INTEGUMENTARY L-HISTIDINE TRANSPORT IN A EURYHALINE POLYCHAETE WORM: REGULATORY ROLES OF CALCIUM AND CADMIUM IN THE TRANSPORT EVENT

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Summary

Integumentary uptake of L-[^{3}H]histidine by polychaete worms (*Nereis succinea*) from estuarine waters of Oahu, Hawaii was measured in the presence and absence of calcium and cadmium using a physiological saline that approximated the ion composition of 60 % sea water. In this medium 1 µmol l^{-1} cadmium significantly increased (*P*<0.01) the uptake of 10 µmol l^{-1} L-[^{3}H]histidine, while 1 µmol l^{-1} cadmium plus 25 µmol l^{-1} L-leucine significantly decreased (*P*<0.01) amino acid uptake. L-[^{3}H]histidine influx was a sigmoidal function (*n*=2.21±0.16, mean ± S.E.M.) of [L-histidine] (1–50 µmol l^{-1}) in the absence of cadmium, but became a hyperbolic function with the addition of 1 µmol l^{-1} cadmium. A decrease of calcium concentration from 6 to 0 mmol l^{-1} (lithium substitution) significantly increased (*P*<0.01) amino acid influx in the presence and absence of cadmium. Calcium significantly

reduced (*P*<0.01), and cadmium significantly increased (*P*<0.01), L-[^{3}H]histidine influx J_{max}, without either divalent cation affecting amino acid influx K_t. Variation in external sodium concentration (0–250 mmol l^{-1}) had no effect on 10 µmol l^{-1} L-[^{3}H]histidine influx, but amino acid entry was a sigmoidal function of both [cadmium] (*n*=2.34±0.44) and [lithium] (*n*=1.91±0.39) in the absence of calcium. A model is proposed for transapical L-[^{3}H]histidine influx by a transporter that resembles the classical sodium-independent L-system carrier protein that is regulated by the external divalent cations calcium and cadmium.

Key words: epithelial transport, integument, polychaete worm, *Nereis succinea,* Annelida, transport regulation, calcium, cadmium, heavy metal.

Introduction

Over the course of the last century, researchers found that soft-bodied marine invertebrates displayed the capacity to absorb low molecular weight organic compounds from their surroundings by transport across epithelial apical membranes of their gills and integument (Pütter, 1909; Krogh, 1931; Stephens and Schinske, 1961; Jørgensen, 1976; Wright, 1988). Once absorbed, these compounds contributed to nutritional needs, osmoregulatory processes, and were involved in developmental life-cycle changes. Membrane-transport processes responsible for transferring these compounds from seawater to integumental epithelial cells were similar to those described for animal cells across phyla, except that integumental transport systems displayed extremely high apparent binding affinities due to very low environmental substrate concentrations, and sodium-dependent mechanisms, which employed multiple cotransported sodium ions to transfer organic solutes into the cells against extremely steep concentration gradients (Stevens and Preston, 1980a–c; Preston and Stevens, 1982).

The integument of marine polychaete worms is one tissue that has been extensively studied in this regard and the marine

annelids were, in fact, among the numerous groups of organisms initially shown to display this physiological property (Stephens and Schinske, 1961). The polychaete integument absorbs carbohydrates (Ahearn and Gomme, 1975; Gomme, 1981a,b, 1985), a wide assortment of amino acids (Stephens, 1963, 1964; Reish and Stephens, 1969; Stevens and Preston, 1980a–c; Preston and Stevens, 1982; Qafaiti and Stephens, 1988) and fatty acids (Testerman, 1972) from micromolar concentrations in sea water. The transport of these substances, in many instances, is dependent upon the salinity of their environment (Stephens, 1964; Siebers and Bulnheim, 1977; Wagner, 1981), and occurs by cotransport with environmental sodium ions, using the integumentary transapical sodium gradient to power concentrative nutrient uptake (Preston and Stevens, 1982). While it is now clear that environmental sodium ions are extremely important for nutrient uptake by several entry pathways in marine invertebrates, less is known about potential roles played by other ions in sea water in these nutrient absorption processes.

The present investigation is a study of L-[^{3}H]histidine uptake

by the integument of the euryhaline polychaete worm, *Nereis succinea*, and the effects of the environmental divalent cations, calcium and cadmium, on the transport of this amino acid. Results suggest that divalent cations may play an important regulatory function in organic solute transport in invertebrate integumentary epithelium. This work has recently appeared in abstract form (Ahearn et al., 2000).

Materials and Methods

Nereis succinea (10–25 mg, dry mass) were collected from the brackish waters of the Diamond Head end of the Ala Wai Canal, Honolulu, Hawaii; sample size ranged from approximately 40–50 worms per visit. Worms were transferred into artificial sea water (ASW) made with the following salts in mmol l^{-1}: NaCl (410.5), KCl (9.6), CaCl$_2$.2H$_2$O (10.2), MgCl$_2$.6H$_2$O (53.5), Na$_2$SO$_4$.10H$_2$O (28.2), NaHCO$_3$ (2.3), NaBr (0.8) and H$_3$BO$_3$ (0.07). Worms were transported from the collection site to the laboratory in 60 % ASW. All experiments reported here were conducted at 60 % ASW made by dilution of 100 % ASW with deionized water. All worms were acclimated for 1-2 days at 22 °C to this salinity before experimentation. During this acclimation period, *Nereis* were in plastic containers that were supplied with numerous glass tubes for the worms to irrigate.

L-[2,5-^{3}H]histidine (1.89 TBq mmol^{-1}) was obtained from Amersham Life Science (Elk Grove, Illinois, USA). A small volume of isotope was added to ASW along with unlabelled L-histidine to achieve the desired labelled concentration. Stop Solutions at 0 °C, used to rinse the worms following an exposure to radiolabelled ASW and reduce nontransported isotope to a minimum, were made from the same salts used to prepare incubation media. Animals were incubated in isotopically labelled incubation medium at 22 °C for predetermined time intervals ranging from 5 to 30 min. Various calcium or sodium conditions were obtained by replacing calcium or sodium with an equivalent molarity of lithium. All metals were added as chloride salts. Following incubation the worms were removed from the labelled medium, rinsed for 15 s in ice-cold Stop Solution of the same % ASW as the incubation medium and dropped into 3 ml 70 % ethanol for overnight extraction. After this extraction time interval less than 5 % of the total radioactivity in the 3 ml ethanol was recovered from a tissue digest of the animals, suggesting that little incorporation of labelled amino acid into ethanol-insoluble compounds had taken place during transport measurements. A sample (1 ml) of the ethanol extraction was removed and isotope content assessed in a Beckman LS8100 liquid scintillation counter. Activity (as cts min^{-1}) was converted to pmol of amino acid using the isotopic specific activity of the incubation medium. Control experiments were conducted with ethanol extracts of worms varying in size to determine the extent of ^{3}H quenching by extractable compounds. Over the range of worm sizes used in these experiments no discernible effects of quenching were observed.

