water bath. The purple end product was compared colorimetrically at 570 nm to a glycine standard curve. Urea and ammonia levels were determined with test kits (640 and 170-UV, respectively) from the Sigma Chemical Company, St. Louis, Mo.

Total NPS was also measured in samples of body wall. Pieces of body wall tissue weighing 0.1–0.15 g were scraped clean of nerve and gut and homogenized in 3.0 ml H_2O. Proteins were precipitated with 10 vol. of 95% ethanol, samples centrifuged, and the supernatant was assayed as described above.

Results are presented as means + 2 SE. Differences between means were analyzed by Student's *t*-test and considered significant if $P < 0.05$.

RESULTS

Under steady-state conditions *Lumbricus terrestris* regulates both the water content of the body and the osmotic pressure of the coelomic fluid over a wide range of salinities. The osmotic pressure of the coelomic fluid is hyperregulated at approx 150 mOsm in salinities of 150 mOsm and below (Fig. 1). The lower limit of regulation, the lower critical salinity (Oglesby, 1965), is below 2.7 mOsm, but the animals cannot survive extended immersion in distilled water. In hypertonic media the osmotic pressure of the coelomic fluid begins to rise until it follows the line of isotonicity, but remains slightly hypertonic to the medium.

Table 1 shows that 84% of the wet weight of worms in APW and in 100 mM NaCl is water. The constant hydration state exhibited over this range of salinities apparently results from changes in the distribution of body water in different media (Table 2). Greater proportions of the body water are distributed extracellularly in more dilute media. The extracellular water content decreases by about ten percent of the total body water when worms are transferred from APW to isotonic media (150 mOsm). The observed differences approach the level of statistical significance ($P = 0.06$).

One possible mechanism for volume regulation in different salinities is the reduction of integumental permeability to water in dilute media, which would reduce the osmotic influx of water. As shown in Table 3, *Lumbricus* uses this mechanism. Water influx in APW is 20% lower than that in 100 mM NaCl, representing a significantly reduced hourly water exchange fraction, *K*.

Organic solutes also appear to play an important role in volume and osmoregulation in these worms (Fig. 2). Concentrations of total ninhydrin-positive

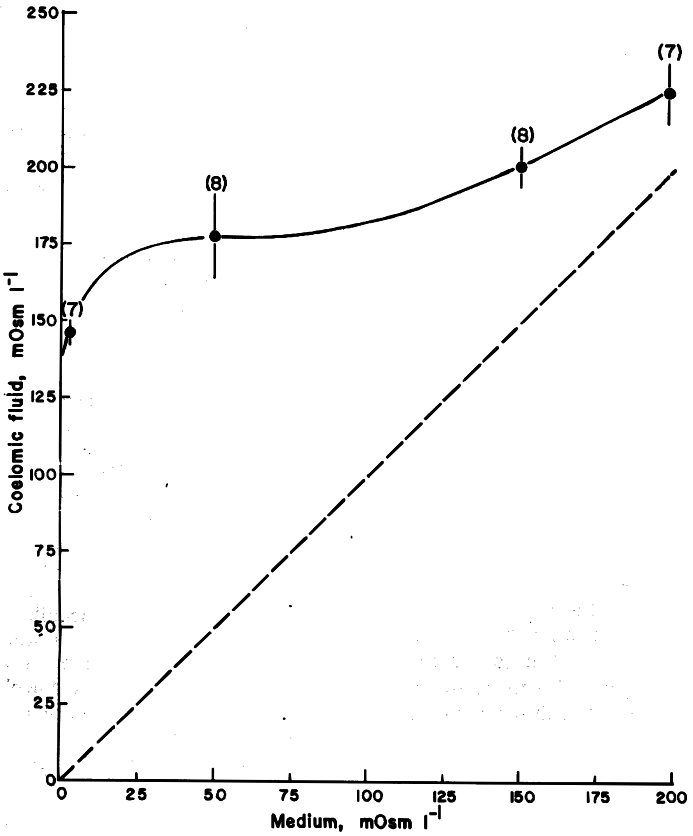

Fig. 1. Osmotic pressure of the coelomic fluid of *Lumbricus terrestris* as a function of external salinity. Dashed line is line of isotonicity. Osmotic pressure is regulated from below 3 mOsm to 150 mOsm. Vertical bars are ±2 SE; figures in parentheses are numbers of worms tested at each salinity.

