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Phosphodiester amine, taurine and derivatives, and other osmolytes in Vesicomyid bivalves: correlations with depth and symbiont metabolism

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Introduction

Marine invertebrates use organic solutes such as free amino acids and methylamines to maintain cell volume against the osmotic pressure of sea water. Unlike salts, these "compatible osmolytes" can be present within the cell at high levels without disrupting macromolecules. Importantly, some osmolytes also have non-osmotic roles as protein stabilizers, counteracting perturbing effects of urea, salt, and temperature (Yancey, 2001). The most common osmolytes in shallow marine invertebrates are usually taurine (a sulphur-based amino acid), betaine (a trimethlyamine), and glycine. However, in deep-sea animals different osmolytes have been found, some of which have been proposed to be adaptations to hydrostatic pressure (Yancey, 2001). The compound trimethylamine oxide (TMAO) is an osmolyte found in increasing concentrations in parallel with depth in bony fishes and some invertebrates (Kelly & Yancey, 1999); TMAO has been shown to stabilize proteins against perturbation by hydrostatic pressure (Yancey et al., 2001). Other recent studies have shown that bivalve molluscs and vestimentiferans from hydrothermal vents and cold seeps have high levels of unusual compounds such as hypotaurine, thiotaurine, N-methyltaurine and sarcosine. It has been hypothesized that these osmolytes have non-osmotic roles as adaptations either to depth (e.g., pressure), or to the sulphide-based metabolism that microbial symbionts in these animals use (Pruski et al., 2000a, 2000b; Yin et al., 2000). The goal of the present study was to test these hypotheses in more detail.

Vesicomyid clams, common invertebrates at cold seeps, were chosen as a unique system for testing non-osmotic roles of osmolytes, for two reasons. First, these animals rely on sulphide metabolism with prokaryotic symbionts, and have osmotically significant levels of hypotaurine and thiotaurine in gill tissue, the site of symbiont metabolism (Pruski et al., 2000b). The presence of thiotaurine (a product of hypotaurine and HS) has been proposed to be an indicator of H_2S metabolism, since it is present in clams and mussels with sulphide-using symbionts and absent in mussels with methylotrophic symbionts (Pruski et al., 2000b). Second, species in this family are found over a wide range of depths, so that any depth trends in osmolytes might be related to pressure rather than metabolism. In fact, unusual compounds have been found at high levels in the deep vesicomyid clam *Calyptogena phaseoliformi*s Métivier et al. 1986, from 5900 m depth (Japan Trench). In particular a unique, partially identified phosphodiester amine compound (with serine, phosphate, ethanolamine, and another unidentified moiety) was reported at very high concentrations, and proposed to be related to symbiont metabolism (Alberic & Boulègue, 1990). However, it could be involved in depth adaptation. By analysing the osmolytes of this very deep species and other vesicomyid clams at a variety of depths, we hoped to determine whether this and other unusual osmolytes are adaptations to depth and/or to symbiont-related metabolism.

Material and methods

Adult vesicomyid bivalves (*Calyptogena/Vesicomya* spp) used for the analyses were obtained from a variety of depths. *Calyptogena pacifica* Dall, 1891, were taken from a depth of 510 m at the Eel River seeps off Northern California, by a remotely operated vehicle (ROV). Specimens of an undescribed *Calyptogena* species were obtained from the Okinawa Trough at 1050 m by ROV. *Calyptogena soyoae* Okutani and Egawa, 1985, were taken at 1150 m from Sagami Bay by ROV. *Vesicomya gigas* Dall, 1896, were collected from the Oregon Margin methane seeps using an otter trawl at 2000 m, and *Calyptogena phaseoliformis* were collected from 4400 m and 6400 m

from the Alaska and the Japan Trenches, respectively, by ROV. Non-vesicomyid intertidal clams (*Saxidomus giganteus* Conrad, 1837; Veneridae) were obtained live from a Seattle fish market.