All data were normalized to unit surface area of worms by multiplying the $W^{2/3}$ ratio by the calculated pmol of L-[^{3}H]histidine in the worms, where W is worm dry mass, to provide surface-specific uptake, since previous work (Gomme, 1981a) showed that integumentary transport of dissolved substances in these worms followed a surface area relationship better than a strict mass function. Following isotopic counting the worms were removed from their vials, placed on preweighed aluminum boats and desiccated at 80 °C overnight. Following this period the dry masses of the worms were determined and isotopic uptake was then expressed per unit dry mass using the above $W^{2/3}$ ratio.

'Blank' uptake values, or an estimate of adhering, nontransported isotope, was obtained by incubating worms in external labelled medium for 15 s followed by the normal 15 s rinse in ice-cold Stop Solution. This 15 s uptake represented isotope adhering to a thin film on the exterior or the animal as well as surface binding. These binding values were approximately 3 % of the uptake over 5–30 min of exposure to the same isotope content. A curve-fitting procedure was used throughout this study applying Sigma Plot (Jandel) software to values presented in the figures to obtain the best fits to the data. Statistical significance was determined between values using Student's *t*-test with $P=0.05$ taken as the level of significant difference between means. Values are means ± S.E.M.

Results

Effects of 1 µmol l^{-1} cadmium and 25 µmol l^{-1} L-leucine on the time course of uptake of 10 µmol l^{-1} L-[^{3}H]histidine

Triplicate samples of worms were exposed to three experimental treatments for 10, 20, 30, 45 and 60 min in 60 % ASW containing 10 µmol l^{-1} L-[^{3}H]histidine. The treatments were: medium with no cadmium or L-leucine (control), medium with added 1 µmol l^{-1} cadmium and medium with added 1 µmol l^{-1} cadmium plus 25 µmol l^{-1} L-leucine. As shown in Fig. 1, L-[^{3}H]histidine uptake over this time course followed first-order kinetics, approaching equilibrium at time points in excess of 60 min. From the results of this experiment, an incubation period of 5 min was chosen for subsequent estimations of unidirectional amino acid influx. Addition of 1 µmol l^{-1} cadmium alone to the external medium resulted in a significant increase ($P<0.01$) in the uptake of amino acid into the worm integumentary epithelium compared to the control condition lacking the heavy metal. Addition of both 1 µmol l^{-1} cadmium plus 25 µmol l^{-1} L-leucine to the uptake medium significantly reduced ($P<0.01$) the uptake of L-[^{3}H]histidine at each time point to values less than those of the control lacking either metal or L-leucine. These results suggest that a transporter with similar characteristics to the classical L-system (Stevens et al., 1984) was largely responsible for L-[^{3}H]histidine uptake by the worm integumentary epithelium and that the heavy metal cadmium stimulated this uptake process.

Kinetics of L-[^{3}H]histidine influx in the presence and absence of 1 µmol l^{-1} cadmium
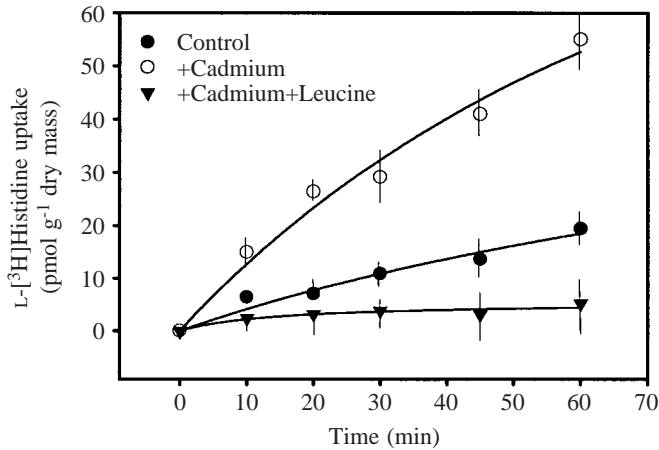
Triplicate samples of worms were incubated for 5 min in

Fig. 1. Effects of $1\,\mu mol\,l^{-1}$ cadmium and $25\,\mu mol\,l^{-1}$ L-leucine on influx of $10\,\mu mol\,l^{-1}$ L-[³H]histidine in *Nereis succinea*. Triplicate samples of worms (means ± S.E.M.) were incubated for the times shown in a 60 % artificial seawater saline (ASW). Cadmium was added as CdCl₂. Lines drawn through the means are best-fit curves using Sigma Plot software, assuming hyperbolic time-course relationships.

60 % ASW containing 0.5, 1, 10, 25 and $50\,\mu mol\,l^{-1}$ L-[³H]histidine concentrations in the presence and absence of $1\,\mu mol\,l^{-1}$ cadmium. Fig. 2 indicates that a sigmoidal relationship occurred between amino acid influx and external amino acid concentration in the control condition lacking cadmium. Under these conditions the entry rate of the uninhibited amino acid followed the Hill equation for cooperativity between identical binding ligands:

$$J_H = J_{max}[H]^n/K_t + [H]^n, \tag{1}$$

where J_H is amino acid influx rate in $pmol\,mg^{-1}$ dry mass per 5 min, J_{max} is apparent maximal amino acid influx rate, K_t is an apparent affinity constant adjusted for multisite cooperativity and is the concentration of amino acid at half maximal velocity, and $[H]$ is the amino acid concentration. The exponent n is a cooperativity coefficient describing multisite binding of amino acid molecules to transport proteins. A curve-fitting program, Sigma Plot (Jandel, Inc.), was used to obtain estimates of these transport constants under control conditions lacking the addition of heavy metal to the external medium. This program provided the following transport constants for these conditions: $J_{max}=53\pm3\,pmol\,mg^{-1}$ dry mass per 5 min, $K_t=25\pm2\,\mu mol\,l^{-1}$ and $n=2.2\pm0.2$. These results suggest that at least two L-histidine molecules crossed the membrane per transport cycle in the worm integumentary epithelium under these experimental conditions.

