

INTEGUMENTARY AMINO ACID TRANSPORT AND METABOLISM IN THE APODOUS SEA CUCUMBER, *CHIRIDOTA RIGIDA*

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SUMMARY

1. The apodous sea cucumber, *Chiridota rigida*, was found to transport exogenous amino acids to intracellular free amino acid pools in the integument, the gastrointestinal epithelium making only a minimal contribution to total animal uptake.

2. Amino acids entering the integumentary free amino acid pool were either completely catabolized to CO_2 , incorporated into large molecular weight compounds, such as proteins, or left unchanged. The latter fraction was available for efflux through both apical and basal cellular membranes through saturable or non-saturable transport processes.

3. Influx of natural (glycine) and analog (AIB) amino acids into the integumentary epithelium was achieved by a combination of saturable and non-saturable transport mechanisms: the former displaying Michaelis-Menten kinetics, being Na^+ -dependent, and transporting both glycine and AIB; the latter involving a simple diffusion of amino acid across the apical cell membranes and/or through the intercellular septate desmosomes.

4. Preloaded AIB exhibited both homo- and hetero-exchange diffusion with exogenous amino acids when a relatively large internal analog pool had been established. With a small internal pool of AIB, homo-exchange diffusion persisted, but high external concentrations of glycine inhibited the washout of the analog.

5. Transintegumentary influx of AIB to the coelomic fluid was a linear function of the exogenous amino acid concentration and was unchanged when choline chloride was used as a substitute saline. At steady-state the coelomic fluid analog concentration was maintained at a much lower level than the incubation medium or the epithelial free amino acid pool, due to apparent absorptive processes of the dermis and muscle layers of the integument, as well as by possible carrier-mediated re-uptake mechanisms on the basal membranes of the epithelial cells.

6. A tentative model of integumentary amino acid transport and metabolism in *C. rigida* is presented.

INTRODUCTION

It has long been known that marine and brackish-water invertebrates possess high intracellular concentrations of free amino acids (Fredericq, 1901). Many cellular processes involved with synthesis and degradation continually tap this pool of reactive substrates. In addition, these compounds play an important role in cellular osmo-regulation, serving as osmotic effector substances within the cell to balance the high extra-cellular concentrations of inorganic ions (Florkin, 1956, 1962, 1966; Schoffeniels, 1967; Lange, 1968; Florkin & Schoffeniels, 1969; Schoffeniels & Gilles, 1970). Because cellular amino acids of aquatic animals are involved in such a large number of biologically important processes, factors which affect the composition of the free amino acid pool and the concentrations of its individual amino acids may have profound effects upon the organism as a whole.

One factor which might influence the cellular free amino acid pool of aquatic animals is the transport of these compounds across cell membranes. Numerous studies since the classic publication of Pütter (1909) have indicated that marine and brackish-water invertebrates exhibit the ability to transfer exogenous organic molecules from their surroundings to their intracellular pool of metabolic substrates. These investigations have been reviewed on several occasions (Krogh, 1931; Jørgensen, 1966; Stephens, 1968, 1972; Wong, 1971). A recurrent theme in many of these studies is that the transport of exogenous compounds to intracellular pools provides the organisms with a supplemental source of nutrition. However, since these animals have a very large concentration gradient for substances such as amino acids across their cellular membranes, the net outward flow of these materials to the environment also seems possible. It is, therefore, of considerable nutritional interest to characterize the patterns of organic molecule flux between intracellular pools and the surrounding medium.

Stephens (1968), Wong & Stephens (1970) and Wong (1971) compared amino acid influx and efflux in polychaete worms and concluded that uptake rates were likely to exceed leakage rates by a large margin. Furthermore, these investigators could find no evidence for exchange diffusion between intracellular and extracellular amino acids and stated that the processes of amino acid exchange between the animal and its environment consisted of carrier-mediated entry mechanisms (either facilitated diffusion or active transport) and simple diffusional leakage.

The present investigation is an analysis of integumentary amino acid transport and metabolism in the sea cucumber, *Chiridota rigida*. The natural amino acid, glycine, was used to establish some of the major metabolic pathways available to exogenous compounds once within the intracellular free amino acid pool. In addition, the non-metabolizable analog amino acid α -aminoisobutyric acid (AIB) served as a means of detecting the occurrence of specific transport processes without the involvement of cellular metabolism. A preliminary report of the present findings has recently been published (Townsend & Ahearn, 1973).

MATERIALS AND METHODS

Collection and maintenance of animals

Chiridota rigida, a small (2–4 cm) apodous sea cucumber, lives buried in the upper one or two centimetres of sand in shallow intertidal waters throughout the Hawaiian

Islands. Animals were collected by sieving aliquots of sand through hand-held screens and were maintained in the laboratory at 24 °C in an artificial seawater medium proposed by Nicol (1960). This saline solution had a chlorinity of 19‰, a freezing point depression of -1.872 °C and a pH of 8.1. In addition, this medium had a total Na⁺ concentration of 471 mM and an osmotic pressure of approximately 1000 m-osmoles/l. This artificial seawater medium was used both for laboratory acclimation of the animals (24 h before experimentation) as well as for their incubation with selected radioactively labelled compounds. When Na⁺-free incubation medium was used, choline chloride and choline bicarbonate (Sigma Chemical Co.) were substituted for NaCl and NaHCO₃, MgSO₄ replaced Na₂SO₄ and NaF was deleted. The remaining salt composition was adjusted to maintain normal seawater ionic ratios and total osmotic pressure.

Experimental procedures

Acclimated animals were gently blotted dry with Kleenex tissue, weighed to the nearest 0.1 mg on an analytical balance, and transferred individually to 5 ml of artificial seawater solution in test tubes maintained at 24 °C. After approximately 5–10 min each animal was transferred to another test tube, at the same temperature, containing 5 ml of seawater medium to which was added either [¹⁴C]glycine (U), [¹⁴C]α-aminoisobutyric acid (3-¹⁴C) (New England Nuclear, Corp.), or [³H]α-aminoisobutyric acid (methyl-³H) (ICN Pharmaceutical, Inc.) together with inactive glycine or α-amino-isobutyric acid (AIB) to final concentrations between 1 and 100 μM and activities of 0.01–1.7 μCi/ml. Medium samples (taken before and after animal incubation) in most cases gave similar radioactivity measurements, indicating that the cucumbers had not significantly altered the concentration of amino acid in the solution during the exposure interval (only during the relatively long 2 h exposures of animals to labelled glycine did a significant decrease in medium amino acid concentration occur). Following incubation the animals were removed, rinsed rapidly (2–3 sec) in unlabelled seawater medium and extracted for 48 h in 5 ml of 70% ethanol at room temperature (24 °C). Samples of the extracts were counted in liquid scintillation spectrometers (Beckman LS100 and LS230) using a toluene-based cocktail and the external standard method for quench correction. The volatile fraction of ethanol extracts, most likely representing the terminal products of amino acid catalysis (¹⁴CO₂ or ³H₂O), was measured as the difference in activity before and after evaporation of a known volume of radioactively-labelled extract. When non-extractable radioactivity (protein, etc.) was to be measured, the animals were dissolved in Protosol tissue solubilizer (New England Nuclear, Corp.) after two 48 h ethanol extractions and subsequently counted in the scintillation counters as described above.

Samples of coelomic fluid were obtained by puncture of the body wall with a tapering-tipped glass capillary tube as described elsewhere (Ahearn & Gomme, 1975). After fluid withdrawal, the sample volume was measured to the nearest microlitre using a graduated Hamilton syringe. Coelomic fluid samples were added directly to the toluene-based scintillation cocktail and counted for radioactivity.