Table 1. Water contents of *Lumbricus terrestris* in hypotonic and hypertonic media

Medium	N	Water Content (% Wet weight)	Hydration state (g H_2O g dry weight^{-1})
APW	30	84.1 ± 0.6	5.39 ± 0.26
100mM NaCl	10	84.3 ± 0.6	5.37 ± 0.28

Values (mean ±2 SE) for water content and hydration state do not differ between media.

Table 2. Distribution of body water in *Lumbricus terrestris* in hypotonic and isotonic media

Medium	N	Extracellular fluid (measured, % total H_2O)	Intracellular fluid (calculated, % total H_2O)
APW	23	63.4 ± 6.3	36.6
75mM NaCl	18	54.7 ± 6.3*	45.3

Values are mean ±2 SE.
* Probability of a greater difference, $P = 0.06$.

substances (NPS) in the coelomic fluids are at their lowest levels in isotonic media and rise markedly in both more concentrated and more dilute media. In APW the NPS may comprise as much as one-third of the total solutes in the coelomic fluid. In order to determine the composition of the NPS we performed separate analyses of the concentrations of ammonia nitrogen and urea nitrogen in the coelomic fluid. As shown in Fig. 3, ammonia concentrations are quite low in all media. Urea concentrations appear to remain rather steady in the several media tested. The mean values of 5–18 mM urea do not show any consistent trend with increasing external solute concentration. The balance of the NPS not accounted for by ammonia and urea is presumed to be free amino acids.

Table 3. Hourly water exchange fractions (K) of *Lumbricus terrestris* in hypotonic and hypertonic media

Medium	N	K
APW	24	1.29 ± 0.09
100mM NaCl	23	1.56 ± 0.17*

Values are mean ±2 SE.
* Differs from APW, $P < 0.05$.

Because free amino acids may be important in volume regulation of the coelomic fluid, we also measured the total NPS of the cells of the body wall to examine any possible role of organic solutes in

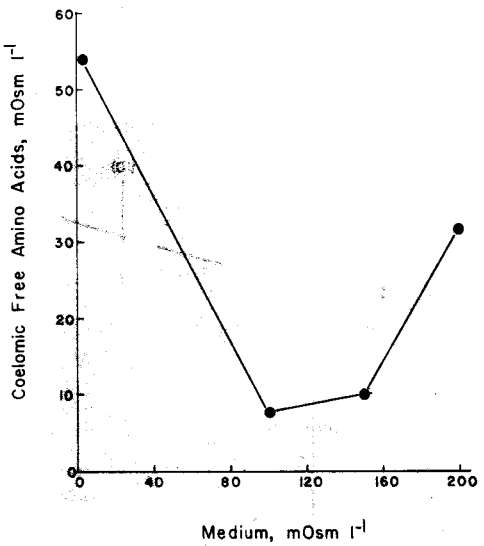

Fig. 2. Free amino acids (NPS) in the coelomic fluid of *Lumbricus terrestris*. NPS are lowest in isotonic media and rise significantly as salinity is increased or decreased. Points are means of triplicate samples of pooled coelomic fluid from 5 worms.

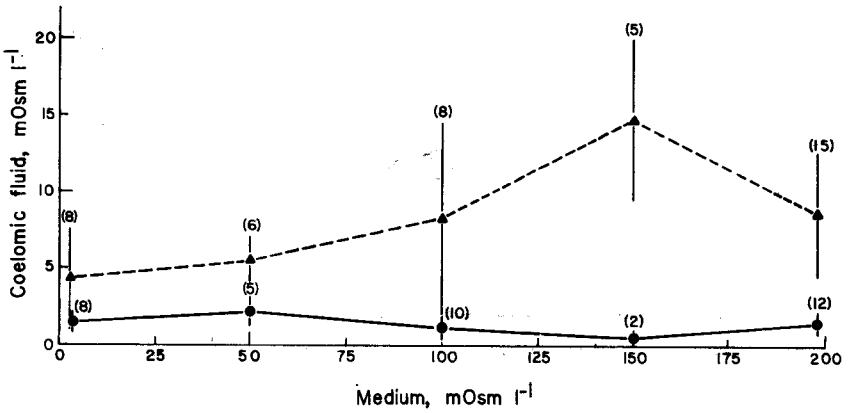

Fig. 3. Ammonia (●——●) and urea (▲----▲) in the coelomic fluid of *Lumbricus terrestris*. These ions appear not to be importantly osmoactive. Vertical bars are ±2 SE and numbers in parentheses are numbers of worms tested at each point.

