DISTRIBUTION AND TRANSPORT OF D-AMINO ACIDS IN MARINE INVERTEBRATES

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There is preliminary evidence that D-amino acids occur in the free amino acid pools of marine invertebrates (Preston, submitted for publication). About 30% of 30 species previously examined contained amino acids at concentrations ranging from .05 mM to 40 mM. We have also shown that in the coelomocytes of the bloodworm, Glycera dibranchiata, there is a transport system for D-alanine which is preferentially competitively inhibited by other D-amino acids. In addition, the D-alanine absorbed was ultimately metabolized into at least two other substances. It is not known whether D-amino acids are primarily synthesized within invertebrate tissues or are absorbed from the environment. A few measurements have been made on sea water which suggest that D-amino acids occur in sea water at roughly 10% of the concentration of the total dissolved free amino acids (Bada and Lee, Marine Chem., 5, 527-534; Lee and Bada, Limnol. and Oceanogr., 22, 502-510). Nothing is known about the physiological role of D-amino acids except the suggestion that they may be involved with osmoregulation (Matsushima, et al., Mar. Biol. Lett., 5, 217-225).

The objective of the present investigation was to survey a wide range of invertebrate species for the presence of D-amino acids to provide baseline data for further investigation of the absorption and physiological role of D-amino acids. In addition, transport experiments were done with the gills of mollusc Mya arenaria for comparison to the earlier observations made with Glycera.

Methods: Most of the organisms were collected in Salsbury Cove or in the mudflats near Thompson Island. The soft tissues were weighed and then extracted in 80 percent ethanol. Aliquots of the extracts were dried under an air stream and then resuspended in glass distilled water. D-amino acids were analyzed using a D-amino acid oxidase (DAO) assay technique. Typical assay conditions were: 2.4 ml tetrasodium pyrophosphate buffer (0.02M, pH 8.5) containing 0.007% o-dianisidine (w/v), 0.02 ml horseradish peroxidase (EC 1.11.1.7) solution (0.05 mg/ml), 0.05 ml D-amino acid oxidase (EC 1.4.3.3) solution (0.25 mg/ml), and 0.5 ml sample. The concentration of colored reaction product was measured by reading A_{436} in a spectrophotometer. Standard curves prepared using D-methionine were repeated every time new samples were analyzed. In most tissues D-amino acid concentration was calculated on the basis of total tissue water which was estimated by comparing fresh tissue wet weight with tissue dry weight after drying 24 h at 100°C. In a few cases where these data could not be obtained, it was assumed that the tissue water comprised 75% of tissue wet weight.

The transport of ^{14}C-D-alanine was measured by incubating sections of gill (typically 100 mg) from Mya arenaria in sea water (NaSW) or in artificial sea water in which the NaCl was replaced with choline chloride (CSW). The concentration of D-alanine was usually 10 µM. In some experiments 200 µM or 50 mM D or L-methionine and alanine was added to measure competitive inhibition. After incubation the tissue was thoroughly rinsed in ice-cold NaSW or CSW and extracted with 1.0 ml 0.1 N HCl. Ten ml of fluor was added and the amount of label was evaluated by liquid scintillation spectroscopy.

Results and Discussion: Twenty-five species were analyzed for the presence of D-amino acids. Table 1 shows the results. Twelve species (48%) were found to contain D-amino acids at apparent concentrations ranging from 0.08 mM to 3.3 mM. Thirteen other species apparently did not contain D-amino acids. There appeared to be no phyletic correlation with the occurrence of D-amino acids since different species from a single phylum typically appeared in both the positive and negative groups. The concentrations listed should be considered minimum values for several reasons: first, some D-amino acids (D-serine, D-lysine, D-aspartate) are relatively poor substrates for DAO. D-Methionine, the standard utilized for these measurements, was an excellent substrate for DAO and therefore provides conservative estimates of apparent tissue concentrations. Second, in most cases whole animals were extracted for these measurements. If only particular tissues contained D-amino acids the local concentrations would be higher. For example, in Glycera dibranchiata the red blood cells contain about 20 mM D-amino acid whereas the body wall apparently contains none. Third, in earlier experiments I observed that some tissues contain endogenous inhibitors which drastically reduce the sensitivity of the DAO assay. These data confirm that D-amino acids are widely distributed in marine invertebrates in at least 5 phyla.

The apparent concentrations are relatively low compared with the total free amino acid pool which in most marine invertebrates ranges from 100 mM to 300 mM. The suggestion that D-amino acids play a unique role in osmoregulation is not supported by these data since their contribution to the total osmotic pool is barely significant. However, as noted above, it is possible that the true intracellular D-amino acid concentrations are considerably higher. In the event that all factors favored decreased assay sensitivity, the D-amino acid concentration could be as much as 10 times higher than the values given in Table 1 and under these conditions it remains possible that D-amino acids play some role in osmoregulation. Accurate quantitation of D-amino acid pool size requires direct chemical analysis using chiral HPLC. This technique is presently being adapted for biological tissues in my laboratory.