Inulin space, representing surface medium adherence plus gastrointestinal volume, was measured by incubating the animals in artificial seawater medium containing trace amounts of [³H]inulin (G) (New England Nuclear, Corp.). After exposure for

predetermined intervals, the animals were removed from this solution, rinsed in unlabelled seawater, and digested in Protosol. Digest samples were added to Aquasol scintillation cocktail (New England Nuclear, Corp.) together with distilled water in proportions which formed thick gels and prevented the precipitation of labelled inulin (Bray, 1970). Radioactivity counting of the digest samples was conducted as previously described. Inulin space (expressed as percentage tissue wet weight) was calculated according to Neame & Richards (1972).

Labelled compounds in ethanol extracts were separated and identified by thin-layer chromatography on plastic silica gel sheets (Eastman Kodak Co.). Extract samples were de-salted by passage through a Dowex-2 anion exchange column (chloride form). The eluant was evaporated to dryness and redissolved in 20 μ l 70 % ethanol for application to the silica gel sheets. The solvent system used was butanol/acetic acid/water (80:20:20). Sheets were scanned for distribution of radioactivity by cutting out 0.5 \times 3.0 cm units of the separation path and transferring them directly into liquid scintillation vials for counting. On each chromatograph sheet an inactive glycine standard was run, its position being determined by spraying with ninhydrin.

Animals were prepared for autoradiography by exposing them for 60 min to 1 μ M [14 C]glycine in the artificial seawater medium. Following this incubation interval the animals were rinsed and fixed in 1 % glutaraldehyde in seawater using a phosphate buffer to maintain a pH of 7.5. The fixed animals were embedded in paraffin and sectioned at 8 μ m. Transverse sections of whole animals were covered with Kodak NTB liquid emulsion and kept in the dark for three weeks for film exposure. Unlabelled animals were treated in the same manner for controls. Both stained and unstained slides were prepared.

RESULTS

Evaluation of inulin space

To determine the volume of incubation medium adhering to the body surface and ingested by the animals during their exposure to radioactively labelled amino acids, the cucumbers were exposed to [3 H]inulin for periods ranging from 5 to 60 min. During this period the inulin space of the whole animal gradually increased from 3.4 to 7.3 % of the wet weight (Fig. 1). Since this increase may have resulted from cellular penetration by the extracellular space marker of both the epithelial cell layers of the body wall and gastrointestinal tract, the true value of adherent + gut volume was estimated by extrapolating the inulin space curve back to zero time. The initial inulin space, derived in this manner, was approximately 3 % of the whole animal wet weight. All subsequent amino acid transport data, except those from the washout experiments, were corrected for this extracellular inulin space.

Time course of glycine uptake and metabolism

Animals were exposed to 1 μ M [14 C]glycine in artificial seawater medium for periods ranging from 5 to 30 min and uptake of the radioactive label into volatile ethanol soluble, non-volatile ethanol soluble, and Protosol digest fractions monitored (Fig. 2). Although total animal activity continued to increase over the 30 min incubation period, the rate of increase in ethanol-soluble activity exhibited a considerable reduction by 30 min, indicating the approach to steady-state conditions between th

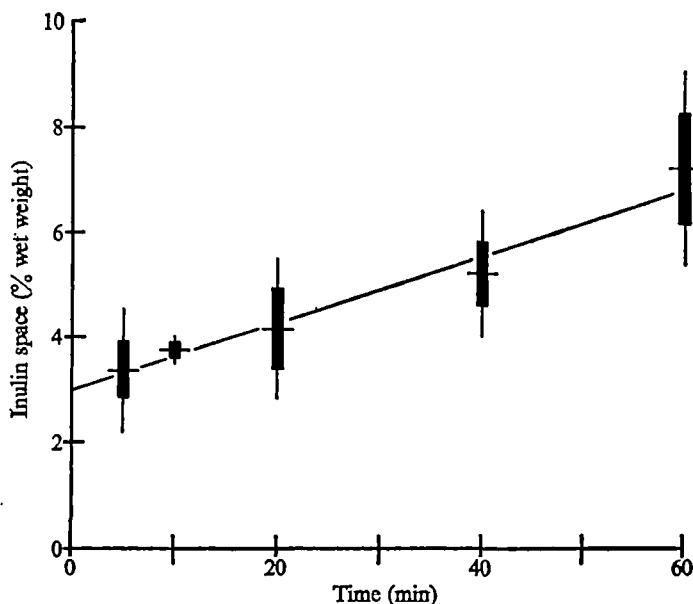


Fig. 1. Time course of $[^3\text{H}]$ inulin uptake into *C. rigida* in seawater incubation medium. In this and subsequent illustrations the horizontal lines indicate the means of results from five animals, vertical bars ± 1 standard error of the mean and vertical lines ± 1 standard deviation.

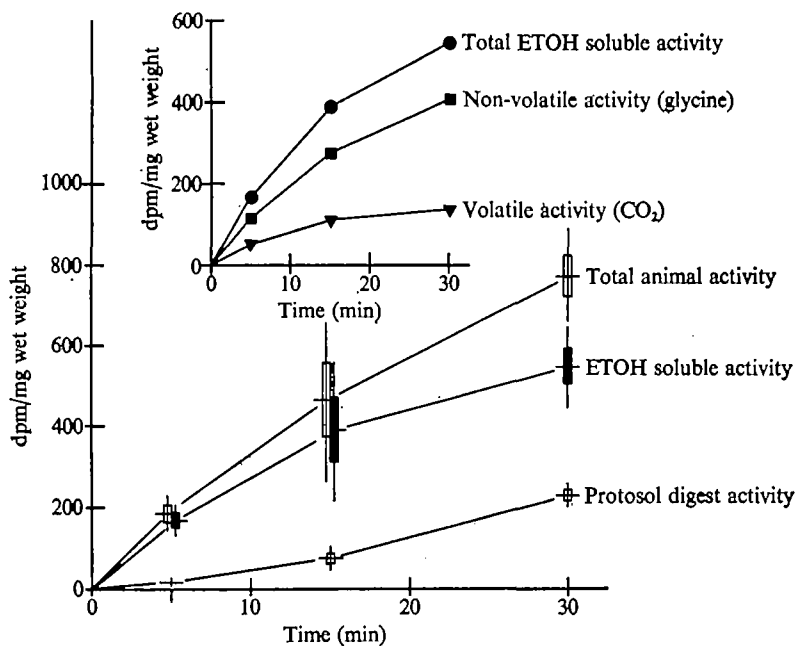


Fig. 2. Time course of $1 \mu\text{M}$ $[^{14}\text{C}]$ glycine uptake into ethanol soluble and ethanol insoluble (Protosol digest) activity fractions. The data in the inset represent the time course of the mean uptake ($n = 5$) into volatile and non-volatile fractions of ethanol soluble activity. The glycine fraction of ethanol soluble activity was determined by subtraction.