intracellular volume regulation. NPS form a significant component of the intracellular fluids of *Lumbricus* (Fig. 4). Intracellular NPS concentrations increase with increasing external salinity up to isoosmotic media. Above 150 mOsm, NPS levels fall considerably; at 200 mOsm external concentration, NPS are only 66% of peak levels.

DISCUSSION

Because of their origin from freshwater ancestors (Stephenson, 1930), earthworms might be expected to exhibit volume regulation. And, at least to a limited extent, such is the case. As early as 1925 Adolph (1927) showed that earthworms lost weight upon immersion in NaCl solutions, but were able to regain this weight in solutions as concentrated as 200 mOsm. In more concentrated media animals were unable to compensate for lost water, although weight loss was halted after an initial adjustment period. Interestingly, this response, which is also seen in $NaHCO_3$ solutions, is markedly reduced in other media, and only slight weight changes were observed in very dilute KCl, urea, or sucrose solutions. Maluf (1939) confirmed Adolph's observation that worms placed in dilute NaCl solutions can maintain volume. However, Maluf also had worms which maintained weight levels in dilute solutions of glucose and urea. In contrast, one animal which he labelled "dying" showed a steady weight gain for 24 hr following immersion. The

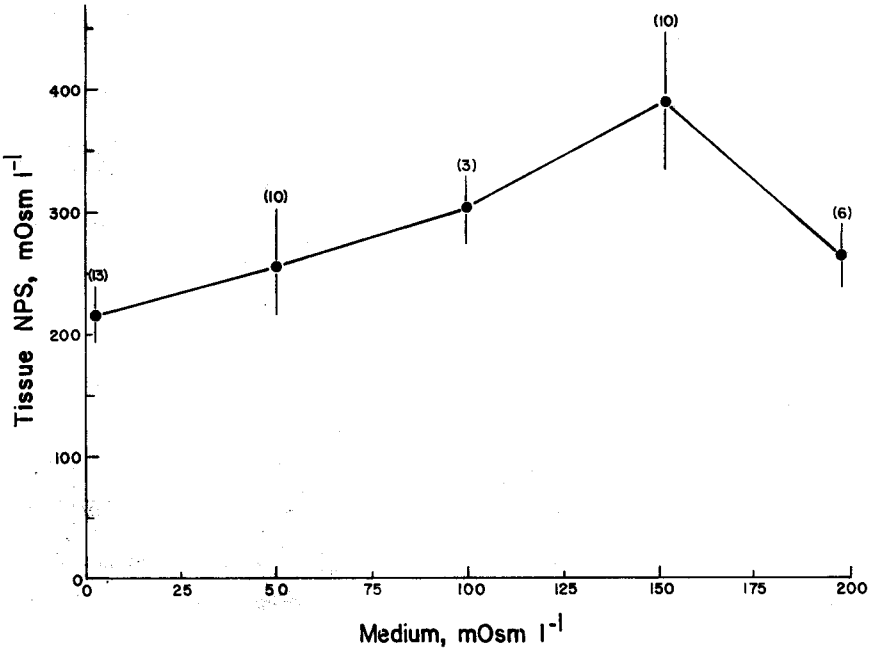

Fig. 4. Ninhydrin-positive substances in the body wall tissues of *Lumbricus terrestris* are correlated with external salinity. Symbols as in Fig. 1.

results of these authors are corroborated by a different analysis in this study. Total water contents of animals in APW and 200 mOsm NaCl are both 84% of total body weight. If we presume no change in the amount of dry matter per worm between the two media, the constancy of the ratio of water/dry weight suggests that worms in both media regulate volume at the same levels.

