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EFFECTS OF SALINITY AND STARVATION ON RELEASE OF DISSOLVED FREE AMINO ACIDS BY *DUGESIA DOROTOCEPHALA* AND *BDELLOURA CANDIDA* [PLATYHELMINTHES, TURBELLARIA] [1]

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The tissues of marine invertebrates contain very high levels of free amino acids (FAA) which are believed to serve an intracellular osmoregulatory function (*e.g.*, Florkin and Schoeffeniels, 1965). Potts (1967) suggested that as a consequence of these high FAA levels and the tendency of these compounds to leak across the body wall into the water, marine invertebrates probably lose more FAA to the water than do comparable freshwater invertebrates. Consistent with this hypothesis was our observation that both tissue FAA levels and FAA release rates of the marine turbellarian, *Bdelloura candida* increased with increasing salinity (Johannes, Coward and Webb, 1969).

Relative *respiratory* costs of coping with marine *versus* freshwater environments have been calculated (Potts, 1954; Potts and Parry, 1964). If differences in FAA release rates between marine and freshwater invertebrates exist as a consequence of differences in tissue FAA levels, this implies another type of differential energy loss related to osmoregulation—one in which studies of the respiratory energy costs of osmoregulation do not take into account. Here we compare FAA release rates of *Bdelloura* and its freshwater relative (*Dugesia dorotocephala*), on an energetic basis.

In addition, we use our data to demonstrate how some differences of opinion in the literature concerning FAA release rates by marine invertebrates can perhaps be reconciled if the relationships between release rates and the rate of feeding and time since feeding are taken into consideration.

Methods and Materials

The freshwater planarian *Dugesia dorotocephala* used in these experiments were from a permanently sexual strain originally collected in Oklahoma and maintained in the laboratory on a diet of chicken liver. Specimens of *Dugesia* were kept in a dilute artificial saline medium (Coward, 1968) for both maintenance and experimental situations. All procedures, including aseptic conditions, involving *Dugesia dorotocephala* and *Bdelloura candida* were as previously described (Johannes, Coward and Webb, 1969), with appropriate use of the dilute saline (0.35‰) for *Dugesia* rather than seawater: FAA samples of 2.5 hour duration were taken at precise 24 hour intervals with time zero being the cessation of feeding; the time zero incubation sample contained some presumably egested material and mucus (which were removed by the routine Millipore filtration step) but the subsequent samples did not. FAA samples were analyzed as previously described (Johannes,

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Coward and Webb, 1969). The bound amino acids from the 80% ethanol insoluble residues of chicken liver were determined after 20 hours of 6 N HCl hydrolysis at 100° C.

Values for caloric content of most amino acids ($-\Delta H^0_c$, K cal/mole) were obtained from Hutchens (1970). The value for taurine (382.9) was obtained from Kharasch (1929). The values for proline (649) and histidine (747) were determined by bomb calorimetry and the values for ornithine (722) and lysine (879) were calculated by the method of Kharasch (1929).

Respiration of *Bdelloura* was measured in a 25 ml respirometer equipped with Ag-Pt electrodes and a paddle type stirrer operating at about 120 rpm. The continuously recorded signals, representing the oxygen concentrations, were linear during the one hour determination at 25° C. Calibration was by Winkler oxygen determination.

Results

Respiration, FAA release rates and FAA in tissues of *Dugesia* after periods of starvation are presented in Table I. Respiration rates dropped 50% over a five-day period, whereas FAA release rates dropped by almost three orders of magnitude.

Comparison of caloric equivalence of released FAA and of respiration for *Dugesia* and *Bdelloura* is presented in Table II. Caloric cost of FAA loss is positively correlated with salinity for *Bdelloura* and was higher at all salinities than the caloric cost of FAA loss in the freshwater *Dugesia*. Weight specific respiration rates were similar for the two species.