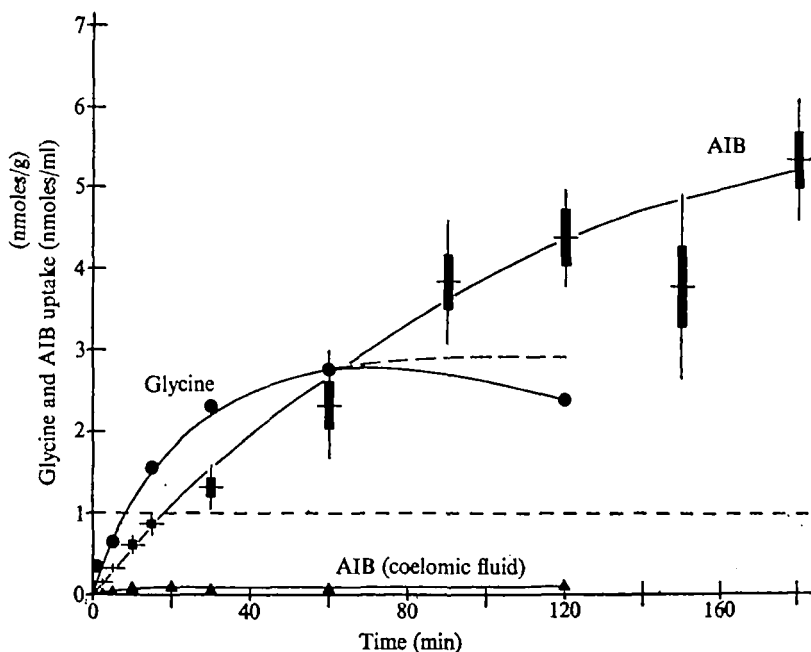


Fig. 3. Time course of $1 \mu\text{M}$ $[^{14}\text{C}]$ glycine and $[^{14}\text{C}]$ AIB uptake into intact animals and $1 \mu\text{M}$ $[^3\text{H}]$ AIB uptake into coelomic fluid of *C. rigida* in artificial seawater medium. Values for the mean $[^{14}\text{C}]$ glycine uptake ($n = 5$) were calculated as described in Fig. 2. Values for $[^3\text{H}]$ AIB uptake into coelomic fluid are means ($n = 5$) with their variability encompassed by the symbols. The broken line across the graph represents the amino acid concentration in the incubation medium during the exposures, except for the 2 h $[^{14}\text{C}]$ glycine incubation where a slight decrease in external amino acid concentration occurred. The extension of the $[^{14}\text{C}]$ glycine uptake curve, indicated by the broken line, represents the estimated steady-state animal concentration at a constant external amino acid concentration.

activity in the latter fraction and that in the incubation medium. In contrast, the activity of the Protosol digest showed an exponential increase suggesting that it was largely the increased rate of activity incorporation into this fraction which accounted for the sustained overall increase in total animal activity. While the volatile portion of ethanol soluble activity ($^{14}\text{CO}_2$) had apparently reached steady-state by 30 min of incubation, the non-volatile component of this fraction continued to increase over the exposure period (inset, Fig. 2). Thin-layer chromatograms of the non-volatile ethanol soluble fraction of total animal radioactivity yielded a single activity peak with an R_f corresponding to that of standard glycine, indicating that no measurable conversion of $[^{14}\text{C}]$ glycine to other amino acids had occurred during the 30-min uptake process. Therefore, the majority of total radioactivity in animals exposed to $1 \mu\text{M}$ $[^{14}\text{C}]$ glycine for up to 30 min proved to be the unaltered amino acid, although both incorporation into presumably large non-diffusible compounds and total catabolism to $^{14}\text{CO}_2$ had also occurred during this time period.

Further experiments characterizing the time course of $[^{14}\text{C}]$ glycine uptake in *C. rigida* utilized incubation intervals of 60 and 120 min duration. Chromatography of the non-volatile ethanol soluble fraction showed $[^{14}\text{C}]$ glycine to be the only labelled amino acid present. After subtraction of activity present in the $^{14}\text{CO}_2$ and Protosol digest fractions, the results showed that $[^{14}\text{C}]$ glycine in the animals had reached steady

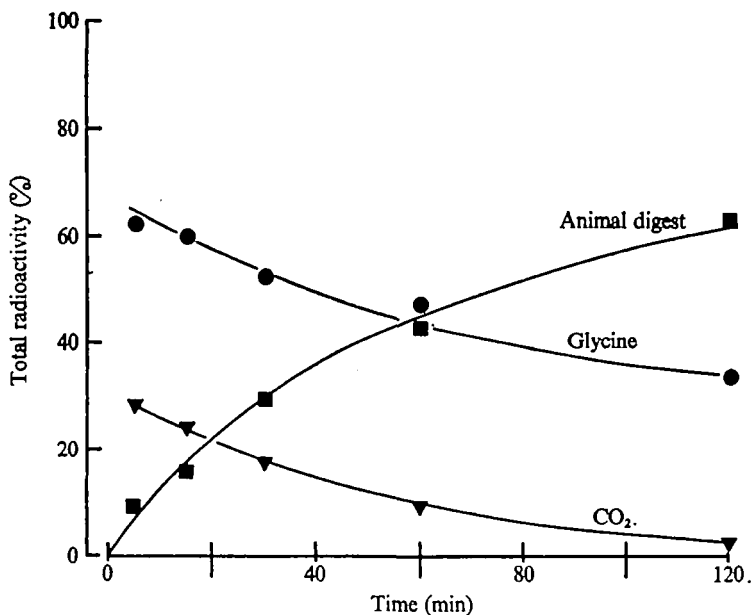


Fig. 4. Per cent distribution of total animal activity among the Protosol digest, [^{14}C]glycine, and $^{14}\text{CO}_2$ fractions of sea cucumbers exposed to $1\text{ }\mu\text{M}$ [^{14}C]glycine in artificial seawater medium. Values presented at each time period are means ($n = 5$). Animal digest and $^{14}\text{CO}_2$ fractions were measured directly, while the [^{14}C]glycine fraction was determined as indicated in Fig. 2.

state with the labelled amino acid in the incubation medium by 60 min (Fig. 3). The steady-state concentration of [^{14}C]glycine in the animals was three times that of the incubation medium suggesting the presence of a transport mechanism capable of transferring the amino acid against a concentration gradient.

During exposure to $1\text{ }\mu\text{M}$ [^{14}C]glycine in artificial seawater medium, for periods ranging from 5 to 120 min, both the [^{14}C]glycine and $^{14}\text{CO}_2$ fractions decreased their relative contribution to the total animal activity, while the digest fraction exhibited a continual rise (Fig. 4). During short exposures (5–10 min) to the labelled amino acid, [^{14}C]glycine recovered from the animals accounted for approximately 60% of the total radioactivity present, while the $^{14}\text{CO}_2$ and Protosol digest portions each contributed 30 and 10%, respectively. However, after 120 min the digest fraction accounted for more than 60% of the total activity, the contributions of the [^{14}C]glycine and $^{14}\text{CO}_2$ being reduced to 35 and 5%, respectively. The relative decrease in percent contribution to total animal radioactivity shown by the $^{14}\text{CO}_2$ and [^{14}C]glycine fractions appears to result from their attainment of a steady-state after 30–60 min of incubation (Figs. 2 and 3) and thereby not contributing to any further increase in total activity. Incorporation of exogenous [^{14}C]glycine into non-diffusible compounds, therefore, apparently continued at a sufficiently high rate to account for the increased accumulation of radioactivity above the levels set by the diffusible fractions in steady-state.