Of particular interest in this context is the change in tissue hydration in different media (Table 2). The apparent decrease in intracellular fluid in dilute media is indicative of several possible volume-regulating mechanisms. Jackson (1926) has shown that the coelomic cavity of *Lumbricus* loses a relatively greater proportion of its water during desiccation (equivalent to hyperosmotic media) than the gut tissue and body wall. Cell volumes did not change significantly, suggesting some form of cell volume regulation. As the gut is important in osmoregulation (Maluf, 1939; Oglesby, 1978), there may be exchanges of water between the intestine and coelomic fluid which do not involve the body wall. One probable cause for the observed changes in tissue water is the increase in coelomic free amino acids and the decrease in intracellular free amino acids in the body wall during immersion in APW (Figs 3 and 4, respectively). The decrease in cellular osmotic pressure would result in constant or reduced hydration of the tissues, while water would be osmotically drawn into the coelom. This behavior differs from that of the polychaete *Nereis* (= *Neanthes*) *succinea*, whose tissues become more hydrated when the animal is transferred to more dilute media (Freel *et al.*, 1973), suggesting that isoosmotic cell volume regulation is better developed in polychaetes than in oligochaetes.

One of the principal mechanisms for volume regulation in earthworms is active transport of ions across the body wall. Separate transport systems for sodium and chloride have been demonstrated by Dietz (1974) and Dietz & Alvarado (1970). Because these two ions are selectively taken up from the medium, *Lumbricus* can maintain large concentration gradients and hyper-regulate its coelomic fluid in dilute media (Fig. 1). The failure of active transport probably caused the steady weight gain exhibited by Maluf's "dying" worm (Maluf, 1930). Stephens and his colleagues (Stephens, 1964) have shown that freshwater invertebrates do not actively transport organic molecules across the body wall, which correlates with Adolph's finding that earthworms cannot regulate volume in solutions of sucrose and urea.

Although, as is true of other freshwater animals, water exchange in earthworms is lower than it is marine polychaetes, these animals transport significant quantities of water (Carley, 1982). The ability to regulate water influx in dilute media is a major aspect of volume regulation in many euryhaline animals (Smith, 1976). Carley (1975, 1978a) has already demonstrated that *Lumbricus* can reduce water exchange in terrestrial conditions, which could conserve water in a desiccating environment. Table 3 shows that water exchange is significantly lower in APW than in 100 mM NaCl, a hyperosmotic medium. In addition to regulating solute concentration, the reduction of water exchange will result in an important reduction of osmotic water influx.

Small organic anions also appear to play a significant role in volume and osmoregulation of earthworms. As Cl^- occurs at only about 60% of the Na^+ concentration in the coelomic fluid of *Lumbricus* (Kamemoto *et al.*, 1962; Dietz & Alvarado, 1970), amino acids and other organic molecules would be expected to make important contributions to osmotic pressure in all salinities. In the leech *Hirudo medicinalis*, for example, Zerbst-Boroffka (1970) found that organic acids including fumarate, succinate, and lactate accounted for 21.7% of the total hemocoelic osmolarity, enough to completely balance the anion deficiency caused by low Cl^- concentrations. McLaughlin (1971) reported only trace quantities of fumaric and succinic acids, and 2.2 mM amino acids in the blood of *Eisenia*. As his worms were not maintained in controlled media, it is not possible to assess the importance of these ions in osmoregulation.

In the only previous report of free amino acids in the coelomic fluid of a lumbricid oligochaete, Takeuchi (1980c) found that levels of free amino acids in *Eisenia* ranged from nearly zero in isosmotic media to almost 150 mOsm in strongly hypertonic NaCl solutions. As seen in Fig. 2, the first measurement of ninhydrin-positive substances (NPS) in *Lumbricus*, coelomic content of NPS is high in both hypo- and hyperosmotic media. Dietz & Alvarado (1970) reported an increase in the non-NaCl constituents of the coelomic fluid of worms in media above 125 mOsm total solute, and Takeuchi (1980c) suggested such hyperosmotic regulation in *Eisenia foetida* is brought about by free amino acids. We have confirmed this result and extended the observation to include osmoregulation in dilute media. The eightfold increase in coelomic NPS between 100 mOsm NaCl and APW represents a clear response to reduced environmental salinity. Clark (1968a, b) demonstrated similar trends in amino acid nitrogen content of several species of polychaetes. Basal levels vary from 5 mg/100 ml in subtidal species to 150 mg/100 ml in intertidal forms. In *Neoamphitrite robusta* coelomic amino acid nitrogen rose from 22.61 mg/100 ml in 100% sea water to 63.69 mg/100 ml in 50% sea water. Although *Lumbricus* maintains steady-state levels of Na^+ and Cl^- in APW and 5 mM NaCl solutions, other ions, for example K+, are continually lost to the medium (Dietz & Alvarado, 1970). Mobilization of free amino acid stores could be expected to balance losses and contribute increasingly to the total osmotic pressure. There are, however, limits to this compensation. Both Dietz & Alvarado (1970) and Carley (1974) have shown that *Lumbricus* cannot survive in distilled water. The lower critical salinity, as determined by this study (Fig. 1) and supported by Dietz & Alvarado (1970), is less than 3 mOsm. The one report of a lower critical salinity of 13 mOsm (Takeuchi, 1980c) cannot be confirmed here or in the literature.