All clams were transported in dry ice and stored in freezers at -70º C. Frozen tissue samples from bivalve gill and mantle were homogenized either with 1M perchloric acid and processed according to Wolff et al. (1989), or with 70% ethanol and processed according to Pruski et al. (2000a). Ethanol extraction was used to analyse thiotaurine, which converts to hypotaurine and H_2S in acid (Pruski et al., 2000a). Final samples were analysed by an ion-exchange HPLC (Wolff et al., 1989). Aliquots of unidentified solutes were collected from the HPLC and tested for quaternary amines (fully substituted methylamines) using the Dragendorff procedure (Stumpf, 1984), and for amine groups using a standard ninhydrin procedure. TMAO was analysed as previously reported (Kelly & Yancey, 1999). To calculate dry weights, tissues were weighed, dried for 72 hr at 60˚C, then re-weighed.

Results

In general, the shallowest vesicomyids were dominated by the common osmolytes taurine, betaine and glycine, differing little from the non-vesicomyid intertidal clam. But the osmolyte compositions in the deepest species were considerably different (Table 1). The deepest species (the two *C. phaseoliformis* groups) contained high amounts of the serine phosphodiester amine first identified by Alberic & Boulègue (1990), and also had small amounts of myo-inositol. In addition, two unidentified solutes were discovered: a ninhydrin-positive compound in the Oregon clams, and a quaternary amine (Dragendorff positive) compound in both *C. phaseoliformis* groups. No species contained TMAO.

The tissues varied considerably in their total osmolyte contents, presumably due to differing amounts of non-cellular and intracellular spaces. Since organic osmolytes are largely found intracellularly, comparisons of actual contents (Table 1) among species can be misleading. Therefore, we analysed each osmolyte as a percentage of the total osmolyte pool (Figs. 1 and 2).

Table 1. Contents of major organic osmolytes in mantle tissues of a venerid and several vesicomyid bivalves. Values are in mmol kg^{-1} dry wt. with standard deviations. Not all osmolytes are shown. Full species names are in Materials and methods; *gig.* = *giganteus*; *pac.* = *pacifica*; *soy.* = *soyoae*; *phaseo* = *phaseoliformis*. N.D. = not detected.

Species, depth	Hypo-taurine	Taurine	Betaine	Glycine	Phospho-diester
S. gig. 0 m (n=4)	N.D.	262 + 7.7	268 + 8.1	146 + 4.3	N.D.
C. pac. 510 m (n=5)	65.6 ± 11.8	174.9 ± 19.9	161 ± 16.9	211 ± 8.9	N.D.
C. spp 1050 m (n=2)	20.7 ± 9.2	201 ± 38.8	242 ± 47	523 ± 15.6	N.D.
C. soy. 1150 m (n=3)	61.8 ± 2.5	198 ± 10	254 ± 17	603 ± 82	N.D.
V. gigas 2000 m (n=2)	19.6 ± 6.8	105 ± 4.2	204 ± 13.9	488 ± 37.4	21.1 1.2
C. phaseo. 4400 m (n=5)	77 ± 11	23.8 ± 7.0	313 ± 96	114 ± 21	318 ± 41
C. phaseo. 6400 m (n=2)	32.6 ± 0.1	8.0 ± 1.8	60.8 ± 14.7	31.4 ± 9.7	498 ± 34

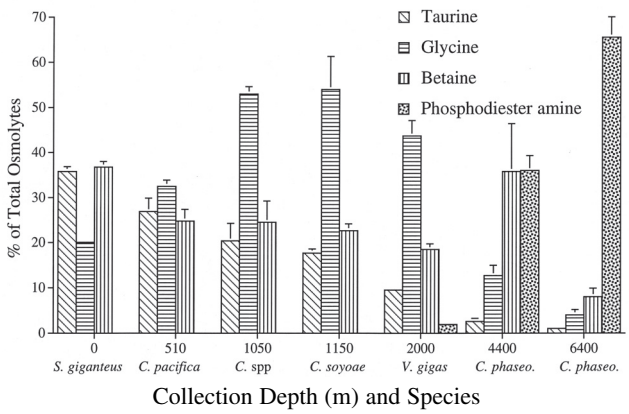

Figure 1. Contents of major organic osmolytes in mantle tissues of vesicomyid bivalves collected from 510 to 6400 m, and in an intertidal venerid bivalve. Values are expressed as percentage of total osmolyte pool, which includes some osmolytes (mostly minor) not shown in Table 1. For complete species names, see Materials and methods (*phaseo* = *phaseoliformis*). Error bars represent standard deviations.