To examine the internal distribution of incorporated [^{14}C]glycine in *C. rigida*, autoradiograms were prepared from animals which had been exposed to the labelled amino acid for 60 min. The greatest degree of ^{14}C -incorporation occurred in the body wall epithelium, but significant activity was also present in the epithelial layer of

certain portions of the gastrointestinal tract (Fig. 10, Plate 1). The density of silver grains appeared to be relatively uniform over the entire extent of the body wall epithelium except in the tentacles surrounding the mouth which appeared to be the sites of maximal [^{14}C]glycine incorporation. The gastro-intestinal tract appeared to be both morphologically and physiologically differentiated into three distinct regions, in its manner of amino acid incorporation (assuming that each gut region had equal access to labelled incubation medium). One portion of the gut which possessed long villus-like projections exhibited a relatively high intracellular density of silver grains. Another region which possessed short projections showed fewer silver grains distributed within the cells, but considerable blackening occurred extracellularly along the luminal margin of these processes. The third distinct gut area lacked finger-like extensions and only exhibited background grain density, suggesting that little [^{14}C]glycine incorporation had occurred in this region.

Since it appeared that some exogenous amino acid was swallowed and thereby became available for transport by the epithelial cells of the gut, an experiment was conducted to determine the extent to which gut absorption contributed to total animal absorption during short incubation periods when influxes were being measured. Two animal groups were exposed to $1\text{ }\mu\text{M}$ [^3H]AIB for 10 min. In one group, thin cotton thread secured around the head and tail ends prevented connexion between the incubation medium and the gastrointestinal tract. The other group, which was not ligated, served as the control. Influx rates of the analog amino acid for the ligated and control groups under these conditions were 0.054 ± 0.009 ($\bar{x} \pm 1\text{ S.E.M.}$; $n = 7$) and 0.051 ± 0.008 ($\bar{x} \pm 1\text{ S.E.M.}$; $n = 5$) nmole/g \times min, respectively. Since the gut was sealed in the ligated group, no correction was made for the activity present in the inulin space of these animals (this assumes that most of the inulin space was confined to the gut and not adhering to the body surface). These results suggest that transport of amino acids by the gut did not significantly contribute to whole animal absorption, and further establish the body-wall epithelium as the main site of exogenous amino acid transport and metabolism in these animals.

AIB uptake and transport kinetics

The time course of $1\text{ }\mu\text{M}$ [^{14}C]AIB and [^{14}C]glycine uptake is shown in Fig. 3. Thin-layer chromatography showed that only labelled AIB was present in ethanol extracts of whole animals which had been exposed to the analog for three hours. Furthermore, activity associated with the volatile and digest fractions was insignificant indicating that little metabolic alteration of the compound occurred during its uptake. Although both amino acids were accumulated within whole animals to concentrations exceeding those of the incubation media, the rate of [^{14}C]glycine uptake was considerably more rapid than that of [^{14}C]AIB. The initial uptake rates, measured over the first 5 min for [^{14}C]glycine or 10 min for [^{14}C]AIB, represent glycine and AIB influxes of 0.24 and 0.06 nmole/g \times min, respectively. These results indicate that the natural amino acid was transported four times faster than the analog under similar conditions. In addition, [^{14}C]glycine reached steady-state by 60 min of incubation, while the whole animal concentration of [^{14}C]AIB was still increasing after an exposure interval of 3 h.

Uptake of $1\text{ }\mu\text{M}$ [^3H]AIB into the coelomic fluid for incubation intervals of 2.5–120 min, was very rapid (apparent steady-state being reached between 10 and 15 min).

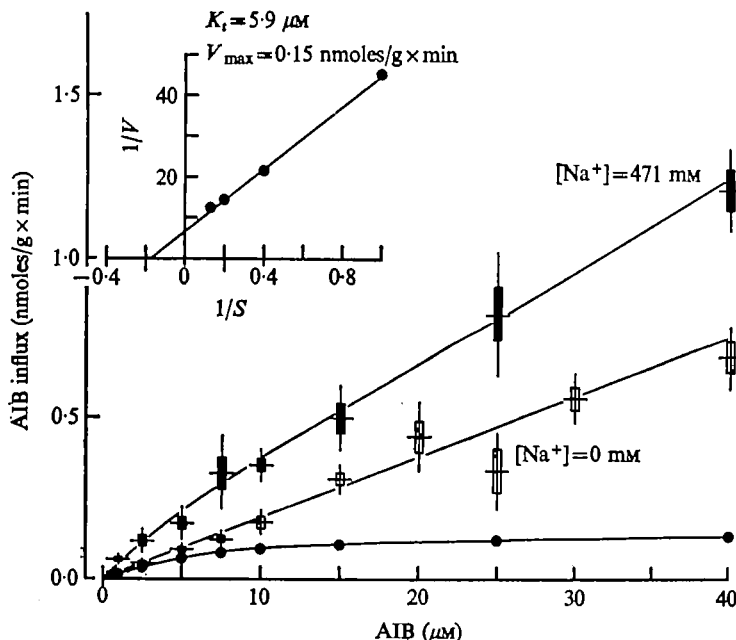


Fig. 5. $[^{14}\text{C}]$ AIB influx into *C. rigida* as functions of external amino acid concentration in artificial seawater medium ($[\text{Na}^+] = 471 \text{ mM}$) and choline chloride medium ($[\text{Na}^+] = 0 \text{ mM}$), respectively. Closed circles represent the calculated facilitated transport process for AIB as described in text. The inset is a Lineweaver-Burk plot of the data for carrier-mediated AIB entry.

compared to the uptake of this compound into the whole animal (Fig. 3). However, the apparent steady-state concentration of the analog in the coelomic fluid only amounted to 0.09 nmole/ml or 9% of its concentration in the incubation medium. The transintegumentary influx of $1 \mu\text{M}$ $[^3\text{H}]\text{AIB}$ into the coelomic fluid, measured over the first 5 or 10 min of uptake, while the transfer was approximately a linear function of time, was $0.007 \text{ nmole/ml} \times \text{min}$ (Fig. 3). Therefore, at this concentration only approximately 12% of the analog entry into whole animals was delivered to the coelomic fluid, the remainder being presumably distributed intracellularly. These results, therefore, suggest a marked cellular accumulation of AIB with little transfer of the analog from the cellular compartments to the coelomic fluid.

The influx of $[^{14}\text{C}]\text{AIB}$ (10 min exposures) into *C. rigida* in artificial seawater was a biphasic function of external AIB concentration ($1\text{--}40 \mu\text{M}$), being curvilinear below $10 \mu\text{M}$ and linear above this level (Fig. 5). The biphasic nature of AIB influx indicated that at least two transfer processes were operating to remove the analog amino acid from the incubation medium: a saturable mechanism functioning at the lower amino acid concentrations, which reached a maximal transport rate at approximately $10 \mu\text{M}$ AIB, and a non-saturable system maintaining a constant increase in transfer rate with increasing external AIB concentration. This type of transport across a biological membrane, through the combination of saturable (following Michaelis-Menten kinetics) and non-saturable mechanisms is described by the following equation:

$$J = \frac{J_{\max}[S]}{[S] + K_t} + k[S], \quad (1)$$

where \bar{J} is total AIB influx rate (nmole/g \times min), \bar{J}_{\max} the maximal transport rate of the saturable system, K_t the AIB concentration resulting in half maximal transport rate through saturable influx, k the non-saturable transport constant, and $[S]$ the AIB concentration in the incubation medium. From the data presented in Fig. 5 it cannot be ascertained whether non-saturable AIB influx results from simple diffusion or represents a second facilitated system which does not exhibit saturation kinetics (high K_t) over the concentration range examined. Influx rates for [^{14}C]AIB have been measured over the concentration range of 40–200 μM and were found to increase in a linear manner, not deviating significantly from the linear relationship determined at the lower concentrations. Therefore, non-saturable AIB influx in *C. rigida* will tentatively be referred to as the apparent diffusional influx of this amino acid, although at this point in time, the existence of a second facilitated transport mechanism with very low affinity for AIB cannot be totally excluded. The rate of this apparent diffusional entry, as estimated by the slope of the linear portion of the influx curve, is 0.028 nmole/g \times min \times μM exogenous AIB. By subtraction ($\bar{J} - k[S]$) a measure of the facilitated [^{14}C]AIB influx at each amino acid concentration was obtained and plotted according to the method of Lineweaver & Burk (1934) (inset, Fig. 5). From this graphical analysis the influx K_t and \bar{J}_{\max} were found to be 5.9 μM and 0.15 nmole/g \times min, respectively. The solid circles shown in the main body of Fig. 5 represent saturable AIB influx calculated in this manner.