The major constituent FAA of both the tissues and release products of *Bdelloura*, and their caloric values are reported in Table III in order of increasing caloric content of the amino acid. A number of FAA individually $\leq 1\%$ of the total have been omitted from both Tables III and IV. The relatively calorically poor amino acid glycine, makes up a much greater proportion of the released FAA than of the tissue FAA. This difference in proportions of FAA between the tissues and release products reinforces our confidence that the animals are not being damaged during handling with a concomitant non-specific release of FAA (Corner and Cowey, 1968).

Table I

Effect of starvation on caloric equivalence of respiration and free amino acid (FAA) release by Dugesia. *Dashes indicate values not determined*

Hours starved	Respiration* (cal $\times 10^{-3}$/g wet wt/hr)	FAA released (cal $\times 10^{-3}$/g wet wt/hr)	FAA loss caloric (equivalent as % respiration)	Tissue FAA (μM/g wet wt)
0	2250	967.0	43.00	28.3
24	1600	9.3	0.58	13.5
48	1400	3.79	0.27	—
72	1350	3.05	0.23	—
96	—	—	—	9.2
120	1250	1.52	0.12	—

* calculated from Hyman (1919).

TABLE II

Caloric equivalence of respiration and free amino acid (FAA) released by Dugesia *and* Bdelloura *after 24 hours starvation*

	Respiration* (cal $\times 10^{-3}$/g wet wt/hr)	FAA released (cal $\times 10^{-3}$/g wet wt/hr)	FAA loss caloric (equivalent as % respiration)	Tissue FAA (μM/g wet wt)
Dugesia				
0.35‰ salinity	1600*	9.3	0.58	13.5
Bdelloura				
12‰ salinity	1620	12.5	0.77	4.2
19‰ salinity	2350**	18.1	0.77	11.6
26‰ salinity	1610	24.3	1.51	11.6
33‰ salinity	1710	23.0	1.35	32.0

* Calculated from Hyman (1919).
** Animals were extremely active.

The *Bdelloura* tissue FAA and FAA release values are reported as means (Table III) of determinations made at four salinities (Johannes, Coward and Webb, 1969); mole percentages of the various FAA were essentially the same at different salinities (12, 19, 26 and 33‰), unlike those for some other marine invertebrates that have been examined in this connection (*e.g.*, Virkar and Webb, 1970).

Relative proportions of FAA in *Dugesia* tissue and release products after starvation are reported in Table IV. The FAA and bound amino acid content of chicken liver upon which both *Dugesia* and *Bdelloura* were fed are included in Table III. Immediately after feeding, the relative proportions of most of the FAA in the release products (Table IV) and the food (Table III) are similar for *Dugesia*. Proportions in the tissues change with starvation, but do not approximate those of the release products.

TABLE III

Comparison of mole per cent of free amino acids (FAA) in food, tissues and release products of Bdelloura. *Animals starved 24 hours; ± one standard deviation in parenthesis; constituents ≤ 1% omitted*

	$-\Delta H°c$ (Kcal./mole)	Food		*Bdelloura*	
		FAA	Bound amino acid*	Tissue	Released
Glycine	230.5	8.7	8.3	1.7 (0.33)	52 (11.0)
Serine	347.7	7.9	5.1	1.9 (0.24)	7 (2.0)
Taurine	383	11.5	0	0.91 (0.18)	0
Aspartic acid	382.6	7.1	8.4	4.6 (2.0)	3.1 (0.78)
Alanine	387.1	8.8	7.0	18.1 (3.3)	6.7 (1.2)
Threonine	409.7	5.4	4.5	2.8 (1.2)	2.7 (0.37)
Glutaminc acid	536.4	13.3	9.3	4.0 (1.1)	3.4 (0.79)
Ornithine	722	0.4	6.9	0.1	2.8 (1.2)
Arginine	893.5	2.6	3.9	7.4 (1.3)	5.1 (1.2)

* Bound FAA are 6 N HCl hydrolysates of 80% ethanol insouble material.

