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THE EFFECT OF HEAVY METALS ON THE UPTAKE OF
L-HISTIDINE BY THE POLYCHAETE *NEREIS SUCCINEA*

A THESIS SUBMITTED TO THE GRADUATE DIVISION OF THE
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ABSTRACT

Integumentary uptake of ^3H -L-Histidine by *Nereis succinea* was measured in the presence and absence of selected heavy metals and inhibitors in 60% artificial seawater (ASW). At low concentrations of L-Histidine (10 μM), metals stimulated L-Histidine uptake from ASW. Higher concentrations of metal inhibited L-Histidine uptake. In amino acid kinetic experiments, 0.5 μM Zn^{2+} significantly ($P < 0.003$) increased both L-Histidine influx J_{max} (control: 4.7 ± 0.4 ; treatment: 15.3 ± 1.7 nmol/g dry weight x 15 min), and K_m (control: 23.8 ± 5.1 ; treatment: 44.0 ± 8.8 μM). Fe^{3+} (0.5 μM) stimulated influx of 10 μM L-Histidine ($J_{\text{max}} = 6.9 \pm 0.4$ nmol/g dry wt x 15 min; $K_m = 86.7 \pm 12.3$ μM), but neither Ag^+ nor Al^{3+} significantly ($P > 0.05$) altered amino acid influx. L-Leucine (25 μM) reduced Zn^{2+} -stimulated L-Histidine influx, suggesting a possible role of the Na-independent L-transport system in metal-stimulated L-Histidine transport by worm integument.

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CHAPTER 1 INTRODUCTION

Initial Research of Epithelial Transport of Dissolved Organic Matter

The suggestion that aquatic animals could utilize dissolved organic matter (DOM) as an available food source was first proposed in the late 1800's. It was suggested that organisms, which lived at the bottom of the ocean with no apparent food source and animals lacking a fully developed digestive system, were absorbing DOM as their nutritional resource.¹⁸ This theory led to much debate within the scientific community for a majority of the next century.

August Pütter was the first person to critically examine the theory of DOM utilization by aquatic organisms.²⁵ Pütter hypothesized DOM, created from the biological and chemical breakdown of larger particles,³⁶ could be absorbed across the integument of aquatic animals and utilized as an important food source.²⁵ Pütter began quantitatively researching his hypothesis by measuring an animal's oxygen consumption (nutritional needs) and comparing that value to the amount of energy available to the animal from planktonic food alone. Pütter concluded an animal would not be able to meet its metabolic needs on plankton and algae alone. Pütter continued to conduct research for the next several years attempting to verify an animal's ability to make up its nutritional deficit through the absorption of DOM.²²

Unfortunately, Pütter's work was greatly criticized by the scientific community; not only because of his controversial theory, but also for his experimental procedures. Several scientists who revisited Pütter's work found many oversights and mathematical errors in his research. Scientists considered there to be no scientific basis behind the

theory that aquatic animals could absorb any substance in any concentration across their integument.²² Even as more precise methods for identifying small organic molecules arrived and DOM was found to occur in all aquatic environments,¹⁶ the scientific community remained skeptical of Pütter's theory.³¹ Active transport had not been fully realized and scientists concluded even if an animal's epithelium was permeable to DOM, a sufficient concentration gradient for diffusion did not exist.⁴²

In the early 1930's, August Krogh compiled all of Pütter's research and conducted an extensive review of Pütter's work. After assessing Pütter's findings, Krogh responded, "It cannot, therefore, be doubted that the water contains a number of substances essential as building material in the animal body, but it must be remembered on the other hand, that most of these are present in the very high dilution of one in a hundred million or less and that the non-nitrogenous substances do not appear to be in forms which can easily be absorbed or utilized by animals."²² Additionally, scientists in general were completely aware of the fully developed internal digestive systems most aquatic organisms had and they saw no reason why such an animal would not ingest particulate food for its total nutritional needs.³¹

August Krogh recognized that the current methods for determining DOM uptake were inadequate¹⁸ and he ultimately concluded there was not sufficient evidence to support Pütter's theory that aquatic animals could significantly utilize DOM.²² Independent research conducted by Krogh in 1934 on freshwater mussels and fish,²³ further strengthened his conclusion that DOM was not an available resource for aquatic animals.

Epithelial Transport of Dissolved Organic Matter, Revisited

After August Krogh published his review of Pütter's work and the results of his own research,^{22,23} the debate of epithelial DOM absorption remained relatively unexamined until the early 1960's.⁵⁴ When new experimental techniques, specifically radioisotope-labeling techniques,^{52,54} became available, Grover Stephens revisited the subject to reexamine the potential for marine invertebrates to absorb amino acids and sugars from their environment.^{32,33,34,35} Using C¹⁴-labelled glucose and amino acids, Stephens ultimately determined essentially all soft-bodied marine invertebrates were capable of removing small organic compounds, at varying rates,³⁶ through a process that did not involve the digestive tract.³⁹

To disprove some scientists' suspicions that water was being swallowed for nutrient absorption by the gut,²² experiments were conducted where the mouth and anus of the organism were blocked.^{15,17,36} Results of these experiments showed no significant difference between uptake values of DOM with or without the mouth and anus blocked. DOM epithelial absorption was also reconfirmed through the use of an Ussing-type chamber, where an animal's integument could be isolated and worked on independent of the animal itself. Uptake values measured for amino acid transport during these Ussing chamber experiments were found to be comparable to those uptake values measured for the whole worm.⁴² And although only certain amino acids were used during these experiments, it was assumed that other amino acids were also capable of being transported across invertebrate epithelium.³⁴

However, criticism still existed. Scientists knew, in many cases, experiments involved only one substrate and results were simply determined by the organism's ability

to remove the substrate from solution. This only allowed for nutrient uptake to be assumed, not proven, and told nothing about the mechanism of transport.⁴² Other criticisms that existed were due to the use of environmentally unrealistic substrate concentrations used during experiments⁵⁵ and the lack of metabolic and weight data collected.³⁴ Furthermore, still no definitive explanation could be given as to why filter feeders, scavengers, and carnivores would need additional nutrients aside from general microorganism consumption.³¹

The Energy Component of Epithelial Transport

Scientists knew DOM existed at concentrations as low as 10^{-7} and 10^{-6} M and therefore recognized marine invertebrates were accumulating nutrients against extreme concentration gradients.⁵⁴ Scientists began to research the foreseeable fact that the epithelial transport was dependent upon the input of energy.⁴³ Initial transport experiments on the gills of soft-shelled clams showed the transport of L-alanine was decreased in the presence of metabolic inhibitors,⁴⁰ offering proof epithelial transport required energy. Furthermore, because of the limited ability of freshwater invertebrates to conduct DOM uptake,^{35,36,53} scientists began to explore the possibility that sugars and amino acids were transported across an organism's integument coupled with sodium. Scientists hypothesized the transport of sodium into an organism, along its electrochemical gradient, could provide the necessary energy to facilitate the transport of sugars and amino acids into the organism against extreme concentration gradients.³⁹