Whole animal influx of [^3H]AIB (10 min exposures) in Na^+ -free choline chloride saline was a linear function of amino acid concentration from 1 to 40 μM (Fig. 5). These data suggest that the facilitated transport mechanism for this amino acid observed in artificial seawater exhibited a strict Na^+ -dependency and was totally eliminated when this ion was removed from the bathing solution. In addition, non-saturable AIB influx in choline chloride was only two-thirds of that which occurred in seawater medium ($k_{\text{choline}}[S] = 0.019$ nmole AIB/g \times min \times μM exogenous AIB), suggesting an effect of the substitute cation on the apparent diffusional permeability of *C. rigida* to the analog amino acid. Alternatively, the decrease in non-saturable AIB influx in choline chloride saline may represent the loss of a second Na^+ -dependent facilitated transport process which did not exhibit saturation kinetics over the range examined.

Transintegumentary influx of [^3H]AIB into coelomic fluid from seawater and choline chloride media as a function of external amino acid concentration is shown in Fig. 6. The analog entry rates into the coelomic fluid in both saline solutions increased linearly from 1 to 40 μM AIB. Furthermore, these rates of increase were found to be virtually identical (0.006 nmole/ml \times min \times μM AIB). These results suggest that the decrease in whole animal non-saturable [^3H]AIB influx observed in choline chloride medium (Fig. 5), may have been due to an effect induced by the substitute cation on a non-saturable process not directly involved in amino acid exchange between the incubation medium and the coelomic fluid. During transintegumentary influx the coelomic fluid amino acid concentrations varied between 5 and 10% of those in the bathing media for all external AIB concentrations, indicating the presence of steep concentration gradients for the analog across the body wall. Since coelomic fluid AIB steady-state appeared to be attained by about 15 min of exposure (Fig. 3), the transintegumentary concentration gradients for the analog observed during influx were, to a large degree, probably also present at steady-state.

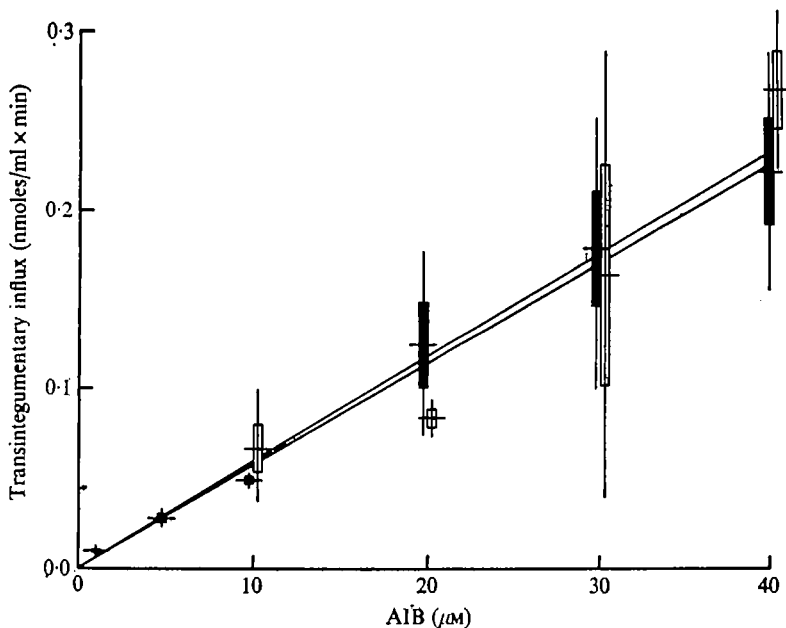


Fig. 6. Transintegratory influx (10 min exposures) of [^3H]AIB from seawater (filled rectangles) and choline chloride (open rectangles) media as functions of external amino acid concentration.

Amino acid inhibition of AIB influx

The effects of increasing exogenous glycine concentrations (0–100 μM) on 1 μM [^{14}C]AIB influx and 5 μM [^3H]AIB influx into whole animals are shown in Fig. 7. At both analog concentrations a marked decrease in AIB influx rate occurred in the presence of glycine, reducing entry to approximately half of that observed in the uninhibited condition. Once this reduced level of analog entry was attained, however, further increases in glycine concentration did not produce any additional decrease in AIB influx. The uninhibitable portion of AIB influx at 1 and 5 μM AIB (0.028 and 0.140 nmole/g \times min, respectively) corresponded with the previously determined non-saturable analog entry at these two concentrations (Fig. 5). These results suggest that external glycine totally inhibited the influx of AIB through the saturable transport mechanism, but failed to decrease the analog entry by way of the non-saturable process. To determine the mechanism of glycine inhibition, the data are plotted according to Dixon (1953) in the inset of Fig. 7 after subtraction of the uninhibitable component of AIB influx. The dissimilar horizontal axis intercepts of the two linear plots indicate that competitive inhibition between the substrates had occurred and the point of intersection was used to estimate the glycine inhibitory constant (K_i —0.6 μM). The equation describing AIB influx in the presence of exogeneous glycine acting as a competitive inhibitor to saturable analog entry is given below:

$$\mathcal{J}_i = \frac{\mathcal{J}_{\max} [S]}{[S] + K_t (1 + ([I]/K_i))} + k[S], \quad (2)$$

where \mathcal{J}_i refers to whole animal analog entry under inhibited conditions (nmole/g \times min), \mathcal{J}_{\max} , K_t , k and $[S]$ have their previous meanings, $[I]$ is the glycine concentration and K_i is the glycine inhibitory constant.

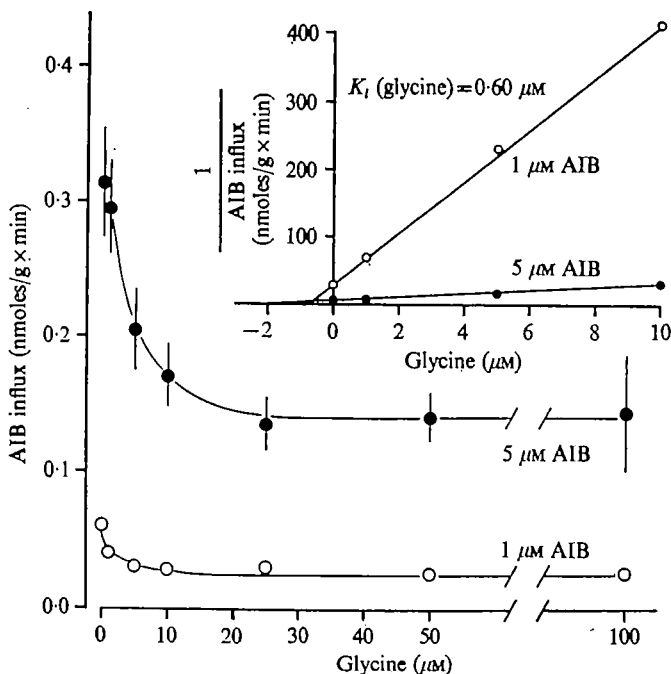


Fig. 7. Effects of increasing external glycine concentration on 1 μM [14 C]AIB influx and 5 μM [3 H]AIB influx in *C. rigida*. Circles represent means ($n = 5$). Vertical lines for 5 μM [3 H]AIB influx refer to ± 1 S.E.M., while the variability associated with 1 μM [14 C]AIB influx is encompassed by the symbols. The inset is a Dixon plot of the data after subtraction of the uninhibitable components of AIB influx.