Addition of $1\,\mu mol\,l^{-1}$ cadmium to the external medium during measurement of the uptake of L-[³H]histidine resulted in a significant change in the relationship between L-[³H]histidine influx and external L-histidine concentration. When cadmium ions were present in the external medium, amino acid influx was a hyperbolic function of amino acid concentration and the curve representing this relationship was shifted to the left of that describing L-histidine influx in the
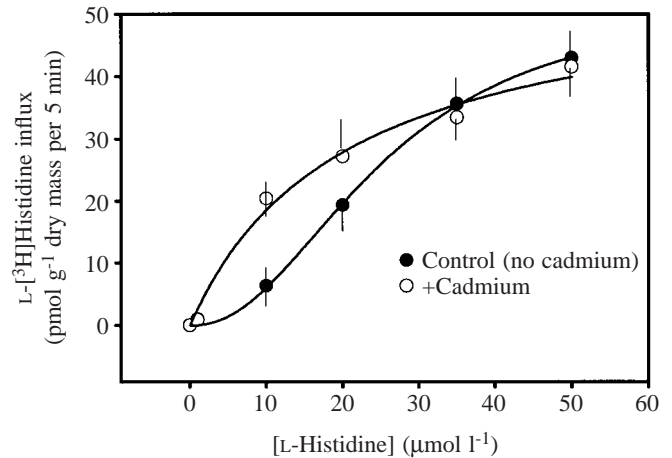

Fig. 2. Effect of $1\,\mu mol\,l^{-1}$ cadmium on $10\,\mu mol\,l^{-1}$ L-[³H]histidine influx in the presence of $6\,mmol\,l^{-1}$ CaCl₂. Triplicate worm samples (means ± S.E.M.) were incubated for 5 min in the presence and absence of $1\,\mu mol\,l^{-1}$ cadmium in 60 % ASW containing $6\,mmol\,l^{-1}$ calcium and 0.5, 1, 10, 25 and $50\,\mu mol\,l^{-1}$ L-[³H]histidine. Lines drawn through the means are best-fit curves using Sigma Plot software and applying equations 1 or 2 to the data. In both cases r^2 values for the fits were 0.99, indicating highly significant fits to the data.

absence of the metal (Fig. 2). L-[³H]histidine influx kinetics in the presence of $1\,\mu mol\,l^{-1}$ cadmium followed the Michaelis–Menten equation given below:

$$J_H = J_{max}[H]/K_t + [H], \tag{2}$$

where J_H is amino acid influx in $pmol\,g^{-1}$ dry mass per 5 min, J_{max} is apparent maximal influx rate, K_t is an apparent affinity constant and is the concentration of L-histidine at one-half maximal influx velocity, and $[H]$ is the external concentration of L-histidine. The Sigma Plot curve-fitting program was applied to the influx data obtained in the presence of cadmium and resulted in the following values: $J_{max}=56\pm6\,pmol\,g^{-1}$ dry mass per 5 min and $K_t=20\pm5\,\mu mol\,l^{-1}$ L-histidine. A comparison of the L-histidine influx kinetic constants (J_{max} and K_t) in the presence and absence of $1\,\mu mol\,l^{-1}$ cadmium suggests that no significant difference ($P>0.05$) occurred between the values of these constants as a result of metal exposure, but that considerable alteration in the extent of substrate cooperativity took place when the metal was present. The shifting of the influx curve from a sigmoidal to a hyperbolic relationship in the presence of the metal suggests that cadmium converted the multiple amino acid entry process into a single amino acid entry process. Because of the shift to the left of the hyperbolic influx curve for L-[³H]histidine in the presence of cadmium, the amino acid uptake rate at $10\,\mu mol\,l^{-1}$ was significantly greater ($P<0.05$) than in the absence of the metal, as described in Fig. 1.

Interactions between calcium and cadmium during L-[³H]histidine influx

Fig. 3 describes the effect of variation in external calcium concentration on the influx of $10\,\mu mol\,l^{-1}$ L-histidine in the

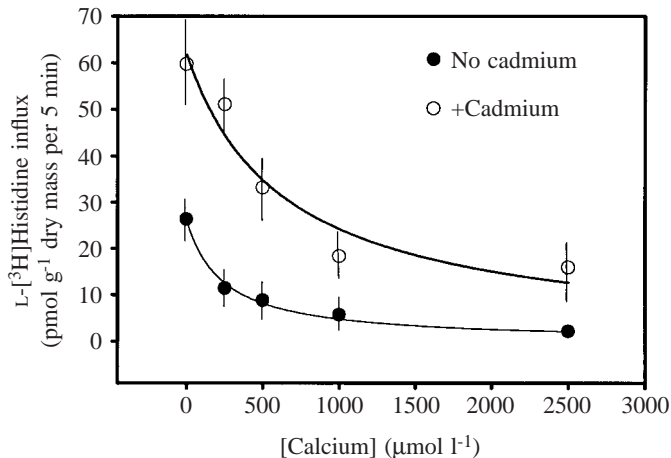Fig. 3. Effect of $1\,\mu mol\,l^{-1}$ cadmium on calcium dependence of $10\,\mu mol\,l^{-1}$ L-[^{3}H]histidine influx. Triplicate samples of worms (means $\pm$ S.E.M.) were exposed for 5 min to radiolabelled amino acid in the presence and absence of cadmium in a 60 % ASW in which calcium was replaced by equimolar lithium (0–$2.5\,mmol\,l^{-1}$) and all other ions remained unchanged. Lines drawn through the means are best-fit curves using Sigma Plot software and applying equation 3 for a hyperbolic decay relationship. In both cases r^2 values for the fits were greater than 0.94, indicating a high degree of significance to the fits.

Fig. 4. Effect of $1\,\mu mol\,l^{-1}$ cadmium on L-[^{3}H]histidine influx kinetics in the absence of calcium. Triplicate samples of worms (means $\pm$ S.E.M.) were exposed for 5 min in the presence and absence of $1\,\mu mol\,l^{-1}$ cadmium in 60 % ASW lacking calcium (lithium replacement) containing 0.5, 1, 10, 25 and $50\,\mu mol\,l^{-1}$ L-[^{3}H]histidine. Lines drawn through the data are best-fit curves using Sigma Plot software and applying equation 2 to the data. In both cases r^2 values for the fits were greater than 0.96, indicating a high degree of significance to the fits.