The dependence of amino acid uptake on sodium transport was already well documented within vertebrate systems.³⁹ Scientists suggested invertebrate integument

might have a similar sodium-coupled transport mechanism. Stevens and Preston researched, in great detail, the effect of sodium ions on L-alanine transport across marine polychaete integument by measuring alanine influx as a function of alanine at varying sodium concentrations. They found the influx of L-alanine was obligatorily coupled to a sodium gradient and in the absence of sodium, L-alanine influx occurred primarily through diffusion.^{38,39,40} Further research by Gomme,¹⁶ using phlorozine and harmaline (competitive inhibitors of D-glucose transport), showed the influx of D-glucose across invertebrate epithelium to be sodium dependent. Over the next several years, researchers generated considerable data detailing the mechanisms behind the influence of sodium ions on the transport processes of several invertebrate species.^{2,16,26,37,42,53} Sodium dependent transporters similar to those in mammalian nutrient absorbing epithelium were found to exist in tapeworms,^{16,30} lobster antennal glands,⁸ eel intestinal brush-border membranes,²⁹ and cuttlefish integument.¹³

The use of sodium coupling to drive intracellular nutrient accumulation appeared to be a convenient and conservative evolutionary strategy utilized by marine invertebrates.³⁸ However, doubts were soon raised regarding the ability of a sodium gradient to fully account for all trans-epithelial nutrient accumulation by marine invertebrates.^{17,54,55} A number of mammalian, invertebrate, and bacterial transport systems had been found to accumulate amino acids independent of sodium^{42,55} suggesting the possibility a directly energized transport mechanism.⁴² Researchers began to find, contrary to previous studies, some amino acids were transferred across invertebrate integument through a sodium independent transport process.^{3,49} A transport classification system for sugars and amino acids was adapted for marine invertebrates and today, six

fundamental categories of invertebrate sodium-dependent and sodium-dependent transporters have been described.^{12,41}

Current Research Involving Epithelial Transport

Over the past several decades, scientists have worked towards better understanding the specific mechanisms involved in nutrient absorption by marine invertebrates. Researchers have identified numerous transport mechanisms and have now begun to examine these transport mechanisms on a much smaller, more detailed scale. Behnke et. al.⁸ have recently studied the mechanism behind glucose transport in lobster antennal glands and Klein and Ahearn²¹ have taken a closer look at the calcium transporter in lobster hepatopancreatic mitochondria cells. Research recently conducted by de Eguileor examined the uptake of L-leucine across the arms of cuttlefish in the presence and absence of sodium using several innovative techniques, including autoradiography.¹³ Still, other researchers are beginning to examine the molecular biology of transport mechanisms^{5,14} while others have moved beyond amino acids and sugars to other compounds readily available to be absorbed.^{4,10,44,45,46}

More so, whether or not DOM plays an important role in the nutrition of marine invertebrates still remains an active area of study.⁵⁴ Scientists are continuing to conduct research to establish the biological importance of DOM utilization by marine invertebrates. Several ideas for DOM utilization have been proposed including: growth spurts,⁵³ nutritional support during winter months or other periods of food shortage,^{13,31} osmotic regulation after food consumption,¹³ or direct metabolism by the organism's integument and/or muscles.¹³

Statement of Purpose

As mentioned previously, most research involving epithelial transport has been directed towards identifying the physiological mechanisms involved in the uptake of nutrients by soft-bodied aquatic invertebrates. Today, considerable literature and research exists that defines the basic properties and mechanisms of nutrient transport by invertebrate integumentary epithelia. However, what has yet to be fully investigated is the effect environmentally occurring heavy metals have on these known nutrient transport systems. To date, relatively few experiments have been conducted involving the transport of amino acids in a metal contaminated environment.^{7,24} Interestingly enough, these results suggest that, at low concentrations, heavy metals stimulate amino acid uptake into the organism.

The purpose of this investigation is to obtain estimates of unidirectional ³H-L-histidine influx in order to determine the effect of various heavy metals on the transmembrane entry rate of L-histidine in the polychaete, *Nereis succinea*. From this study, estimates of the Michaelis-Menten influx kinetics constants, K_m (apparent binding affinity) and J_{max} (apparent maximal amino acid influx rate) of the transport event, in the presence and absence of heavy metals, will be identified. These results could help to further characterize the mechanism involved in L-histidine transport across polychaete integument.

CHAPTER 2 METHODS AND MATERIALS

Euryhaline polychaete worms, of the genus and species *Nereis succinea*, ranging in size from 10mg to 300mg, were collected during low tide from the banks of the Ala Wai Canal (Diamond Head end) in Honolulu, Hawaii. The worms were transported from the collection site to the laboratory in a plastic cooler containing approximately 2L of 60% artificial seawater (ASW) at room temperature. (The 60% ASW was made by diluting 100% seawater that consisted of (per L of deionized water): 23.991g of NaCl, 0.72g of KCl, 1.503g of CaCl₂•2H₂O, 10.883g of MgCl₂•6H₂O, 9.099g of Na₂SO₄•10H₂O, 0.197g of NaHCO₃, 0.085g of NaBr, and 0.027g of H₃BO₃) with deionized water. At the laboratory, the worms were cleaned of mud and transferred to an artificial environment consisting of a 10L plastic basin, 30-40 small glass capillary tubes (for the worms to irrigate), and approximately 2L of 60% ASW at room temperature. The worms were kept in their artificial environment in the dark for at least 24 hours in order to allow sufficient time for the worms to become acclimated. Injured worms or worms failing to burrow into a glass capillary tube were not used.

Experimental solutions of L-histidine stock solution, which were prepared daily, and experimental solutions of metal stocks, which were prepared and stored for the duration of the experimental period, were prepared in 100mL volumetric flasks using the appropriate weights of chemicals and volumes of deionized water to achieve desired concentrations. Designated volumes from these prepared stock solutions were then added to 50mL beakers containing designated volumes of 60% ASW and ³H-L-histidine

(specific activity of $37.5\mu\text{Ci}/\mu\text{L}$) to create the experimental incubation solution that the worms would be placed in. Two $100\mu\text{L}$ aliquots were taken from each beaker of incubation solution and placed into a 5mL plastic scintillation vial. 3mL of scintillation fluid was then added. These samples (blanks) were used to calculate the specific activity of the experimental incubation solutions. The specific activity of the incubation solution was found using the following equation:

$$(\text{cpm of sample}/\mu\text{L of sample}) / [\text{L-histidine}] (\text{pmol}/\mu\text{L})$$

Glass test tubes (10mL) were filled with 3mL of incubation solution. Using small forceps, each worm was transferred from its artificial environment to a 10mL glass test tube. The incubation times for each worm varied from 10 minutes to 60 minutes and were timed using a stop clock. A stop solution, composed of 5mL of 60% ASW at 0°C , was kept on ice throughout the experiment in 10mL glass test tubes. At the end of each worm's specified incubation time, each worm was placed into the stop solution and shaken vigorously for 15 seconds to stop any further transport of $^3\text{H-L-histidine}$ from taking place and to wash off any excess radioactivity that was adhered to the surface of the worm's epithelium. Each worm was then placed in a 5mL plastic scintillation vial containing 3mL of 70% ethanol, capped, and allowed to incubate for 24 hours in order to extract the $^3\text{H-L-histidine}$ that had been transported across the polychaete's epithelium. After the 24-hour extraction period, a $250\mu\text{L}$ aliquot was taken from each worm's ethanol extract and placed into a 5mL scintillation vial. 3mL of scintillation fluid was then added into each of the sample vials and the vials were capped and placed into a Beckman

scintillation counter where the samples were counted for radioactivity in counts per minute (cpm).