The transport of ^{14}C-D-alanine at an external concentration of 10 μM by the gills of Mya arenaria proceeded linearly for at least 10 minutes (data not shown). Influx in Na free CSW was drastically reduced (85% inhibition) compared with the NaSW control (Table 2). To estimate the fraction of ^{14}C-D-alanine uptake due to diffusion, influx was measured in the presence 50 mM D-methionine in both NaSW and CSW (Table 2). In NaSW only 6% of the D-alanine influx remained (presumably nonmediated) in the presence of 50 mM D-methionine. In CSW the largest fraction of the flux appeared to be mediated since addition of 50 mM D-methionine reduces this flux by 84%. In NaSW the influx of ^{14}C-D-alanine (at 10 μM) was more strongly inhibited by L-methionine and L-alanine (at 200 μM) than by equivalent concentrations of the D analogues (Table 3). This suggests that in contrast to D-alanine transport by Glycera coelomocytes which occurs via a system which preferentially transports D-amino acids, the transport of D-alanine by Mya gill appears to be by an L-amino acid transport system with relatively broad stereospecificity.

In conclusion these data confirm that D-amino acids occur in diverse marine invertebrate species. In this survey about 50% of the species tested showed detectable levels of D-amino acids. Including previous data, a total of 55 species have been tested, 40% of which contain D-amino acids. The presence of Na dependent D-alanine transport indicates (at least in Mya gill) that some of the D-amino acids may be obtained from the environment. Additional studies are being conducted to examine the metabolism and cellular role of D-amino acids.

TABLE 1: OCCURRENCE OF D-AMINO ACIDS BY PHYLUM

Species With D-AA	(n=3) Conc. mM ± S.E.	Species Lacking D-AA
Cnidaria		Cnidaria
Metridium senile	0.096 ± 0.020	*Aurelia aurita*
		Cyanea capillata
Rhyncocoela		
Cerebratulus lacteus	0.187 ± 0.031	
Lineus ruber	3.19 ± 0.007	
Mollusca		Mollusca
Crepidula fornicata	0.232 ± 0.005	*Illex illecebrosus*
Mya arenaria	1.53 ± 0.046	*Littorina littorea*
		Modiolus modiolus
		Mytilus edulis
		Onchidoris muricata
		Placopecten magellanicus
		Thais lapillus
Annelida		Annelida
Amphitrite johnstoni	0.433 ± 0.045	*Nephthys* sp.
Clymenella torquata	3.29 ± 0.170	
Harmathoe sp.	0.573 ± 0.033	
Nereis virens	3.21 ± 0.54	
Nereis sp. (prob. *pelagica*)	0.457 ± 0.018	
		Arthropoda
		Balanus balanoides
Echinodermata		Echinodermata
Cucumaria frondosa	0.413 ± 0.052	*Asterias vulgaris*
Strongylocentrotus droebachiensis	0.086 ± 0.0009	
		Chordata
		Boltenia ovifera

TABLE 2: D-ALANINE UPTAKE BY MYA GILL IN NaSW AND CSW

Condition	Influx, µMol/kg tissue h	J_{exp}/J_{na}	p
NaSW	410 ± 88	--	--
CSW	63.4 ± 7.1	0.15	p<0.025
NaSW + 50 mM D-Meth	6.89 ± 0.35	0.06	p<0.025
CSW + 50 mM D-Meth	2.57 ± 0.26	0.004	p<0.025

(Flux period, 8 min; ^{14}C-D-alanine conc. = 10 µM; J_{exp}/J_{na} = Exp influx/ influx in NaSW; p values, t-test, exp. values compared with control, n=3 each condition)

TABLE 3: INHIBITION OF D-ALANINE INFLUX IN MYA GILL

Inhibitor	Influx, µM/kg tissue h	J_i/J_o	p
Control	359 ± 6	--	--
D-Methionine	262 ± 20	0.73	p<0.01
L-Methionine	127 ± 5	0.35	p<0.001
D-Alanine	275 ± 30	0.76	p<0.05
L-Alanine	170 ± 31	0.47	p<0.005

(Flux period, 5 min; inhibitor conc. = 200 µM; ^{14}C-D-alanine conc. = 10 µM; J_i/J_o= influx in presence of inhib/control flux; p values, t-test, exp. values compared with control, n=3 each condition; D-alanine inhibition calculated as if isotope conc. = 10 µM)

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