presence and absence of $1\,\mu mol\,l^{-1}$ cadmium. In this experiment external calcium was replaced with lithium ion on an equimolar basis. All other ions were present in the incubation medium at concentrations found at 60 % ASW (except for modifications in chloride concentration). Under both control conditions lacking cadmium and in the presence of $1\,\mu mol\,l^{-1}$ cadmium, an increase in external calcium concentration from 0 to $2.5\,mmol\,l^{-1}$ resulted in significant reductions ($P<0.01$) in the uptake of L-[^{3}H]histidine following an equation for hyperbolic decay:

$$J_H = ab/(b+[Ca]),\qquad(3)$$

where J_H is L-[^{3}H]histidine influx in $pmol\,g^{-1}$ dry mass per 5 min; a is amino acid influx at $0\,mmol\,l^{-1}$ calcium, b is L-[^{3}H]histidine influx at 50 % of maximal entry (at $0\,mmol\,l^{-1}$ calcium), and [Ca] is external calcium concentration in $\mu mol\,l^{-1}$. Implicit in this relationship is the fact that calcium ion acted as a direct or indirect inhibitor of L-histidine influx and that at a finite external calcium concentration, amino acid influx would approach zero. The lines drawn in Fig. 3 were computed using Sigma Plot curve-fitting software and provided the following values for the transport constants in this equation: control treatment: $a=26\pm1\,pmol\,g^{-1}$ dry mass per 5 min; $b=227\pm24\,\mu mol\,l^{-1}$ calcium; cadmium treatment: $a=61\pm5\,pmol\,g^{-1}$ dry mass per 5 min; $b=642\pm184\,\mu mol\,l^{-1}$ calcium. These computed values suggest that addition of cadmium to the external medium increased the influx of L-[^{3}H]histidine at every calcium concentration examined, and that in the presence of cadmium a significantly higher concentration of calcium was needed to reduce amino acid entry to one-half of its maximal value than in the absence of the metal.
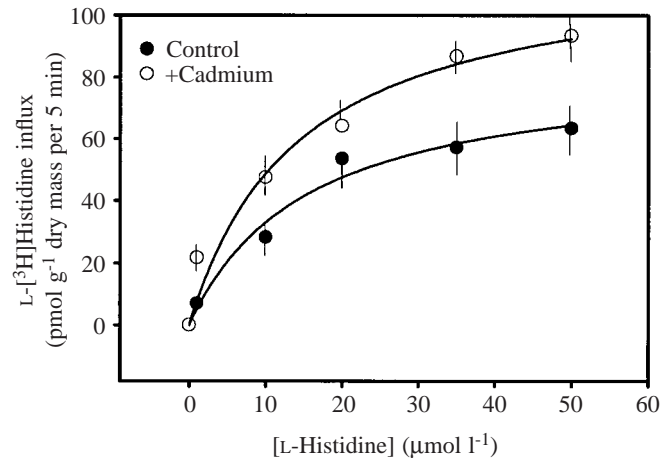
Effects of $1\,\mu mol\,l^{-1}$ cadmium on L-[^{3}H]histidine influx kinetics in the absence of calcium

Because calcium was inhibitory to L-[^{3}H]histidine influx in both the presence and absence of cadmium, the effects of cadmium on the kinetics of amino acid influx were determined in the absence of calcium. Fig. 4 shows that, in the complete absence of calcium in the external medium, L-[^{3}H]histidine influx was a hyperbolic function of external L-histidine concentration and followed equation 2 above, as described previously for amino acid influx in the presence of both calcium and cadmium (Fig. 2). Addition of $1\,\mu mol\,l^{-1}$ cadmium to the external medium, in the absence of calcium, resulted in a hyperbolic increase in amino acid influx over the external L-histidine concentration range used. Significantly more amino acid uptake occurred in the presence of metal than in its absence. Applying Sigma Plot curve-fitting software to the results of this experiment we obtained the lines drawn on Fig. 4. In the absence of cadmium, L-[^{3}H]histidine influx exhibited a K_t of $15\pm5\,\mu mol\,l^{-1}$ L-histidine and a J_{max} of $84\pm10\,pmol\,g^{-1}$ dry mass per 5 min. In contrast, addition of $1\,\mu mol\,l^{-1}$ cadmium resulted in a L-[^{3}H]histidine influx $K_t=14\pm6$ and $J_{max}=119\pm17\,pmol\,g^{-1}$ dry mass per 5 min. In the absence of calcium, therefore, the effect of added cadmium was to increase significantly ($P<0.05$) the maximal rate of L-[^{3}H]histidine influx without significantly ($P>0.05$) altering the apparent half-saturation constant.

Effects of calcium on L-[^{3}H]histidine influx kinetics in the presence of $1\,\mu mol\,l^{-1}$ cadmium

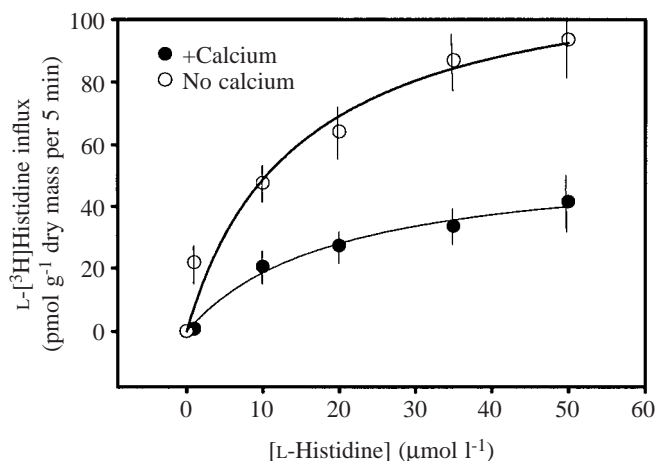Because cadmium was found to increase the apparent maximal transport velocity of L-[^{3}H]histidine influx in the

Fig. 5. Effects of calcium deletion on L-[^{3}H]histidine influx kinetics in the presence of $1\,\mu mol\,l^{-1}$ cadmium. Triplicate samples of worms (means ± S.E.M.) were exposed for 5 min in the presence of $6\,mmol\,l^{-1}$ calcium and absence (lithium replacement) in 60 % ASW containing $1\,\mu mol\,l^{-1}$ cadmium and 0.5, 1, 10, 25 and $50\,\mu mol\,l^{-1}$ L-[^{3}H]histidine. Lines drawn through the data are best-fit curves using Sigma Plot software and applying equation 2 to the data. In both cases r^2 values for the fits were greater than 0.96, indicating a high degree of significance to the fits.