The cpm values were converted into pmol of L-histidine and expressed per gram dry weight in order to standardize for the various weights of the worms used in each experiment. Each worm was placed on a pre-weighed aluminum foil square, inserted into an oven (80°F), and then re-weighed after 24 hours. The gram dry weight of the worm was calculated by subtracting the initial weight from the final weight. The gram dry weight of the worm was then raised to the 2/3 power. The worm's measured cpm value was divided by the specific activity of the incubation solution that the worm was placed in and then multiplied by its gram dry weight raised to the 2/3 power. The calculation is listed below:

$$(\text{cpm} / \text{specific activity}) \times (\text{gram dry weight})^{2/3}$$

CHAPTER 3 RESULTS

Effect of 1 μ M Zinc on the Uptake of 50 μ M 3 H-L-Histidine

A time course experiment was conducted measuring the uptake of 3 H-L-histidine as a function of time. Triplicate sets of worms were incubated for 10, 20, 30, 45, and 60 minutes in 60% ASW solutions containing 50 μ M 3 H-L-histidine in the presence of (experimental) and absence of (control) 1 μ M ZnSO₄ (Zn²⁺). Figure 1 shows the results of this experiment. In the control group, 3 H-L-histidine uptake followed first-order Michaelis-Menten kinetics, approaching equilibrium at time points in excess of 60 minutes. In the experimental group, 3 H-L-histidine uptake had a mean slope close to zero throughout the 60-minute time course. The results show that at low concentrations, Zn²⁺ has noteworthy inhibitory effects on the uptake of high concentrations of 3 H-L-histidine by *Nereis* epithelium. From this experiment an exposure period of 15 minutes (a time at which uptake was still linear) was chosen for all amino acid influx experiments.

Effect of 1 μ M Zinc and 25 μ M L-Leucine on the Uptake of 10 μ M ³H-L-Histidine

Triplicate sets of worms were incubated for 15, 30, and 60 minutes in 60% ASW solutions containing 10 μ M ³H-L-histidine in the presence and absence of 1 μ M zinc and 1 μ M zinc with 25 μ M L-Leucine. Figure 2 shows the results of this experiment. Results show that 1 μ M zinc has little to no effect on the uptake of ³H-L-histidine while 25 μ M L-Leucine has noteworthy inhibitory effects on the uptake of ³H-L-histidine. This inhibitory effect of L-Leucine on L-histidine uptake suggests that both amino acids share the same transport carrier protein.

Kinetics of ³H-L-Histidine Influx

To find the kinetics constants of ³H-L-histidine uptake in the absence of any metal (control), the influx of ³H-L-histidine was measured as a function of varying L-histidine concentrations. Triplicate sets of worms were incubated for 15 minutes in 60% ASW solutions containing 0.5, 1, 10, 20, and 50 μ M ³H-L-histidine. As shown in Figure 3, results follow a hyperbolic pattern plotted using a best-fit curve using Sigma Plot software. Estimates of integument transport values J_{\max} and K_m were calculated through this program. J_{\max} was found to be 4701 +/- 449 pmol g⁻¹ dry mass and K_m was found to be 23.71 +/- 5.02.

Kinetics of $^3\text{H-L-Histidine}$ Influx in the Presence of $0.5\mu\text{M}$ Silver (Ag^+)

Triplicate sets of worms were incubated for 15 minutes in 60% ASW solutions containing 0.5, 1, 10, 20, and $50\mu\text{M}$ $^3\text{H-L-histidine}$ and $0.5\mu\text{M}$ AgCl (Ag^+). As shown in Figure 4, results follow a hyperbolic pattern plotted using a best-fit curve using Sigma Plot software. Estimates of integument transport values J_{max} and K_{m} were calculated through this program. J_{max} was found to be 4289 ± 383 pmol g^{-1} dry mass per 15 minutes and K_{m} was found to be 23.36 ± 4.65 . Transport values suggest that the monovalent cation Ag^+ has no significant effect on L-histidine integument transport at silver concentrations equal to $0.5\mu\text{M}$.

Kinetics of $^3\text{H-L-Histidine}$ Influx in the Presence of $0.5\mu\text{M}$ Aluminum (Al^{3+})

Triplicate sets of worms were incubated for 15 minutes in 60% ASW solutions containing 0.5, 1, 10, 20, and $50\mu\text{M}$ $^3\text{H-L-histidine}$ and $0.5\mu\text{M}$ AlCl_3 (Al^{3+}). As shown in Figure 5, results follow a hyperbolic pattern plotted using a best-fit curve using Sigma Plot software. Estimates of integument transport values J_{max} and K_{m} were calculated through this program. J_{max} was found to be 4117 ± 226 pmol g^{-1} dry mass per 15 minutes and K_{m} was found to be 23.82 ± 2.90 . Transport values suggest that the trivalent cation Al^{3+} has no significant effect on L-histidine integument transport at aluminum concentrations equal to $0.5\mu\text{M}$.

Kinetics of $^3\text{H-L-Histidine}$ Influx in the Presence of $0.5\mu\text{M}$ Iron (Fe^{2+})

Triplicate sets of worms were incubated for 15 minutes in 60% ASW solutions containing 0.5, 1, 10, 20, and $50\mu\text{M}$ $^3\text{H-L-histidine}$ and $0.5\mu\text{M}$ FeSO_4 (Fe^{2+}). As shown in Figure 6, results follow a hyperbolic pattern plotted using a best-fit curve using Sigma Plot software. Estimates of integument transport values J_{max} and K_{m} were calculated through this program. J_{max} was found to be 6886 ± 405 pmol g^{-1} dry mass per 15 minutes and K_{m} was found to be 89.69 ± 12.27 . Transport values suggest that the divalent cation Fe^{2+} has a significant ($p < 0.001$) stimulatory effect on L-histidine integument transport at iron concentrations equal to $0.5\mu\text{M}$.

Kinetics of $^3\text{H-L-Histidine}$ Influx in the Presence of $0.5\mu\text{M}$ Zinc (Zn^{2+})

Triplicate sets of worms were incubated for 15 minutes in 60% ASW solutions containing 0.5, 1, 10, 20, and $50\mu\text{M}$ $^3\text{H-L-histidine}$ and $0.5\mu\text{M}$ ZnSO_4 (Zn^{2+}). As shown in Figure 7, results follow a hyperbolic pattern plotted using a best-fit curve in Sigma Plot software. Estimates of integument transport values J_{max} and K_{m} were calculated through this program. J_{max} was found to be 15259 ± 1711 pmol g^{-1} dry mass per 15 minutes and K_{m} was found to be 44.02 ± 8.77 . Transport values suggest that the divalent cation Zn^{2+} has a significant ($p < 0.003$) stimulatory effect on L-histidine integument transport at zinc concentrations equal to $0.5\mu\text{M}$.

Effect of Various Heavy Metals on ³H-L-Histidine Influx Kinetics

The transport values calculated from all five kinetics experiments were placed into Table 1 for comparison. Of the four experimental metals used (Ag^+ , Fe^{2+} , Zn^{2+} , and Al^{3+}), Zn^{2+} ($J_{\text{max}} = 15259 \pm 1711$ pmol g^{-1} dry mass per 15 minutes) and Fe^{2+} ($J_{\text{max}} = 6886 \pm 405$ pmol g^{-1} dry mass per 15 minutes) significantly ($p < 0.003$ and $p < 0.001$ respectively) stimulated the uptake of ³H-L-histidine across the integument of *Nereis* while Ag^+ ($J_{\text{max}} = 4289 \pm 383$ pmol g^{-1} dry mass per 15 minutes) and Al^{3+} ($J_{\text{max}} = 4117 \pm 226$ pmol g^{-1} dry mass per 15 minutes) had no significant effect. Furthermore, Zn^{2+} had a greater stimulatory effect on the uptake of ³H-L-histidine than Fe^{2+} . Results also show that stimulation of ³H-L-histidine not only stimulated the apparent maximal amino acid influx rate, J_{max} , but also decreased the apparent binding affinity for the amino acid to its transporter, K_m , (Zn^{2+} $K_m = 44.02 \pm 8.77$, Fe^{2+} $K_m = 89.69 \pm 12.27$). Ag^+ and Al^{3+} had no significant effect on the K_m values for L-histidine transport. All values relative to control values, $J_{\text{max}} = 4289 \pm 449$ pmol g^{-1} dry mass per 15 minutes and $K_m = 23.71 \pm 5.02$.