Table 1. Inhibition of [3 H]AIB influx by other amino acids*

Amino acid	AIB influx (nmole/g x min)	% inhibition
AIB	0.061 \pm 0.010†	—
AIB + glycine	0.027 \pm 0.005	56.6
AIB + alanine	0.025 \pm 0.001	58.7
AIB + proline	0.026 \pm 0.007	57.4
AIB + hydroxyproline	0.027 \pm 0.004	56.6
AIB + glycine + alanine	0.027 \pm 0.005	55.4
AIB + glycine + proline	0.028 \pm 0.004	54.4
AIB + glycine + hydroxyproline	0.030 \pm 0.007	50.8

* AIB present at 1 μM, while each inhibiting amino acid present at 50 μM.

† ± 1 S.E.M.

Alanine, proline, and hydroxyproline (50 μM), when present separately in seawater incubation medium, also significantly decreased the entry rate of 1 μM [3 H]AIB into *C. rigida* (Table 1). In all cases the residual analog influx in the presence of these natural amino acids was identical to that observed with exogenous glycine as the inhibitor and equalled the previously determined non-saturable analog influx at this concentration (Fig. 5). Furthermore, when these inhibiting amino acids were used in combination (each at 50 μM), no further decrease in AIB influx was observed over the level which took place when the inhibitors were used separately. Such results suggest (but do not conclusively prove) that the four natural amino acids may each have share

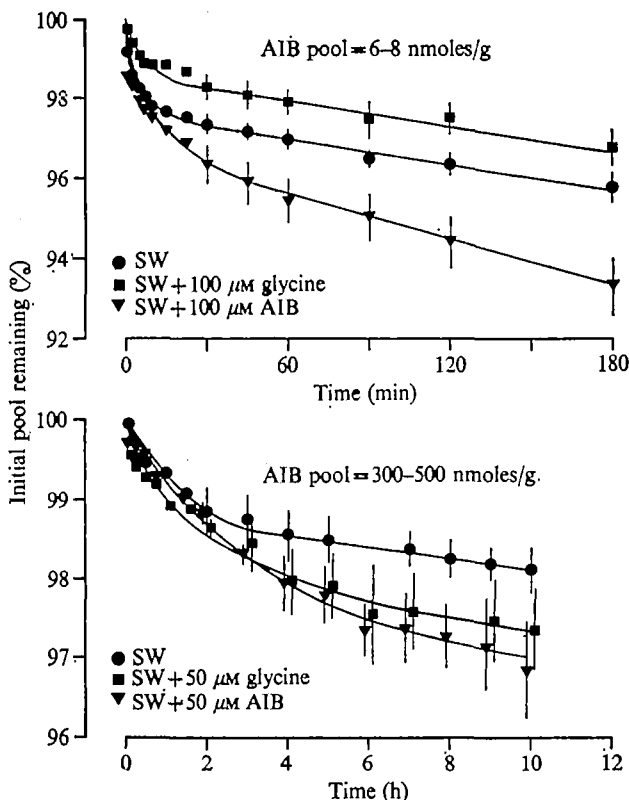


Fig. 8. Washout characteristics of preloaded $3 \mu\text{M}$ $[^3\text{H}]\text{AIB}$ (upper graph) and $100 \mu\text{M}$ $[^{14}\text{C}]\text{AIB}$ (lower graph) from *C. rigida* as a function of washout time. Symbols represent mean values ($n = 3$) and the vertical lines ± 1 S.E.M.

a common facilitated transport system with AIB, but that none apparently had an effect on the non-saturable mode of analog entry.

Washout characteristics of preloaded AIB

To determine if exogenous amino acids influenced the washout rate of a preloaded compound, animals were loaded with either $[^{14}\text{C}]\text{AIB}$ ($100 \mu\text{M}$) or $[^3\text{H}]\text{AIB}$ ($3 \mu\text{M}$) for 60 min, were subsequently rinsed rapidly in unlabelled seawater and were individually placed in 25 ml artificial seawater for efflux of labelled analog. Samples of the washout medium ($250 \mu\text{l}$.) were removed periodically for radioactivity counting and were immediately replaced with an equal volume of unlabelled seawater. Animals loaded in $[^3\text{H}]\text{AIB}$ had initial analog pool sizes between 6 and 8 nmole/g (sum of total activity lost and ethanol extractable activity present after efflux period), while the pool sizes in those specimens incubated in $[^{14}\text{C}]\text{AIB}$ ranged between 300 and 500 nmole/g. Three different washout solutions were used in the present series of experiments: unlabelled artificial seawater with no amino acid additions, seawater medium containing either 50 or $100 \mu\text{M}$ labelled glycine, or seawater medium containing either 50 or $100 \mu\text{M}$ unlabelled AIB.

Unlabelled exogenous amino acids exerted pronounced, but somewhat disparate effects on the rate of analog washout when compared to AIB exodus in seawater

medium alone (Fig. 8). Although unlabelled external AIB accelerated the loss of labelled analog from animals with either a large or small internal pool, exogenous glycine was found to inhibit the rate of AIB exodus from animals with a small analog pool, but accelerated the loss from specimens possessing a large pool. Since AIB and glycine have been shown to share a common facilitated transport process for uptake from the incubation medium (Fig. 7), these washout data suggest that both the accelerative and inhibitory effects of exogenous amino acids on analog washout are due to interactions between the respective amino acids for this common carrier transport system. In addition, the results indicate that AIB washout, like influx, appears to be the sum of at least two independent processes, a carrier-mediated mechanism interacting with other shared amino acids, and an apparently unshared system (residual [^3H]AIB washout in presence of $100\ \mu\text{M}$ glycine) probably representing diffusion.

DISCUSSION

Anatomy of the holothurian integument

The holothurian body wall is a complex structure consisting of a superficial cuticle, a single layer of surface epithelial cells, a thick collagenous dermal region, often delimited into specific zones, a muscular layer and an inner peritoneal lining of the coelomic cavity (Kawaguti, 1966; Menton & Eisen, 1970). In cross section the surface epithelial cells appear 'T' shaped due to apical processes which extend along the inner surface of the cuticle forming a contiguous epithelium. These apical extensions of the epidermal cells are connected to one another by septate desmosomes. Finger-like projections of the apical cell processes penetrate the cuticular lining and may serve as a means of attachment or as a site of solute exchange with the environment. Often the superficial zone of the dermis extends to the septate desmosomes of the apical cell processes and may completely surround the basal portions of the surface epithelial cells. Under low magnification, the integumentary structure of *C. rigida* appears similar to the anatomical descriptions of the body wall of more thoroughly studied species (Fig. 10, Plate 1). Therefore, a model of integumentary amino acid exchange and metabolism in *C. rigida* will be proposed which has its experimental and gross morphological basis in the results of the present investigation, but which also includes ultrastructural information obtained from the literature on closely related species.