Fig. 6. Effect of calcium on cadmium dependency of $10\,\mu mol\,l^{-1}$ L-[^{3}H]histidine influx. Triplicate samples of worms (means ± S.E.M.) were exposed for 5 min in the presence of $6\,mmol\,l^{-1}$ calcium and absence (lithium replacement) in 60 % ASW containing $10\,\mu mol\,l^{-1}$ L-[^{3}H]histidine and 0.1, 0.5, 1 and $10\,\mu mol\,l^{-1}$ cadmium. Lines drawn through the means are best-fit curves using Sigma Plot software and applying equation 1 to the data. In both cases r^2 values for the fits were 0.99, indicating highly significant fits to the data.

absence of calcium (Fig. 4) (without affecting the apparent L-histidine binding affinity, K_t), a comparison was made of the effects of variable external calcium concentration on the influx kinetics for the amino acid when the metal was maintained at a constant concentration. Fig. 5 shows a comparison between kinetic influx curves taken from Figs 2 and 4 under conditions where external cadmium concentration was maintained at $1\,\mu mol\,l^{-1}$, but calcium concentration was either 0 or $6\,mmol\,l^{-1}$. As displayed in the figure, the effect of adding calcium to a medium already containing cadmium was to sharply reduce ($P<0.01$) L-[^{3}H]histidine influx J_{max} (no calcium=119±17; $6\,mmol\,l^{-1}$ calcium=56±6 pmol g^{-1} dry mass per 5 min) without significantly altering ($P>0.05$) the apparent K_t of the transport system (no calcium=14±6; $6\,mmol\,l^{-1}$ calcium=20±5 $\mu mol\,l^{-1}$). The results in Figs 4 and 5, therefore, suggest that the major effect of the divalent cations on L-[^{3}H]histidine influx is to alter the number of transporters moving across the membrane per unit of time or to increase the transfer rate of a constant number of carriers. In each case the binding of the amino acid with the transport system appears unaffected by these cations.

Effect of calcium on cadmium-dependent L-[^{3}H]histidine influx

Results of previous experiments suggested that cadmium had a direct stimulatory effect on the influx of L-[^{3}H]histidine in the absence or at reduced concentrations of calcium. In order to further characterize the nature of this cadmium stimulation, [^{3}H]L-histidine influx was monitored as a function of variable external cadmium concentration in the presence and absence of $6\,mmol\,l^{-1}$ calcium. During this experiment all other buffer
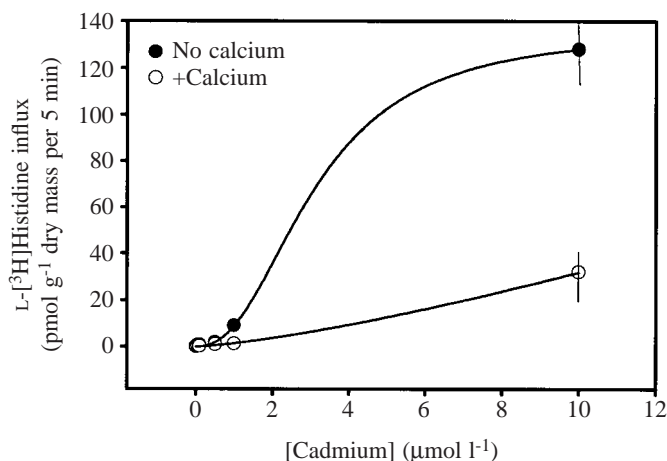
constituents were maintained at levels present in 60 % ASW, as defined previously. Fig. 6 shows that at $0\,mmol\,l^{-1}$ calcium, an increase in external cadmium from 0.1 to $10\,\mu mol\,l^{-1}$ led to a sigmoidal influx of L-[^{3}H]histidine following equation 1, with an apparent maximal uptake value near $10\,\mu mol\,l^{-1}$. Applying Sigma Plot curve-fitting software to the relationship resulted in the following transport constants: $K_t=3.10\pm0.80\,\mu mol\,l^{-1}$; $J_{max}=136.25\pm9.34$ pmol g^{-1} dry mass per 5 min; $n=2.34\pm0.44$. These results suggest that at least two high-affinity cadmium-binding sites are involved in the stimulation of L-[^{3}H]histidine influx.

When L-[^{3}H]histidine influx was measured over the same external cadmium concentration range in the presence of $6\,mmol\,l^{-1}$ calcium, a significant reduction in amino acid uptake at each cadmium concentration was observed, and the entire uptake curve appeared to shift to the right of that measured in the absence of calcium. These results suggest that calcium strongly inhibits the stimulatory effect of cadmium on amino acid influx.

Effect of calcium on the sodium-dependency of $10\,\mu mol\,l^{-1}$ L-[^{3}H]histidine influx

Fig. 7 illustrates the effect of altering external sodium concentration (replaced by equimolar concentrations of lithium) in the presence and absence of $6\,mmol\,l^{-1}$ calcium on the magnitude of $10\,\mu mol\,l^{-1}$ L-[^{3}H]histidine influx. When calcium was present at normal concentrations in 60 % sea water (e.g. $6\,mmol\,l^{-1}$), there was no relationship between the external concentration of sodium and the magnitude of $10\,\mu mol\,l^{-1}$ L-[^{3}H]histidine influx. At every sodium concentration used, the entry rate of $10\,\mu mol\,l^{-1}$ amino acid was approximately 10 pmol g^{-1} dry mass per 5 min. These
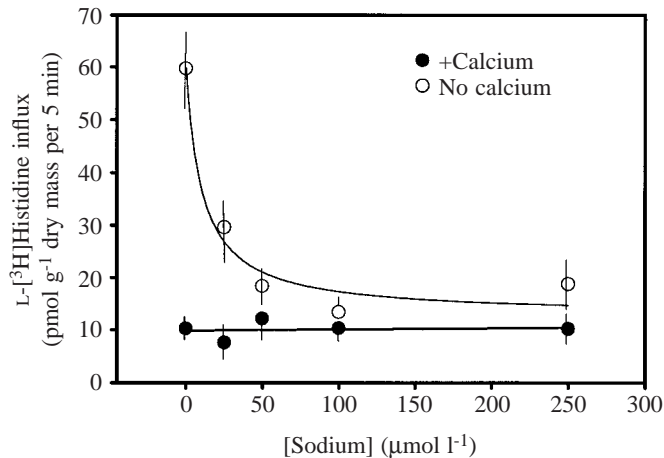
Fig. 7. Effect of calcium on the sodium dependency of $10\,\mu\mathrm{mol\,l^{-1}}$ L-[³H]histidine influx. Triplicate samples of worms (means ± S.E.M.) were incubated for 5 min with $10\,\mu\mathrm{mol\,l^{-1}}$ L-[³H]histidine in the presence of $6\,\mathrm{mmol\,l^{-1}}$ calcium and absence (lithium replacement) in 60 % ASW where sodium was replaced with equimolar lithium (0, 150, 200, 225 and $250\,\mathrm{mmol\,l^{-1}}$) and all other ions remained unchanged. The line drawn through the data lacking calcium is a best-fit curve using Sigma Plot software and applying equation 3 for a hyperbolic decay plus a positive vertical intercept (r^2=0.96). The line drawn through the data with $6\,\mathrm{mmol\,l^{-1}}$ calcium was a regression analysis of the data and yielded an r^2 value of 0.019, suggesting no relationship between the variables.