Table 1. Effect of Heavy Metals on ³H-L-Histidine Influx Kinetics

Condition	K_m	J_{max}
Control	23.71 +/- 5.02	4701 +/- 449
0.5 uM Ag ⁺	23.36 +/- 4.65	4289 +/- 383
0.5 uM Al ³⁺	23.82 +/- 2.90	4117 +/- 226
0.5 uM Zn ²⁺	44.02 +/- 8.77	15259 +/- 1711
0.5 uM Fe ²⁺	89.69 +/- 12.27	6886 +/- 405

Influx measured over 15 minute uptake intervals.

Triplicate worm samples used for each treatment condition.

Values are means +/- 1 SEM.

J_{max} units are in pmol g⁻¹ dry mass

**Effect of 1 μ M Zinc on the Uptake of 50 μ M 3 H-L-Histidine
Across the Integument of *Nereis succinea***

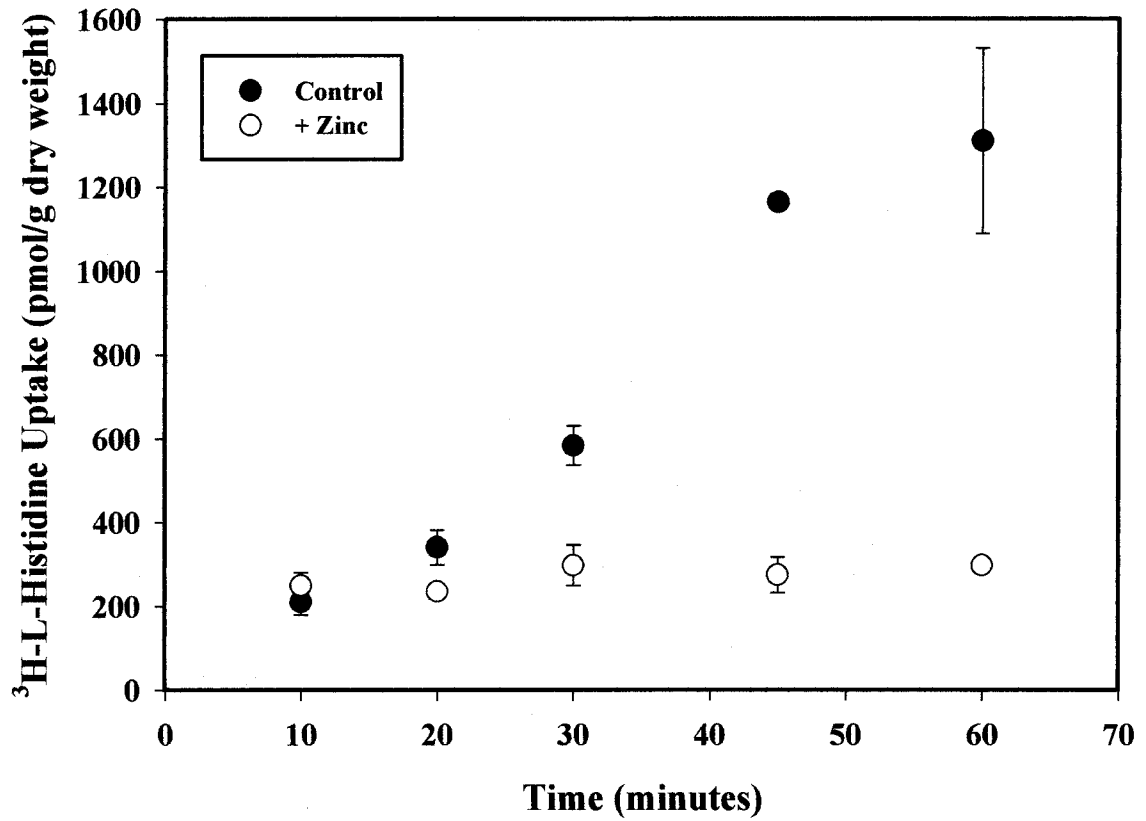


Figure 1. Effect of 1 μ M zinc on the uptake of 50 μ M 3 H-L-histidine in *Nereis succinea*. Triplicate samples of worms (means \pm SEM shown) were incubated for the designated times in 60% artificial sea water for 15 minutes. Zinc was added as ZnSO₄.

Effect of 1 μ M Zinc and 25 μ M L-Leucine on the Uptake of 10 μ M 3 H-L-Histidine Across the Integument of *Nereis succinea*