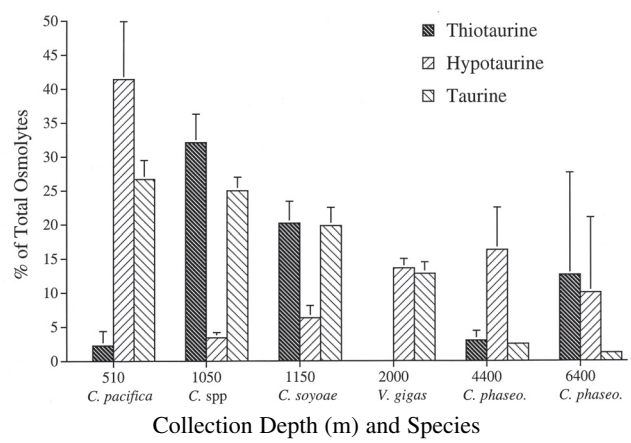

Figure 2. Contents of sulphur-containing osmolytes in gill tissues of vesicomyid bivalves collected from 510 to 6400 m, showing the lack of depth trends. Values are expressed as percentage of total osmolyte pool, which includes some minor osmolytes not shown in Table 1. For complete species names, see Materials and methods (*phaseo* = *phaseoliformis*). Error bars represent standard deviations.

Mantle Tissue. Mantle tissues revealed highly significant depth trends in some osmolytes analysed by percentage (Fig. 1). Contents of taurine, the major osmolyte in shallow bivalves, decreased exponentially with depth from 39% of the total osmolyte pool in intertidal species (0 m) to 1% at 6400 m (exponential curve fit with $p < 0.001$). Glycine increased linearly from 22% at 0 m to approximately 53% at 1050 m, then decreased exponentially from 1050 m to 4% at 6400 m. The unidentified amine made up about 25% of the osmolyte pool in the Oregon clams (not shown in Table 1 or Fig. 1). The phosphodiester amine increased linearly from 2% in the 2000-m species to approximately 65% in the 6400-m species (linear curve fit with $p < 0.01$). Trimethylamines (quaternary amines) were major osmolytes in all species, with betaine high in all but the deepest (6400 m) species (Fig. 1). The deepest clams also had high amounts of the unidentified quaternary amine (15.6% of total; not shown in Fig. 1). Mantle tissues of vesicoymids (but not of the intertidal clam) also contained hypotaurine, but no thiotaurine (Table 1).

Gill Tissue. Gills had osmolyte compositions (Table 2) broadly similar to those of mantle tissue, including the unidentified amine in the Oregon clams, the unidentified quaternary amine in the deepest clams, and a linear increase in the phosphodiester amine in the deepest clams. Figure 2 shows the sulphur-based osmolytes as percentages of total osmolyte pool. The main differences in the vesicomyid gills compared to mantles were: 1) along with lower contents of betaine, taurine and glycine, hypotaurine was a larger osmotic component in many vesicomyid gills, and 2) thiotaurine was present, sometimes at high levels, in most vesicomyid gills. In the two clams from 6400 m, the thiotaurine and hypotaurine contents were very different (Table 2), although the sums of the two solutes were fairly close. The notable exception to the vesicomyid patterns was the gill tissue of the Oregon (2000 m) species, which had no detectable thiotaurine (< 0.05 mM).