Fig. 8. Effect of $1\,\mu\mathrm{mol\,l^{-1}}$ cadmium on lithium-dependency of $10\,\mu\mathrm{mol\,l^{-1}}$ L-[³H]histidine influx. Triplicate worm samples (means ± S.E.M.) were incubated in the presence and absence of $1\,\mu\mathrm{mol\,l^{-1}}$ cadmium for 5 min in saline containing $10\,\mu\mathrm{mol\,l^{-1}}$ L-[³H]histidine, lithium chloride (0, 25, 50, 100, 150 and $250\,\mathrm{mmol\,l^{-1}}$), and sufficient mannitol to maintain a total osmolarity of each medium at approximately that of 60 % sea water. Lines drawn through the means are best-fit curves using Sigma Plot software and applying equation 1 for a sigmoidal relationship between the variables. In both cases r^2 values of 0.99 were obtained, indicating highly significant fits to the data.

results suggest that L-[³H]histidine uptake was due to the transport activity of a sodium-independent process.

When calcium was completely replaced in the external medium with lithium and sodium was varied from zero to $250\,\mathrm{mmol\,l^{-1}}$ (using lithium to replace sodium as well), a strong stimulation of amino acid uptake occurred as the external sodium concentration was reduced so that at $0\,\mathrm{mmol\,l^{-1}}$ external sodium (and $0\,\mathrm{mmol\,l^{-1}}$ external calcium) $10\,\mu\mathrm{mol\,l^{-1}}$ L-[³H]histidine influx was six times the rate observed at the highest sodium concentration (e.g., $250\,\mathrm{mmol\,l^{-1}}$). These results, and those of Fig. 3, suggest that as either sodium or calcium were replaced by lithium, a strong stimulation in the uptake of L-[³H]histidine took place and imply a possibly significant role for lithium in the transport of this amino acid.

Effect of $1\,\mu\mathrm{mol\,l^{-1}}$ cadmium on the lithium dependency of $10\,\mu\mathrm{mol\,l^{-1}}$ L-[³H]histidine influx

Because Fig. 7 suggested a potential stimulatory role for lithium ions in L-[³H]histidine transport, an experiment was conducted where $10\,\mu\mathrm{mol\,l^{-1}}$ L-[³H]histidine influx was measured as a function of variable lithium concentratration (0, 25, 50, 100, 150 and $250\,\mathrm{mmol\,l^{-1}}$ lithium) in the presence and absence of external $1\,\mu\mathrm{mol\,l^{-1}}$ cadmium. In this experiment mannitol was used to maintain the total osmolarity of each experimental medium at approximately that of 60 % sea water. No sodium or calcium was added to the uptake medium.

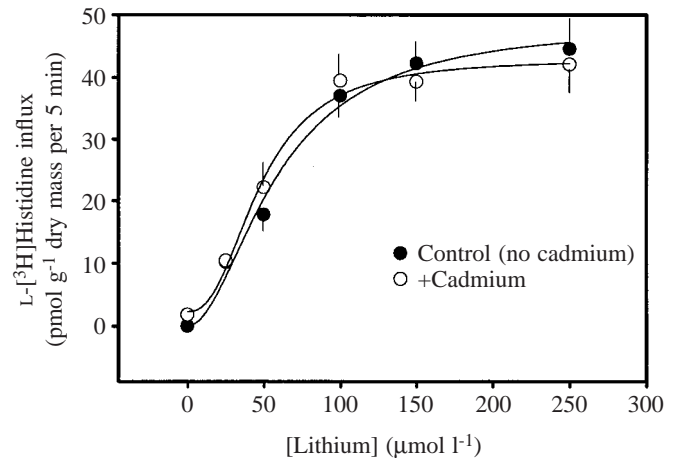Fig. 8 illustrates the relationship between L-[³H]histidine influx and lithium concentration under control conditions lacking cadmium and in the presence of $1\,\mu\mathrm{mol\,l^{-1}}$ cadmium. Under these conditions a sigmoidal relationship existed between the variables in both treatments, which followed the Hill equation (equation 1 above) where lithium ion concentration was varied in the external medium. The Sigma Plot curve-fitting program applied to the influx data resulted in the following kinetic constants for this relationship: control: J_{max}=48±4 pmol g⁻¹ dry mass per 5 min, K_{Li}=59±8 mmol l⁻¹ lithium, sigmoidicity (n)=2±0.4; $1\,\mu\mathrm{mol\,l^{-1}}$ cadmium condition: J_{max}=41±4 pmol g⁻¹ dry mass per 5 min, K_{Li}=48±6 mmol l⁻¹ lithium, sigmoidicity (n)=2.4±0.6.

These results suggest that approximately two lithium ions associated with the L-histidine regulatory site during the transport event in both the control and cadmium treatment conditions. The similarity of the two curves over the entire lithium concentration range used implies a minimal effect of the metal at the concentration used (e.g. $1\,\mu\mathrm{mol\,l^{-1}}$) on the binding of lithium to the L-histidine carrier system.

Discussion

During the second half of the 20th century numerous papers were written describing the capacity of the polychaete worm integumentary epithelium to transport sugars and amino acids from very low concentrations in sea water and to use these transported substrates in a variety of metabolic activities (Ahearn and Gomme, 1975; Gomme, 1981a,b, 1985; Stephens, 1963, 1964; Stevens and Preston, 1980a,b,c; Preston and Stevens, 1982; Qafaiti and Stephens, 1988). A significant

factor affecting the rate of nutrient absorption by these animals is the salinity of their environment. A reduction in external salinity universally led to a drop in the rate of integumentary nutrient uptake. The salinity effect is both an osmotic and sodium ion adjustment. It seems to be due to the combination of both a long-term regulatory response that brings nutrient transport to a new level in a few days and a rapid response (over seconds or minutes) initiated by sodium ion binding to nutrient transport proteins (Gomme, 1982). Most of the nutrients that were studied over the past 50 years were cotransported into the worm epithelium by sodium-dependent carrier processes, which displayed multiple sodium-binding sites to facilitate the concentrative uptake of the respective organic solute while sodium passed down its electrochemical gradient. Very few studies are documented in the literature of invertebrate integumentary carrier-mediated nutrient transport from sea water in the absence of sodium (Wagner, 1981; Stevens and Preston, 1980b; Wright, 1985, 1988), and uptake under these conditions is generally believed to occur largely by simple diffusion into or between epithelial cells.