TABLE IV

Changes in relative molar per cent of free amino acids in Dugesia *tissues and release products with starvation. Constituents ≤ 1% omitted*

	Tissue amino acids			Released amino acids				
	Days starved							
	0	1	4	0	1	2	3	5
Glycine	2.8	1.8	2.4	8.8	15	9.9	12.0	20.8
Serine	5.2	5.1	6.5	2.9	23	20.4	17.8	29.5
Taurine	3.7	5.6	1.3	4.5	16	11.9	13.5	0
Aspartic acid	4.5	9.6	12.9	4.6	6.7	7.1	10.4	11.4
Alanine	9.6	7.3	13.8	11.4	10.9	7.9	trace	trace
Threonine	4.1	2.7	4.1	6.6	5.9	4.5	8.0	0
Glutamic acid	9.2	11.5	15.1	13.2	8.6	10.8	11.0	23.5
Ornithine	1.1	1.7	2.1	0.75	2.7	9.6	8.3	14.8
Arginine	2.3	6.1	5.6	4.0	7.0	trace	0	0

DISCUSSION

Corner, Cowey and Marshall (1965) have shown that ninhydrin positive nitrogen-containing compounds "excreted" by *Calanus* after feeding on *Cricosphaera* declines by nearly an order of magnitude during the first day after feeding. The rate of "excretion" was also related to the level of food input. In the present work, the FAA release rates of *Dugesia* (Table I) dropped by two orders of magnitude during the first day of starvation and by almost another order of magnitude during the next four days. Thus, it appears that the rate of feeding and the elapsed time since eating greatly influence rates of "excretion." These considerations, as well as others (Johannes and Webb, 1970), render the prediction of release rates of compounds by small animals in natural environments difficult. Their recognition, however, may help reconcile some of the controversies in the literature concerning this subject. Corner and Newell (1967) stated that they could not find any evidence for the release of FAA by *Calanus helgolandicus* in either starved or "feeding" animals. Obviously, an animal must have at least as much nitrogen available in the food as it is releasing, otherwise it will become nitrogen starved. The animals used by Corner and Newell either were starved for three or more days after capture or fed at a level which supplied only 23% of their measured nitrogen release. Consequently, their results do not approximate release rates under natural situations.

In Corner and Newell's (1967) criticism of our work (Johannes and Webb, 1965) they correctly report that using our equation, the predicted rate of release of FAA for *C. helgolandicus* at 10° C would be 4 μg alpha-amino N/mg dry body weight/day, or approximately the rate they observed for total nitrogen release from starving animals. Our equation was derived from values obtained on newly captured and presumably unstarved animals. It would thus be more appropriate to compare our predicted value of 4 μg with values obtained for total nitrogen release of actively feeding animals, which are probably within the range of 10–20 μg (Corner, Cowey and Marshall, 1965).

Little and Gupta (1969) imply that the peak FAA release in turbellarians should occur at 24 hours after feeding on the basis that the blind guts would be voided of food remnants approximately 24 hours after feeding (Jennings, 1957, page 67). The suggestion has been made by Little and Gupta (1969) that the release data for *Bdelloura* previously published (Johannes, Coward and Webb, 1969), and in part included here, was misinterpreted because the measurements were made after 24 hours of starvation and thus presumably during egestion. Virtually all visible egesta were voided during the first few hours of starvation by our experimental animals. In addition, maximum release rates of FAA appear to occur at the end of the feeding period in *Dugesia* (Table I) and decline progressively with time of starvation.

The order of magnitude drop of FAA release by *Dugesia* between day 0 and day 1 (Table I) obviously has major ecological implications as well as a direct bearing on the design of experiments to measure release products. It may also bear a relationship to the mechanism(s) involved in the release. Odum (1961) has suggested that the release of ingested radioisotope occurs in two phases, the first consisting of a rapid loss of non-assimilated material, and the second, a slower loss of assimilated or waste material, the loss rate being related to food consumption as well as temperature, growth, and reproduction. In analogous fashion, if the FAA released immediately after feeding are primarily non-assimilated material, the relative proportions of FAA released should be similar to those of the food, as modified by selective absorption. Such appears to be the case, since after taking into account the ratio of 18:1 for bound: FAA in the food, two of the three amino acids (glycine and glutamic acid) most frequent in the release products are also found to be most frequent in the food.