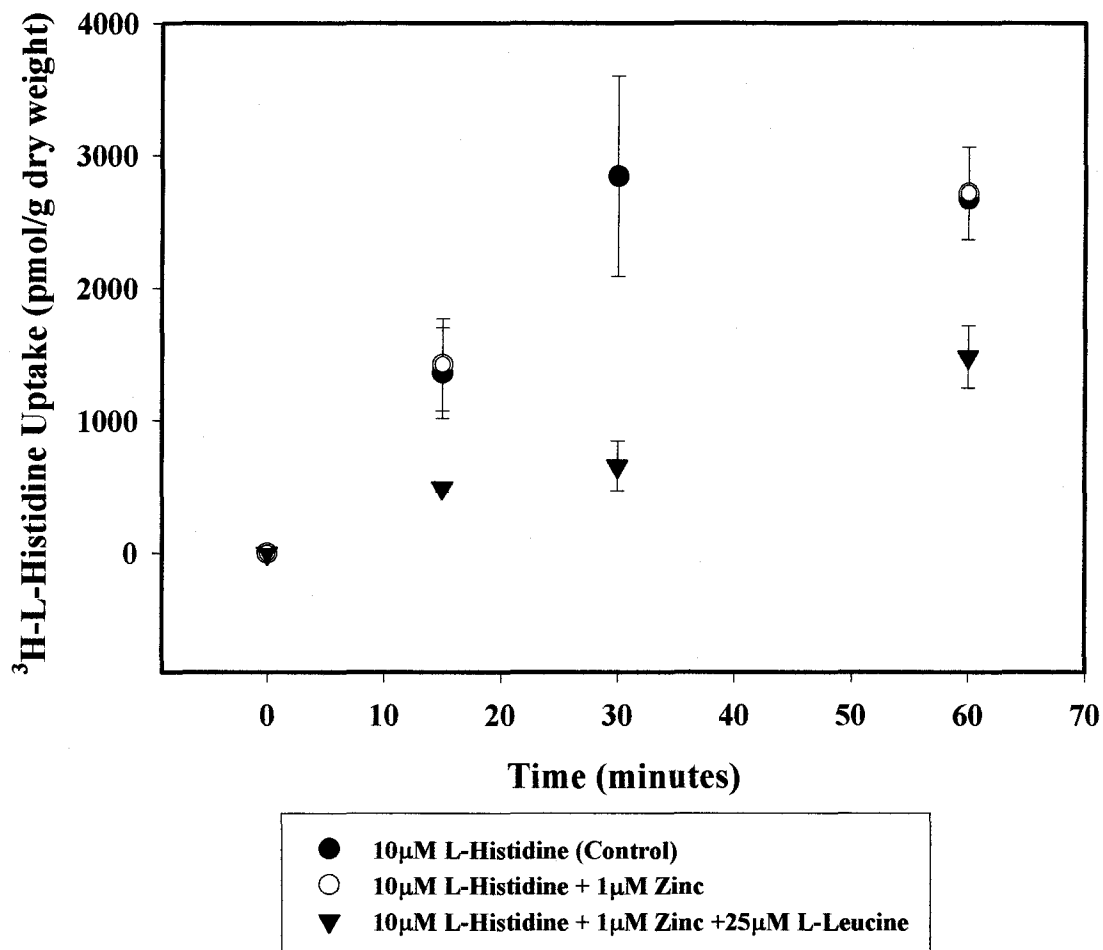


Figure 2. Effect of 1 μ M zinc and 25 μ M L-leucine on the uptake of 10 μ M 3 H-L-histidine in *Nereis succinea*. Triplicate sample of worms (means \pm SEM shown) were incubated at the designated times shown in 60% artificial seawater for 15 minutes. Zinc was added as ZnSO₄.

Kinetics of ^3H -L-Histidine Influx Across the Integument of *Nereis succinea*

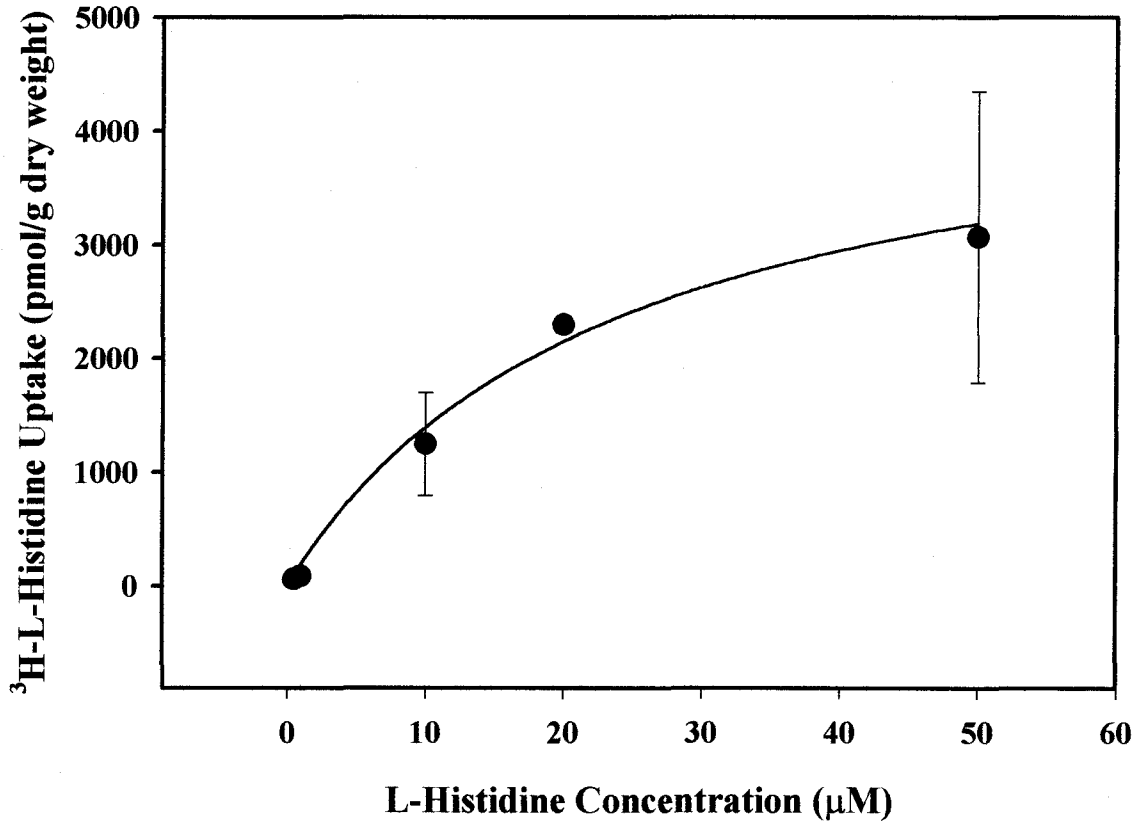


Figure 3. Control kinetics of ^3H -L-histidine influx in *Nereis succinea*. Triplicate samples of worms (means \pm SEM shown) were incubated in the concentrations shown in 60% artificial seawater for 15 minutes. No metals were added. Lines drawn through the data are best-fit curves using Sigma Plot software and kinetics constants were produced using this equation. J_{max} was $4701 \pm 449 \text{ pmol g}^{-1} \text{ dry mass}$. K_m was 23.71 ± 5.02 .

Kinetics of ^3H -L-Histidine Influx in the Presence of $0.5\mu\text{M}$ Silver Across the Integument of *Nereis succinea*

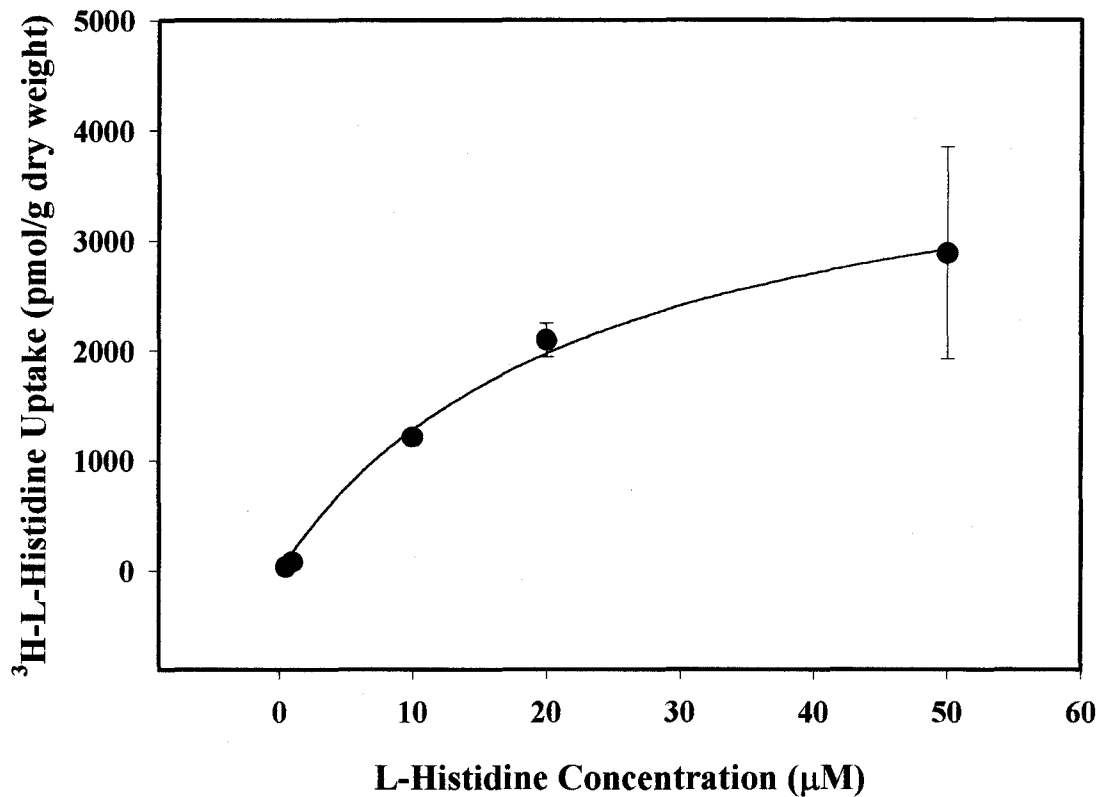


Figure 4. Kinetics of ^3H -L-histidine influx in *Nereis succinea* the presence of $0.5\mu\text{M}$ silver. Triplicate samples of worms (means \pm SEM shown) were incubated in the concentrations shown in 60% artificial seawater for 15 minutes. Lines drawn through the data are best-fit curves using Sigma Plot software and kinetics constants were produced using this equation. J_{max} was 4289 ± 383 pmol g^{-1} dry mass. K_m was 23.36 ± 4.65 . Silver was added as Ag_2SO_4 .