Proposed model of integumentary amino acid transport and metabolism in Chiridota rigida

Dynamics of the intracellular free amino acid pool

The model presented in Fig. 9 represents a highly simplified view of the holothurian integument illustrating the major routes of amino acid transport and metabolism. The model takes account of the observation that both natural and analog amino acids were removed from solution and accumulated within the animals to concentrations considerably in excess of those in the environment (Fig. 3). It also accounts for the observation that a relatively small proportion of the total amino acid entering the entire animal penetrated the body wall and appeared in the coelomic fluid (Fig. 6), suggesting that the accumulative process was intracellular. Autoradiograms of animals exposed to radioactive glycine demonstrated the greatest degree of

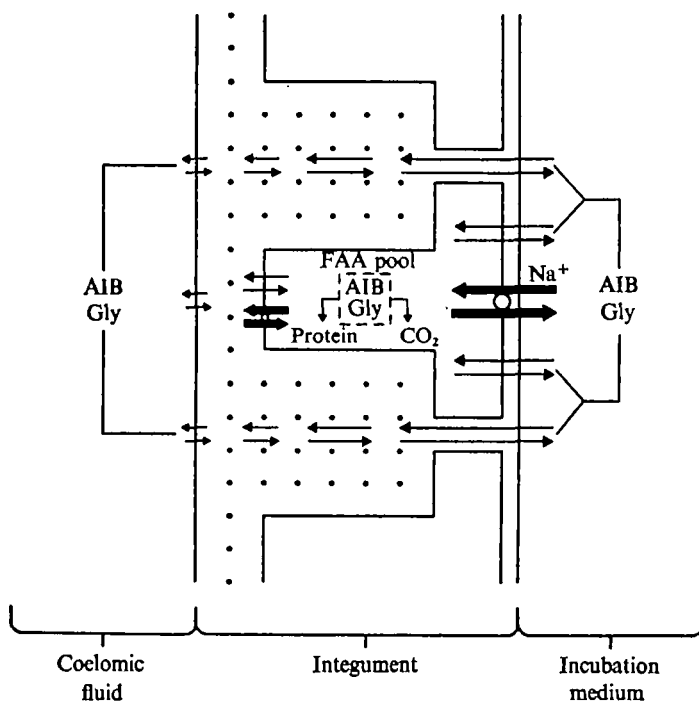


Fig. 9. Proposed model of integumentary amino acid transport and metabolism in *Chiridota rigida*. The 'T' shaped epithelial cells underlying an apparently amino acid permeable cuticle are drawn somewhat out-of-proportion relative to the sub-epithelial integumentary components (dermal and muscle layers) shown here as the stippled region (compare with histology in Fig. 10, Plate 1). Bidirectional arrows represent amino acid exchange pathways with bold arrows identifying carrier-mediated, saturable transport processes and the fine arrows illustrating non-saturable transport across cell membranes and through intercellular spaces. Decreasing size of bidirectional arrows across the stippled region indicates a quantitative reduction in amino acid exchange rates over this integumentary zone due to absorptive processes operating in dermal fibrocytes and muscle cells. Carrier-mediated amino acid transport across the basal epithelial membrane may serve as an additional regulative mechanism controlling the dermal and coelomic fluid amino acid concentrations.

amino acid incorporation in the surface epithelial cells of the body wall (Fig. 10, Plate 1), suggesting that this cellular layer was the major site of exchange and metabolism of exogenous amino acids for the whole animal and probably also represented the anatomical location of a large portion of the intracellular radioactive amino acid pool established during the uptake process. Other investigations with marine or brackish-water invertebrates have also indicated that the surface epithelium of the body wall has a major role in exchange between the organisms' intracellular pools of metabolic substrates and environmental amino acids (Ferguson, 1967, 1970; Little & Gupta, 1968; Southward & Southward, 1968; Péquignat, 1970) or sugars (Ferguson, 1968; Ernst & Goerke, 1969; Ahearn & Gomme, 1975).

As shown in Figs. 2 and 4, metabolizable exogenous amino acids (illustrated by labelled glycine) entering the intracellular free amino acid pool of the surface epithelial cells may be totally catabolized to CO₂ (presumably for direct energy requirements), they may become incorporated into large molecular weight substances such as proteins, or they may remain unchanged within the pool awaiting metabolic demands.

As suggested from Figs. 2 and 4 these three processes probably occur simultaneously and are in a dynamic exchange. During the first few minutes of uptake, much of the amino acid was channeled into total catabolism resulting in a large proportion of the radioactivity appearing as $^{14}\text{CO}_2$. However, as uptake proceeded and the catabolic pathways became saturated with substrate, more of the amino acid entered the incorporation processes. The exponential appearance of ^{14}C -activity in the protosol digest fraction is indicative of a process exhibiting a time-lag (Kipnis, Reiss & Helmreich, 1961), in this case perhaps due to the initial channeling of [^{14}C]glycine to total catabolism. By 60 min both the $^{14}\text{CO}_2$ and [^{14}C]glycine fractions of total animal activity reached steady-state (Figs. 2 and 3), but protein incorporation appeared to continually proceed (Fig. 4). Under such conditions, the rate of [^{14}C]glycine uptake from the environment into the steady-state free amino acid pool would be balanced by the combined loss from the pool to the catabolism, incorporation, and efflux pathways (the latter taking a portion of the intracellular amino acid back into the incubation medium and into the dermis). With continual exposure to exogenous [^{14}C]glycine, the increasing backflux of labelled glycine from the incorporation pathway to the free amino acid pool would presumably equal the flux from the free amino acid pool to the sites of incorporation and the entire animal would be in isotopic steady-state.

Interactions between amino acids for epithelial carrier transport

The model in Fig. 9 indicates that glycine and AIB share a common sodium-dependent, carrier-mediated transport process in the apical membrane of the integumentary epithelium of *C. rigida*. This suggestion is supported by the evidence presented in Figs. 5, 7, and 8 which show that in addition to competitively inhibiting the facilitated influx of AIB from the incubation medium, exogenous glycine also interacted with carrier-mediated efflux of the preloaded intracellular analog. Furthermore, alanine, proline, and hydroxyproline also appeared to inhibit the entrance of AIB into the epithelium (Table 1) through unknown mechanisms, but which may involve shared carrier systems. Studies concerning amino acid transport in other tissues have indicated that AIB can enter the cells of these tissues through a number of distinct carrier transport mechanisms (Christensen, 1962; Oxender & Christensen, 1963; Finerman & Rosenberg, 1966; Sriver & Mohyuddin, 1968; Mohyuddin & Sriver, 1970; Crawhall & Davis, 1971). In these investigations AIB mainly utilized the imino acid-glycine transport system (proline, hydroxyproline, glycine) and the so-called A-site transport system (Christensen, 1962; Oxender & Christensen, 1963), the latter serving the short-chain neutral amino acids such as glycine, alanine and serine. Heterogeneity of AIB influx in *C. rigida*, involving multiple carrier systems, has not been clearly established. Although alanine and proline, representing substrates for reportedly different carrier systems in other tissues, were both effective inhibitors of AIB influx in this animal, a single transport process with a wide range of substrates could adequately provide the inhibitory effects seen.