The transport of L-histidine across animal cell membranes has been investigated in vertebrates and invertebrates alike (Stevens et al., 1984; Giordana et al., 1985; Wright, 1985; Liou and Ellory, 1990; Horn et al., 1995). In most animal cells, L-histidine is transported across plasma membranes by the combination of two sodium-independent carrier processes, the L- and y$^+$ systems (Stevens et al., 1984; Liou and Ellory, 1990), although some interaction between this amino acid and certain sodium-dependent transport systems does appear to occur in some species (Neame, 1965, 1966; Wright, 1985). Furthermore, in lepidopteran insect midgut, where sodium concentrations are very low and potassium concentrations very high, a potassium-dependent uptake of L-histidine has been reported (Giordana et al., 1985). It is likely, therefore, that in the present investigation, most, if not all, carrier-mediated L-[^{3}H]histidine uptake into *Nereis succinea* integumentary epithelium occurred by way of one or more sodium-independent transport processes. At the pH used in this study (e.g. pH 7.2), however, L-histidine was approximately 90-95 % zwitterionic, so its use of the sodium-independent y$^+$ basic amino acid transport system that is used by L-lysine, L-arginine and L-histidine in their cationic forms was probably minimal. The tentative assumption is therefore that most amino acid influx measured in the present investigation took place largely through a sodium-independent system resembling the classical L-system.

This investigation concentrated on the effects of the divalent cations, calcium and cadmium, on integumentary transport of L-[^{3}H]histidine in euryhaline animals generally maintained in a salinity approximating 60 % sea water. As clearly shown in Fig. 1, addition of 1 µmol l^{-1} cadmium to the external medium significantly ($P<0.01$) stimulated the uptake of 10 µmol l^{-1} L-[^{3}H]histidine, and that further addition of 25 µmol l^{-1} L-leucine to the medium containing cadmium led to a significant ($P<0.01$) reduction in the time course of amino acid uptake. These data suggest that cadmium stimulated the uptake of L-[^{3}H]histidine by an L-leucine-shared carrier process in the

integument of these animals, as discussed above for membrane transport of L-histidine in vertebrate plasma membranes.

The data presented for the interaction between L-histidine transport and the divalent cations, calcium and cadmium, can best be discussed by considering the model presented in Fig. 9. At present, however, indirect intracellular effects of the various external metals on unknown biochemical processes that may alter L-histidine transport independently of the processes described in Fig. 9 cannot be ruled out. That said, Fig. 9 suggests that L-histidine was transported across the worm integumentary apical membranes by a sodium-independent transport exchange protein similar to the classical L-system, where intracellular substrates of this process such as L-leucine are capable of exchanging with external substrates in sea water. In the absence of a transmembrane sodium-electrochemical driving force for this transporter, as would be present in sodium-dependent carrier systems, the major driving force for L-histidine/L-leucine exchange on the L-system would be the outwardly directed concentration gradient of L-leucine (or other intracellular substrate using this carrier system). Data presented in Fig. 2 suggest that under control buffer conditions with 6 mmol l^{-1} calcium, two L-histidine molecules (sigmoidicity, n=2.2) exchange with internal substrates per transport event. Fig. 9 suggests that a single calcium ion associates with an inhibitory regulatory binding site (e.g. site I) on this carrier process when this ion is present in the external medium. Addition of 1 µmol l^{-1} cadmium to the calcium-containing incubation medium converted the sigmoidal relationship between L-[^{3}H]histidine influx and L-histidine concentration to a hyperbolic relationship (Fig. 2). These results, and those of Fig. 6 showing a cadmium binding sigmoidicity n of 2.3, are interpreted to mean that two cadmium ions bind to an activator regulatory binding site (e.g. site A) on the transport protein and in so doing block the binding of one of the two L-histidine molecules that would associate with the protein under control conditions. As shown in Fig. 9, when both calcium and cadmium are present in the same experiment, hyperbolic influx of L-[^{3}H]histidine occurs, but uptake in the presence of cadmium alone is greater than when calcium is added (Figs 4, 5). Fig. 9 also shows that in reduced concentrations of calcium, or in its total absence, addition of lithium stimulated the uptake of amino acid in a manner similar to that displayed by cadmium binding. Fig. 8 indicates that, in the absence of calcium, two lithium ions associate with the carrier protein during the transport event and Fig. 4 shows that cadmium ion provides a greater stimulatory effect on amino acid influx than does lithium. As shown in the model, the overall effect of calcium was to reduce the transport rate of L-[^{3}H]histidine, while the overall effects of cadmium or lithium were to stimulate amino acid uptake. Sodium ion had no effect on the transporter (Fig. 7).