Table 2. Contents of major organic osmolytes in gill tissues of a venerid and several vesicomyid bivalves. Values are in mmol kg-1 dry wt. with standard deviations. Not all osmolytes are shown. Full species names are in Materials and methods; *pac.* = *pacifica*; *soy*. = *soyoae*; *phaseo* = *phaseoliformis*. N.D. = not detected; n values are the same as in Table 1.

Species, depth	Thio-taurine	Hypo-taurine	Taurine	Betaine	Glycine	Phospho-diester
C. pac. 510 m	20.1 ± 18.8	369 ± 76	238 ± 25	137 ± 29	127 ± 24	N.D.
C. spp 1050 m	252 ± 33	26.5 ± 6.1	196 ± 16	123 ± 15	163 ± 11	N.D.
C. soy. 1150 m	188 ± 30	58.7 ± 16.5	184 ± 25	135 ± 23	363 ± 30	N.D.
V. gigas 2000 m	N.D.	96.0 ± 10.0	91.0 ± 12.3	98.1 ± 14.3	274 ± 30	16.1 ± 0.5
C. phaseo. 4400 m	22 ± 20	119 ± 45	18.5 ± 2.8	226 ± 38	94.9 ± 14.1	246 ± 31
C. phaseo. 6400 m	18.5, 176*	146, 40*	11.1 ± 0.7	84.6 ± 19.9	19.4 ± 2.8	516 ± 48

*values for 2 individuals are shown separately due to very large differences

Discussion

The depth trends in two unidentified osmolytes in these seep bivalves suggest that these solutes have pressure-related properties. Levels of taurine, a major osmolyte in shallow bivalves, declined exponentially with depth, a trend previously reported for species down to about 3000 m (Pruski et al, 2000a). Glycine contents also declined below 1150 m, a trend not previously reported. Taurine and glycine were in effect replaced with the unusual phosphodiester amine (and to a lesser extent with the unidentified quaternary amine). The phosphodiester amine was by far the dominant compound at 6400 m, consisting of about 59-65% of the total osmolyte pool in gill and mantle. Since the structure of this compound has not yet been solved, we are unable to relate it to other metabolites in other animals, and its function remains unknown. It is possible that it is an abnormal product of depressurization during collection. However, it seems unlikely that such high concentrations would result from this process, and it would not explain why all the other common osmolytes were at such low levels in the deepest clams. The high concentrations of the phosphodiester amine and its depth trend suggest that this solute, like TMAO, might be used to stabilize proteins at high hydrostatic pressures.

In contrast, the sulphur based osmolytes hypotaurine and thiotaurine (but not taurine) showed patterns more likely related to metabolism and seep chemistry rather than depth. The presence of thiotaurine in symbiont-bearing gills but not mantles, and its occurrence in patterns unrelated to depth (Fig. 2), support other studies that propose this solute to be an indicator of sulphide exposure and/or usage (Pruski et al., 2000b). The values also varied considerably among individuals (see Table 1), consistent with local variations in seep chemistry. Furthermore, the absence of thiotaurine in the Oregon (2000 m) gills suggests that these vesicomyids do not rely on sulphide, but perhaps on methylotrophic symbionts. This clam also had large amounts of the unidentified amine, also suggesting a metabolism different from the other vesicomyids. Support for methylotrophy comes from one report that methane but not sulphide was present in the pore water around clams located near our dredge site (Kulm et al., 1986). Also, the δC^{13} ratio of one vesicomyid specimen from near our dredge site was about -52 per mil, considerably lower than the usual -35 per mil typical of other vesicomyids. This also suggests methane rather than sulphide as an energy source (Kulm et al., 1986). However, other seep animals with sulphide-based symbionts have been reported with low thiotaurine levels (Pruski et al., 2000a). Those animals were living in habitats with low sulphide levels, so it is possible that our Oregon clams do use sulphide-based symbionts but were at an inactive seep site.

In conclusion, this study indicates that vesicomyid bivalves use organic osmolytes for at least two non-osmotic purposes. Some osmolytes could play a crucial role in sulphur metabolism while others may be used as pressure adaptations.

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