Kinetics of $^3\text{H-L-Histidine}$ Influx in the Presence of $0.5\mu\text{M}$ Aluminum Across the Integument of *Nereis succinea*

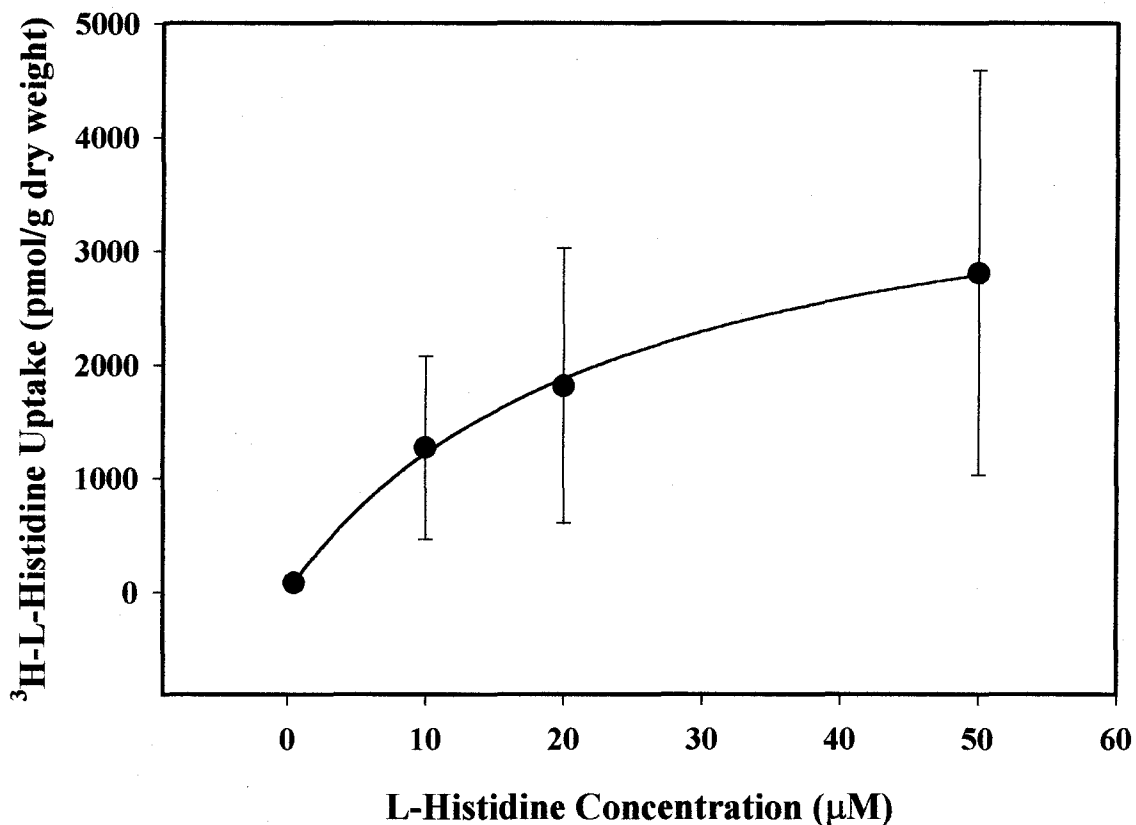


Figure 5. Kinetics of $^3\text{H-L-histidine}$ influx in *Nereis succinea* in the presence of $0.5\mu\text{M}$ aluminum. Triplicate samples of worms (means \pm SEM shown) were incubated in the concentrations shown in 60% artificial seawater for 15 minutes. Lines drawn through the data are best-fit curves using Sigma Plot software and kinetic constants were produced using this equation. J_{max} was 4117 ± 226 pmol g^{-1} dry mass per 15 minutes. K_m was 23.82 ± 2.90 . Aluminum was added as AlCl_3 .

Kinetics of ^3H -L-Histidine Influx in the Presence of $0.5\mu\text{M}$ Iron Across the Integument of *Nereis succinea*