In all experiments presented in this paper involving the addition of cadmium to the incubation medium, the transport of [^{3}H]L-histidine was increased over the control situation. In Fig. 2, at 10 µmol l^{-1} L-histidine, the uptake of the radiolabelled amino acid in the presence of cadmium was more than twice that observed in the absence of the metal. Under
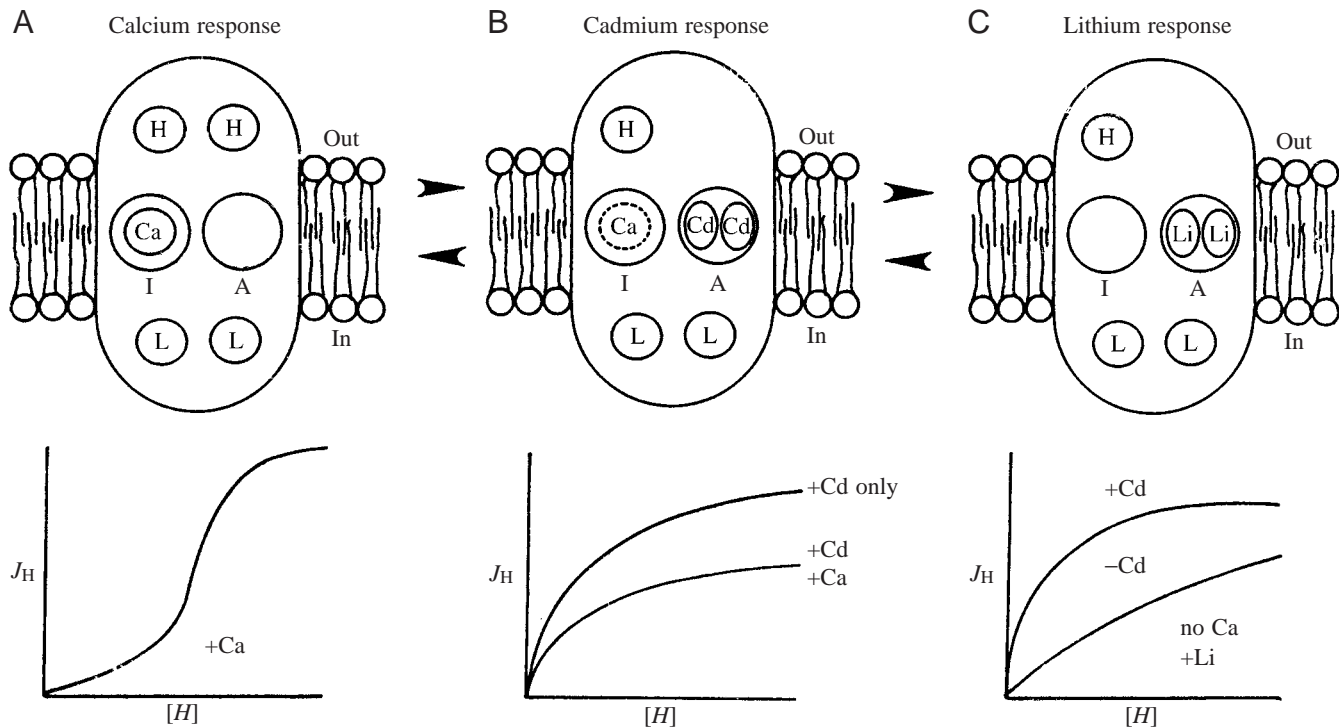
Fig. 9. Model of regulatory action by calcium, cadmium and lithium on sodium-independent L-[^{3}H]histidine influx into polychaete integumentary epithelium. The model suggests that L-histidine influx occurs by way of an L-system carrier process that exchanges with internal substrates of this system such as L-leucine. (A) In normal saline, calcium ion occupies an inhibitory regulatory site (I) on the external transporter surface that allows two molecules of L-histidine (H) to bind during each transport event. Under these conditions a nearby activator regulatory site (A) is not occupied by ligand and the relationship between L-[^{3}H]histidine influx and [L-histidine] is sigmoidal (n=2.0). (B) The transporter configuration in the presence of $1\,\mu mol\,l^{-1}$ cadmium (Cd). Under these conditions the data suggest that two cadmium ions associate with the activator regulatory site (A) in the presence or absence of calcium and convert the sigmoidal relationship between amino acid influx and [amino acid] to a hyperbolic function, due to the functional loss of one L-histidine binding site. The effect of calcium binding to the I site is to reduce the apparent maximal transport velocity of the transporter for amino acid. (C) The transporter configuration in the presence of lithium ion (Li). Under these conditions the data suggest that two lithium ions associate with the A site and act like cadmium to convert sigmoidal amino acid influx to hyperbolic transport. The A site appears to have a higher binding affinity for cadmium than it does for lithium, as hyperbolic L-histidine influx apparent maximal transport velocity is greater with the addition of cadmium than in its absence

conditions of variable calcium concentration, addition of cadmium consistently accelerated the uptake of L-histidine at all calcium concentrations used (e.g. Figs 3–5). Either in the absence (Fig. 4) or the presence (Fig. 5) of calcium, addition of cadmium significantly ($P<0.01$) increased L-[^{3}H]histidine influx J_{max} without significantly ($P>0.05$) influencing the apparent binding of the amino acid to the transporter (K_t). As shown in the model in Fig. 9, the increase in L-[^{3}H]histidine influx J_{max} observed in the presence of cadmium was proably due to the increased positive charge added to the L-histidine carrier protein with the association of two cadmium ions to the regulatory site. This positive charge added to the transport protein resulted in an electrogenicity to the exchange event where both the outwardly directed substrate concentration gradient and the transmembrane electrical potential combined to increase the movement of L-[^{3}H]histidine across the membrane, thereby increasing amino acid influx J_{max}.

Figs 3 and 7 indicate that when control buffer concentrations of calcium or sodium were reduced in transport experiments (e.g. replaced by lithium ions) an increase in the uptake of

$10\,\mu mol\,l^{-1}$ L-[^{3}H]histidine was observed. However, Fig. 7 indicates that a change in external sodium concentration alone, in the presence of control concentrations of calcium, did not alter the uptake of the radiolabelled amino acid. But when calcium was replaced in addition to the deletion of sodium, an enhancement of L-[^{3}H]histidine uptake took place. These data suggest that the stimulation of amino acid uptake observed during the reduction or elimination of sodium or calcium was due to the presence of the replacement ion, lithium.

Lithium is a monovalent cation that has an ionic radius considerably smaller than those of either cadmium or calcium (Li, 0.76 nm; Cd, 0.95 nm; Ca, 1.00 nm). These comparisons suggest that either two lithium ions or two cadmium ions may be able to physically occupy the same activator binding site (e.g. site A). This suggestion is supported by the results in Figs 6 and 8, showing binding sigmoidicity values n of approximately 2.0 for both cadmium and lithium during L-[^{3}H]histidine influx. Therefore the results in Figs 3 and 7 showing a marked increase in the influx of L-[^{3}H]histidine as either calcium or sodium concentrations were reduced is interpreted to be due to the

increased binding of replacement lithium ions at regulatory site A and providing an activation to the amino acid transport.

The overall results of this study suggest that L-[^{3}H]histidine influx across the integumentary epithelial apical membrane in the euryhaline polychaete worm, *Nereis succinea,* appears to be largely due to the operation of a transporter that resembles the classical sodium-independent L-system, which possesses two cation regulatory sites that can be occupied by calcium, cadmium or lithium ions. Under most environmental conditions an inhibitory site (e.g. site I) is probably occupied by a single calcium ion that may impart a small degree of electrogenicity to an otherwise electroneutral amino acid exchange process, bringing two L-histidine molecules into the cell in exchange for one or two intracellular L-system substrates. Addition of the divalent cation, cadmium, to the external medium at the environmentally realistic concentration of $1\,\mu mol\,l^{-1}$ results in the activation of the transport protein and enhanced L-histidine influx J_{max}, due to increased electrogenicity imparted to the carrier by two cadmium ions simultaneously binding to the transporter. Other ions, such as the monovalent cation lithium, also appear to be able to bind to regulatory site A on this transporter and accelerate the uptake of amino acid compared to uptake when calcium alone is the cofactor. It may be that metals other than cadmium, such as zinc, copper and iron, which have essential biological functions in most animals, may have more stimulatory capacity for accelerating amino acid transport than seen in the present study with cadmium.

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