Preloaded intracellular AIB was capable of exhibiting both homo- and hetero-exchange diffusion with exogenous amino acids (unlabelled AIB and glycine) when a relatively large internal pool of the analog had been established (Fig. 8). In contrast, when a small AIB pool had been established intracellularly, the analog exhibited homo-exchange diffusion, but in this instance external glycine inhibited the exodus of

The preloaded compound. This result may best be interpreted as indicating that both AIB and glycine shared a common facilitated efflux mechanism as was seen for their influx (Fig. 7) and that the affinity of this process for glycine was greater than that for the analog. Under conditions where external glycine was at a very high concentration relative to that for internal AIB (Fig. 8, upper graph), all of the shared transport sites would soon be saturated with the 'favoured' amino acid and the resulting analog efflux would be reduced. When internal AIB was at a greater concentration than external glycine (Fig. 8, lower graph), the numerical superiority of the analog appeared to compensate for the affinity differences of the shared efflux process and hetero-exchange diffusion resulted.

In their studies of amino acid efflux in polychaetes, Stephens (1968), Wong & Stephens (1970), and Wong (1971) examined the effect of numerous exogenous amino acids on the exodus of preloaded natural and analog amino acids. Their findings showed that the external amino acids exerted little, if any, influence upon the rate of exodus of the preloaded compounds. However, these investigators did not consider the possible influence of amino acids present in an extracellular inulin space (adherent + gut volume) as contributing to the observed rates of amino acid efflux in preloaded animals in any of their experiments. Such an effect could be considerable when the animals had been preloaded in a seawater medium containing a very high concentration of amino acids. In this case most of the initially measured amino acid loss from the entire animal would be derived from this extracellular space and could mask any exchange diffusion which may simultaneously be occurring across epithelial cell membranes. In the present series of experiments with *C. rigida*, animals which had been preloaded in a relatively high exogenous AIB concentration ($100\ \mu\text{M}$) required between 3 and 4 h of continual efflux to satisfactorily distinguish between the amino acid loss from the inulin space and that derived from the animal itself (Fig. 8, lower graph). In contrast, those animals which were loaded in a much lower AIB concentration ($3.4\ \mu\text{M}$) were found to have a much reduced extracellular contribution to total animal efflux (Fig. 8, upper graph). Species differences in inulin space, however, may have contributed to the disparity of results obtained in the present study and those previously mentioned concerning the presence of amino acid exchange diffusion in marine invertebrates. If polychaetes, in general, have a small inulin space ($0.3\text{--}1.0\%$ for *Nereis diversicolor*, Ahearn & Gomme, 1975), the results of Stephens and Wong may, in fact, indicate that these animals do not exhibit significant exchange diffusion with their environment. Further comparative studies are clearly necessary to determine the prevalence of integumentary amino acid exchange diffusion in marine animals.

Non-saturable and transintegumentary amino acid transport

In addition to carrier-mediated entry across the apical cell membrane of the integumentary surface epithelium, AIB transport also occurred by way of a process which was not saturated over the concentration range used (Fig. 5). Furthermore, this non-saturable influx process was uninfluenced by the presence of other exogenous amino acids in high concentration (Fig. 7, Table 1). Recent studies (Schultz, Frizzell & Nellans, 1974; Ussing, Erlj & Lassen, 1974) have shown that both apical cell membranes and cell junctions of a variety of epithelia represent non-saturable exchange

pathways for ions and small organic molecules and may account for the non-saturable analog entry in *C. rigida* as illustrated by the model in Fig. 9. The model also indicates that transintegumentary exchange of AIB may have occurred through transcellular and intercellular processes producing steep analog concentration gradients across the body wall (Figs. 3 and 6). Under these conditions AIB entering the dermal region in high concentration either from the septate junctions or through the basal portions of the epithelial cells may be accumulated by fibrocyte transport processes in this integumentary zone during transit. In addition, re-uptake of the analog from the dermis through the basal portion of the epithelial cell layer by facilitated transport may also concurrently take place. The resulting coelomic fluid analog concentration would be markedly lower than it was originally in the epithelial cells or in the incubation medium. Steady-state of AIB in the coelomic fluid (Fig. 3), at a concentration considerably lower than that in the epithelial free amino acid pool or in the exposure solution would be maintained when the analog fluxes between the dermal or muscle layers and the coelomic fluid were equal. At present, the relative contribution of either transcellular or intercellular flow of amino acids to overall transintegumentary movements of these compounds is unknown. Since the influx of AIB across the body wall was unaffected by the use of choline chloride incubation medium (Fig. 6), while the carrier-mediated entry process, and hence, the cellular accumulation mechanism, was eliminated under these conditions (Fig. 5), a substantial portion of the transintegumentary flux, therefore, may have been between the epithelial cells.

Nutritional implications of proposed model

The results of the present investigation indicate that the sea cucumber, *C. rigida*, is capable of transporting exogenous amino acids to intracellular compartments, wherein these compounds enter a variety of synthetic and catabolic pathways. In addition, a portion of the entering amino acids remains within the intracellular free amino acid pool and is available for exchange back into the incubation medium by either saturable or non-saturable transport mechanism, or can flow to the dermis through undefined processes. Uptake of both glycine and AIB occurred against concentration gradients with the intracellular amino acids attaining steady-state concentrations considerably in excess of those in the incubation medium (Fig. 3). In the case of AIB the high cellular amino acid concentration indicates that net accumulation of the analog must have occurred during the approach to steady-state. However, since the uptake of radioactive exogenous glycine may have been surpassed by the simultaneous loss of endogenous unlabelled glycine from the intracellular free amino acid pools, an estimation of the extent or direction of its net transport cannot be made at this time. Even though AIB itself appeared to undergo net uptake from the medium, much of this transport may have occurred through hetero-exchange diffusion with endogenous amino acids. If this were the case, there would be no net amino acid accumulation by the overall exchange process. These results indicate, therefore, that no definite conclusions can be made regarding the nutritional significance of the physiological processes characterized in the present study. Should the passive permeability of the surface integumentary epithelium be rather low, and the contribution of facilitated exchange diffusion to influx and efflux be minimal, the processes described in this investigation may provide the epithelium, dermis and muscle layers with a means of

Obtaining significant quantities of metabolic substrates. Further study will be necessary to clarify these points.

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EXPLANATION OF PLATE

Fig. 10. General histology and autoradiography of *Chiridota rigida*

A. Cross-section under low magnification illustrating organs of coelomic cavity and relative thickness of body wall. Note morphological differentiation of gut. ($\times 35$.)

B. Cross-section of body wall under high magnification. Note relative thickness of epithelial and dermal components. ($\times 100$.)

C. Low magnification autoradiographs of longitudinal section cut through oral cavity and tentacular crown. Arrow heads indicate sites of maximal activity incorporation. ($\times 35$.)

D. High magnification autoradiograph of cross section illustrating extensive activity incorporation in integumentary epithelium (arrow heads). Note relative lack of blackening in dermis or peritoneum. ($\times 100$.)

E. High magnification autoradiograph of cross section illustrating incorporated activity in gut regions (g_1 , g_2 , g_3) and body wall epithelium. Arrow head in g_1 shows extensive blackening within villus-like extensions of luminal border. Arrow heads in g_2 point to considerable incorporated activity between villus-like projections. Gut region g_3 illustrates an apparent lack of activity incorporation. ($\times 50$.)

All sections stained with alizarine, toluidine blue and orange G. *g*, gut; *m*, longitudinal muscles; *cc*, coelomic cavity; *i*, integument; *o*, ossicular vesicles; *e*, epithelium; *d*, dermis; *p*, peritoneum; *t*, oral tentacles; *oc*, oral cavity.