A priori, it seems reasonable that the source of released FAA after 5 days of starvation would be the tissue derived FAA. The data of Table IV indicates that such a loss must be quite selective. Alanine, a major tissue FAA, is a minor component of the release products after five days of starvation. However, it is more predominant in the release products after feeding than it is in the food.

Our present data (Table II) plus unpublished data from these laboratories on FAA release by other marine and freshwater invertebrates supports the hypothesis of Potts (p. 33, 1967) that "Marine invertebrates might be expected to lose more amino acids than freshwater ones." Regression analysis of the salinity—FAA release data (Table II) gives a correlation coefficient (r) of 0.95 for the 5 salinities.

Unlike the escape of unassimilated FAA from the gut, loss of FAA by excretion and leakage represents a true loss of metabolic energy by the animal. It appears that this loss is greater in the marine turbellarian *Bdelloura* than in its freshwater relative *Dugesia* and increases with increasing salinity (Table II). This extra energetic cost associated with living in a marine environment is on the order of 1% of the energy loss associated with respiration in *Bdelloura,* and is of the same order as the extra respiratory energy calculated to be expended by freshwater invertebrates in coping with their hypoosmotic environment (Potts, 1954). Thus it appears that the extra respiratory cost of osmoregulation in the hypoosmotic freshwater medium may be more or less balanced energetically by the extra cost, via FAA leakage, of maintaining the high intracellular FAA levels associated with invertebrates living in seawater. Clearly more work must be done before the

relative costs of coping osmotically can be accurately known for seawater *versus* freshwater environments. There may be additional factors complicating this relationship, for example, Sharma (1968) reports that energy loss via urea production by *Orconectes rusticus* increases proportionally to the ambient salinity.

The ubiquitousness of glycine and serine as the major components of samples analyzed for FAA seems worthy of discussion. They are very abundant in human sweat (Oro' and Skewes, 1965, Hamilton, 1965), human urine (Hamilton, 1970), release products of zooplankton (Webb and Johannes, 1967), release products of other aquatic invertebrates (Johannes and Webb, 1970) and in the free form in fresh water, estuaries and seawater (Brehm, 1967; Hobbie, Crawford and Webb, 1968; Bohling, 1970). Glycine is the most abundant amino acid in so-called prebiotic experiments (Fox and Windsor, 1970) and from extraterrestrial sources (Kvenvolden, Lawless, Pering, Peterson, Flores, Ponnamperuma, Kaplan and Moore, 1970). This, perhaps, all seems reasonable because glycine is structurally the simplest amino acid and contains the least amount of covalently bound energy. In the present data glycine and serine make up from 12–60% of the total released FAA (Table III), while they account for only 4–10% of the tissue FAA and only about 14% of the total amino acid in the food used for *Dugesia* and *Bdelloura*. Thus it appears that many species have a relatively low ability to retain these two small molecules of relatively low caloric content. In general, glycine and serine are directly biologically interchangeable; many metabolic pathways terminate in glycine and both glycine and serine are commonly precursors for many syntheses. This seems very reasonable if present biotic systems evolved from abiotic environments with relatively high glycine concentrations and if small molecules served as precursors to an array of larger molecular species. Since glycine is structurally small, calorically poor, and metabolically ubiquitous, it is not surprising that it is both highly available and highly expendable with minimal possible adverse affects on the animal.

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Summary

1. Immediately after feeding, *Dugesia* loses almost half as many calories in FAA release as via respiration. After one day, calorie loss via FAA release drops to less than 0.6% of that lost through respiration.

2. Loss of FAA from 24 hr starved animals of *Dugesia* and *Bdelloura* is highly correlated with environmental salinity ($r = 0.95$).

3. FAA loss appears to occur in two phases, the first consisting of a rapid loss of non-assimilated material, and the second a lesser and gradual loss of (presumably) assimilated material.

4. Glycine or serine, the two lowest energy content amino acids, are predominant among the FAA released by *Bdelloura* and *Dugesia,* respectively.

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