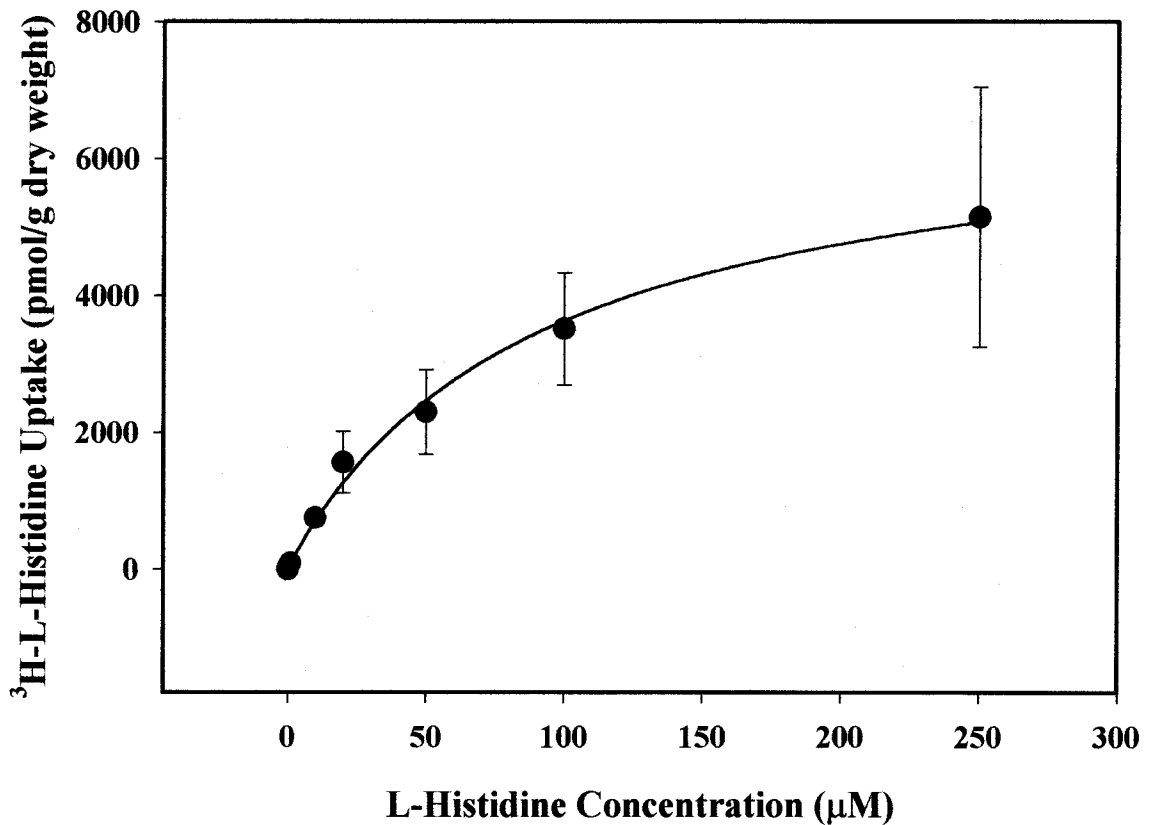


Figure 6. Kinetics of ^3H -L-histidine influx in *Nereis succinea* in the presence of $0.5\mu\text{M}$ iron. Triplicate samples of worms (means \pm SEM shown) were incubated in the concentrations shown in 60% artificial seawater (ASW). Lines drawn through the data are best-fit curves using Sigma Plot software and kinetics constants were produced using this equation. J_{max} was $6886 \pm 405 \text{ pmol g}^{-1} \text{ dry mass}$. K_m was 89.69 ± 12.27 . Iron was added as FeSO_4 .

Kinetics of $^3\text{H-L-Histidine}$ Influx in the Presence of $0.5\mu\text{M}$ Zinc Across the Integument of *Nereis succinea*

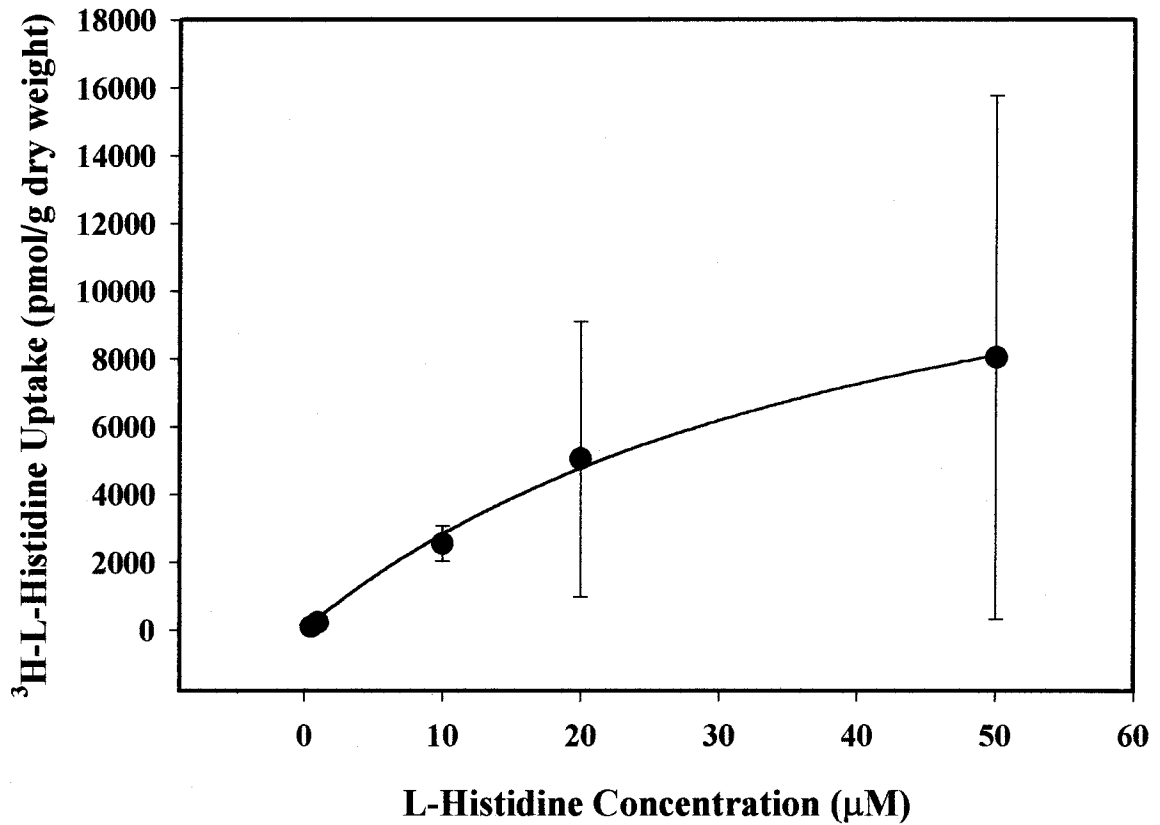


Figure 7. Kinetics of $^3\text{H-L-histidine}$ influx in *Nereis succinea* in the presence of $0.5\mu\text{M}$ zinc. Triplicate samples of worms (means \pm SEM shown) were incubated in the concentrations shown in 60% artificial seawater for 15 minutes. Lines drawn through the data are best-fit curves using Sigma Plot software and kinetic constants were produced using this equation. J_{max} was $15259 \pm 1711 \text{ pmol g}^{-1}$ dry mass. K_m was 44.02 ± 8.77 . Zinc was added as ZnSO_4 .

CHAPTER 4 DISCUSSION

The concept that aquatic organisms were able to absorb dissolved organic matter across their integument was debated within the scientific literature throughout most of the 20th century. With the advent of radioisotope-labeling techniques, it was soon verified that fundamentally, all soft-bodied marine invertebrates had the capacity to absorb organic nutrients from their environment.³⁶ Since then, considerable research has been generated identifying and characterizing the mechanisms involved in this transport process. However, the mechanisms involved in invertebrate nutrient transport still remain greatly under investigated.

The effect of environmentally occurring heavy metals on aquatic animals has become an important area of research in recent years.¹⁰ Aquatic organisms are known to absorb heavy metals from their environment^{6,11,48} and researchers are searching to better understand the impacts these metals have on their biological processes. Through the study of basic ion transporters, we now know heavy metal ions are capable of being transferred across the integument through diffusion or by carrier proteins responsible for the regulation of other cellular constituents.^{1,4,6,21,56,57} Amino acid carrier proteins,^{50,51} $\text{Ca}^{2+}/\text{Na}^{2+}$ exchangers,^{4,56} and Na^+/H^+ exchangers^{1,6,57} are all transporters shown to have the capability to transport heavy metal ions. These heavy metal ions are competitive inhibitors of the ions being transported across the integument of marine invertebrates. When these heavy metal ions are present in high concentrations, the regulation of amino acids, Na^+ , H^+ , and Ca^{2+} can become disrupted leading to deficiencies in growth, development, and possibly death.²⁸

Calcium and zinc have already been well established as regulators of biological processes.^{4,27} However, it is becoming more apparent that other divalent heavy metal ions may also be capable of influencing biological processes. While it is generally known that all metals, in high enough concentrations, are toxic,²⁰ recent papers have suggested certain divalent heavy metal ions, at low concentrations, are capable of stimulating amino acid uptake.^{7,24}