In order to determine the constituents of the NPS, we performed separate analyses of ammonia and urea in the coelomic fluid (Fig. 3). Ammonia occurs in very low concentrations, less than 2 mM, in all media tested and therefore probably is not a significant osmoregulatory ion. Urea is much more variable, ranging from 5 to 18 mM in the different media. Although urea is present in sufficient quantity to form a major component of the coelomic solutes, our

results suggest it is not important in osmoregulation, as the mean values do not differ among salinities tested. Tillinghast (1967, 1968) found urea concentrations in blood and coelomic fluid in *Lumbricus* to be a function of fasting; feeding earthworms are ammonotelic. The worms in our study were not fed during immersion, and thus it is probable that the urea levels we measured were a result of starvation. The remaining NPS, the principal osmoregulatory anions, are assumed to be amino acids, which occur in significant quantities in other earthworms (Oglesby, 1978).

Free amino acids are important in intracellular volume regulation in many euryhaline invertebrates. Free amino acid levels in tissues of the snail *Melanopsis trifasciata* (Bedford, 1971a) and the clam *Rangia cuneata* (Henry and Mangum, 1980; Henry *et al.*, 1980) parallel external salinity and prevent cellular swelling in dilute media. Clark (1968a, b) reported similar trends in the several species of polychaetes she examined. Free amino acids in the cells of the body wall of *Lumbricus* also appear to be a function of external salinity (Fig. 4). There is a 77% increase in NPS from 220 to 390 mOsm between APW and 150 mOsm NaCl. In hyperosmotic media levels of NPS again fall as the worms begin to conform to external salinities. Earthworms appear to regulate levels of free amino acids only in those media wherein they osmoregulate, and not in hyperosmotic media.

The source of free amino acids used in osmoregulation is not entirely clear. Possibilities include *de novo* synthesis, protein catabolism, and transport between fluid spaces in the animal. Henry *et al.* (1980) favor *de novo* synthesis via a modified Embden–Myerhoff pathway in *Rangia*, but *Melanopsis* may use protein turnover (Bedford, 1971b). If tissue proteins are the source of free amino acids, the ratios of different free amino acids should parallel the frequencies of their occurence in proteins. McLaughlin (1971) has shown this to be true of free amino acids in the blood of *Eisenia*, which occur in similar proportions to amino acids in the animal's hemoglobin. Data to determine whether this pattern also holds in the coelomic fluid of lumbricids are presently lacking.

Volume- and osmoregulation of earthworms are complex processes involving many organs, including body wall, nephridia, and gut. The interactions of these organs appear to be finely controlled by neurohormones from the brain. Kamemoto (1964) and Carley (1975, 1978a b) have shown that a neuroendocrine factor from the brain reduces integumental permeability in *Lumbricus*. This same factor may also directly or indirectly affect ion fluxes and concentrations (Kamemoto, 1964; Takeuchi, 1980a). Takeuchi (1980b) has recently shown that the brain may also regulate coelomic free amino acid concentrations in *Pheretima*. Brain removal increases free amino acids from six to thirteen times their concentrations in intact worms. Injection of brain homogenate restores concentrations to levels of control animals. We have shown that *Lumbricus terrestris* regulates the osmotic pressure of its coelomic fluid, the rate of integumental water exchange, and the concentrations of free amino acids both in the coelomic fluid and the intracellular fluid of body wall cells. Strong evidence thus indicates that these several mechanisms for volume regulation are under neuroendocrine control, but the specific nature of this control and the particular target organs of the brain hormone(s) remain to be elucidated.

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