Research conducted by Monteilh-Zoller et. al. involved zinc-stimulated L-proline transport in lobster brush-border membrane vesicles (BBMV).²⁴ Results showed L-proline uptake was significantly stimulated by zinc, at varying concentrations, after a 30-minute pre-incubation of the BBMV to the metal. When no pre-incubation was involved, zinc was shown to inhibit the uptake of L-proline. Monteilh-Zoller et.al. hypothesized the 30-minute pre-incubation period allowed time for zinc ions to diffuse across the brush-border membrane and bind internally, allosterically stimulating the L-proline transport protein. Further research on metal-stimulated amino acid transport was conducted by Ahearn et. al.⁷ Their research attempted to determine the effect of cadmium and calcium on the uptake of L-histidine across polychaete integument. Their investigation found, at low concentrations without pre-incubation, cadmium was capable of stimulating the uptake of L-histidine. Furthermore, they found calcium to play a regulatory role in cadmium-stimulated L-histidine uptake. Unfortunately, the precise mechanism responsible for metal-stimulated amino acid transport in marine invertebrates remains unclear.²⁴ Nonetheless, two basic mechanisms/models have been suggested as explanations of metal-stimulated amino acid transport: 1) The metal forms a complex with the amino acid which more readily binds to the carrier protein than the amino acid

alone,^{50,51} or 2) the metal binds directly to the transporter, allosterically stimulating the amino acid carrier protein.^{7,9,24}

In this study, L-histidine transport by the euryhaline polychaete, *Nereis succinea* in the presence and absence of environmental heavy metals was examined. Experiments were conducted to determine what effect heavy metals (monovalent, divalent, and trivalent) had on the kinetics constants (J_{\max} and K_m) of L-histidine influx and to gather information on how these metals are exerting their effect. Silver (Ag^+), iron (Fe^{2+}), zinc (Zn^{2+}), and aluminum (Al^{3+}) were the heavy metals chosen for use in these experiments. Shown in Figure 1, the uptake of L-histidine across the integument of *Nereis succinea* followed first order Michaelis-Menten kinetics, approaching equilibrium at time points in excess of 60 minutes. From this experiment, a time of 15 minutes was chosen for all kinetics experiments conducted. Also, because low concentrations had been previously found to stimulate amino acid uptake^{7,24} and because 1 μM zinc did not have a significant effect on the uptake of 10 μM L-histidine (Figure 2), a concentration of 0.5 μM was chosen for all heavy metals used during experiments.

L-histidine is transported across the integument of marine invertebrates (while in its zwitterionic state)⁷ by way of a sodium-independent pathway referred to as the L-system. The L-system is known to occur on the brush-border and basolateral membranes of several species and is capable of transporting several amino acids, including leucine, branched amino acids, and ringed amino acids.⁴¹ L-leucine has a extremely high binding affinity for the L-system carrier and, at high concentrations, L-leucine acts as a competitive inhibitor of L-histidine transport. In order to ascertain L-histidine was being transported across the integument through the L-system, L-histidine (10 μM) uptake was

measured in the presence of L-leucine (25 μ M). Figure 2 shows the inhibition of L-histidine transport in the presence of L-leucine, confirming L-histidine epithelial transport through the L-system during this experiment.

Baseline kinetics of L-histidine influx was obtained in the absence of heavy metals (control). Figure 3 shows the results of this experiment. The J_{\max} and K_m constants found from this experiment were compared to all kinetics constants found in the proceeding heavy metal kinetics experiments (see Table 1 for comparisons).

Experimental kinetics influx results for Ag^+ (Figure 4) and Al^{3+} (Figure 5) showed neither metal significantly stimulated nor inhibited the uptake of L-histidine in *Nereis*. However, results showed a significant stimulation of L-histidine influx in the presence of Fe^{2+} (Figure 6) or Zn^{2+} (Figure 7). Fe^{2+} nearly doubled the apparent maximal influx rate of L-histidine (J_{\max}), while Zn^{2+} nearly quadrupled the J_{\max} of L-histidine (see Table 1 for comparisons). These results suggest the possibility of a divalent regulated transport mechanism.

Results from heavy metal kinetics experiments also showed a decrease in the apparent binding affinity (K_m) of L-histidine for its transporter in the presence of Fe^{2+} or Zn^{2+} (Table 1). Again, no effect was seen in the presence of Ag^+ (Figure 4) and Al^{3+} (Figure 5). This again suggests the possibility of a divalent regulated transport mechanism. Furthermore, seeing the effect Fe^{2+} and Zn^{2+} have on the apparent binding affinity of L-histidine influx, it can be hypothesized that these heavy metal ions are exerting their effect directly on the carrier protein. Meaning, the divalent metal ion is binding to the L-histidine transporter causing a conformational change in the carrier protein, stimulating L-histidine uptake. This conformational change reduces the affinity

of the amino acid for its transporter. This finding supports models previously proposed by Monteilh-Zoller²⁴ and Ahearn⁷ for the stimulation of amino acid uptake through the allosteric binding of divalent metals to the amino acid transporter. Wapnir and Stiel^{50,51} had previously examined the absorption of zinc across the ileum of rats in the presence of various amino acids. Their studies found the stimulation of zinc transport to be dependent upon the optimal number of amino acid/zinc complexes formed. At the appropriate ratio of amino acid/zinc complexes, the uptake of zinc was stimulated due to an increase in the binding affinity of the amino acid/zinc complex for the transporter. If the mechanism involved in this current investigation involved an amino acid/metal complex, we would have found the binding affinity of L-histidine to increase in the presence of each metal, not decrease.

In combination with earlier studies involving the effect of heavy metals on nutrient transport mechanisms, the current findings suggest the metal to amino acid ratio is a limiting factor of the inhibition/stimulation of L-histidine transport. For example, Figure 1 shows the inhibition of L-histidine (50 μ M) uptake by zinc (1 μ M) while Figure 2 shows no inhibition of zinc (1 μ M) on the uptake of L-histidine (10 μ M). Monteilh-Zoller²⁴ suggested the inhibition of L-proline from high concentrations of zinc resulted from zinc ions and sodium ions competing for a binding site on the carrier protein responsible for powering the transporter. Unfortunately, due to the sodium independent nature of the L-histidine transporter, his hypothesis does not apply to this investigation. Further studies need to be conducted to determine the mechanism behind heavy metal inhibition of L-histidine.

Overall, this investigation determined that the divalent heavy metal ions Fe^{2+} and Zn^{2+} are capable of stimulating L-histidine uptake by polychaete epithelium, while the monovalent and trivalent heavy metal ions Ag^+ and Al^{3+} are not. Previous experiments have also shown the ability of divalent cations, including calcium^{7,19} and heavy metals,^{6,24,47,57} to regulate and influence marine invertebrate nutrient transport. Combined, these results suggest the possibility that divalent cations play a fundamental role in the regulation of nutrient transport by sodium independent transport systems. This investigation suggests a model for metal-stimulated sodium-independent amino acid transport involving the allosteric binding of the metal ion to a divalent specific binding site on the amino acid transporter. Further experiments should be conducted to examine the potential for other divalent ions (i.e., Cu^{2+} and Pb^{2+}) to stimulate amino acid uptake.

CHAPTER 5 SUMMARY

1. The uptake of L-histidine by the polychaete *Nereis succinea* was examined in the presence and absence of various heavy metals.
2. L-leucine and L-histidine share a common transport carrier protein.
3. Monovalent and trivalent metals, at low concentrations, do not have a significant effect on L-histidine influx.
4. Divalent metals, at low concentrations, increase the apparent maximal influx rate of L-histidine (J_{\max}) and decrease the apparent binding affinity of L-histidine for its transporter (K_m).
5. Divalent metals may stimulate L-histidine uptake by binding allosterically to a divalent specific binding site on the L-histidine transporter.
6. Divalent cations appear to play a fundamental role in the regulation of nutrient transport by sodium independent transport systems.
7. The metal to amino acid ratio may be the limiting factor in the inhibition/stimulation of nutrient transport.

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