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Levels of intracellular free amino acids used for salinity tolerance by oysters (*Crassostrea virginica*) are altered by protozoan (*Perkinsus marinus*) parasitism

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Abstract Free amino acid (FAA) levels were measured from May through October 1991 in gill tissues of two groups of juvenile oysters (*Crassostrea virginica* Gmelin), one transferred from a low salinity field site (8‰) to a field site of high salinity (20‰) and high *Perkinsus marinus* (Mackin, Owen, and Collier) prevalence, the other kept at the low salinity field site. Within 24 h, glycine levels in the oysters transferred to high salinity increased 8-fold, taurine concentrations doubled and the total FAA pool rose from 150 μmol g^{-1} dry wt to 400 μmol g^{-1} dry wt. Taurine levels reached a plateau within 20 d after transfer to high salinity and remained at that level until *P. marinus* infections were detected 85 d after transfer. Taurine and glycine levels declined by ~40% in the high salinity population as infection intensity increased between 70 and 105 d. Total FAA declined by approximately 33% over this period. The oysters kept at low salinity were not infected and continued to grow while the infected high salinity oysters showed no increase in shell length after Day 85. FAA levels in the low salinity group remained relatively constant throughout the experiment except for an initial rise triggered by an increase in ambient salinity from 8 to 12‰. The results suggest that salinity tolerance mechanisms in *C. virginica* may be impaired by *P. marinus* infection.

Introduction

The Eastern oyster, *Crassostrea virginica*, is a euryhaline species capable of acclimating to wide changes in ambient salinity. Cell volume is controlled by regulating a large, intracellular free amino acid (FAA) pool and the quaternary ammonium compound, glycine betaine, to offset changes in extracellular osmotic pressure, i.e., this oyster is an osmoconformer (Pierce et al. 1992). Although the time course of amino acid accumulation has not been measured in oysters exposed to increased salinity, in other bivalves alanine rapidly accumulates and reaches high levels immediately after a hyperosmotic stress. As acclimation proceeds, the glycine concentration rises and within a few days replaces alanine as the major osmotic effector. During the next several days to weeks at high salinity, proline typically appears as a transient peak, beginning to rise slowly after the alanine accumulation peaks and declining as taurine accumulates. Taurine usually becomes the major osmotic effector, often comprising as much as 70% of the FAA pool (Baginski and Pierce 1977). The ability to regulate intracellular amino acids in this way allows *C. virginica* to inhabit estuaries such as Chesapeake Bay. Far less is known about the mechanisms of glycine betaine regulation in response to salinity change, although this usually ignored osmolyte often makes up a substantial portion of the osmolyte pool (e.g. in the horseshoe crab, *Limulus polyphemus*; Dragolovich and Pierce 1993).

Over the last decade a protozoan parasite, *Perkinsus marinus*, has become enzootic in oyster populations in Chesapeake Bay (Andrews 1988; Burreson and Andrews 1988). Infection initially causes reductions in oyster growth and "condition", and eventually results in death (Andrews 1961; Burreson 1991; Paynter and Burreson 1991). Disease prevalence, intensity and mortalities in Chesapeake Bay oysters are higher at higher salinities. While the physiological effects of protozoan parasitism have been addressed by a few studies (Newell 1985; Barber et al. 1988a, b; Ford and Figueras 1988; Newell and Barber 1988), none have examined salinity tolerance. Heavily infected oysters appear wasted, watery and translucent, and the ratio of whole wet tissue weight to dry tissue weight is increased (Paynter and Burreson 1991). In addition, oysters from various locations within the Chesapeake Bay had smaller intracellular FAA pools, almost no glycine betaine, and reduced salinity tolerances compared with conspecific oysters from several locations

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along the Atlantic coast from Georgia to Cape Cod (Pierce et al. 1992). It is likely that all the Chesapeake Bay oysters in that study were parasitized with *P. marinus*. These observations, together with the observed reductions of morbidity and mortality amongst parasitized oysters in lower salinities (Andrews 1988; Burreson and Andrews 1988; Paynter and Burreson 1991), suggest that *P. marinus* infection may have an impact on the salinity tolerance mechanisms of the oyster, a hypothesis suggested many years ago (Soniat and Koenig 1982) but never tested in detail. In order to understand these observations, we examined the effects of both *P. marinus* parasitism and environmental salinity on intracellular FAA concentrations of oyster tissues during a cycle of infection in the field.

Materials and methods

Oysters and field sites

Oyster spat (*Crassostrea virginica*, Gmelin) were produced from oysters collected in Chesapeake Bay in June 1990 at the University of Maryland Horn Point hatchery and reared in an upweller system located on the Wye River, Maryland, USA at a low mean salinity (~10‰) until the beginning of the experiment. The spat showed no evidence of *Perkinsus marinus* (Mackin, Owen, and Collier) infection while held at this low salinity site. Approximately 1000 juvenile oysters (~25 mm in length) were deployed in the field in floating mesh trays at the beginning of the next growing season (9 May 1991) at each of two sites of different mean salinities. The high salinity site was located near the mouth of the York River, Virginia, USA at the Virginia Institute of Marine Science. The mean salinity at this site was 20‰ and did not vary by more than 2‰ over the duration of our study. The low salinity site was located on the Wye River, Maryland, USA where the mean salinity was 12‰ during the study period, although some salinity variation occurred (see "Results"). Ambient salinity and temperature were measured biweekly.

Sampling and measurements

The mean size of the individuals within each group of oysters was determined initially, and at biweekly intervals thereafter, by removing 25 to 50 individuals at once in one or two grabs from a tray and measuring the shell height (from the umbo to the ventral shell margin) of all oysters in the sample and returning them to the tray. Monthly, five oysters were collected for determination of total weight, shell height, shell weight, wet tissue weight and dry tissue weight.

FAAs were quantified (see below) in gill tissue removed from five individuals at Time 0, then daily for the first 7 d after transfer and approximately biweekly thereafter until Day 70. In order to examine the association of infection intensity with changes in FAA, a larger sample of 25 oysters was obtained from each field site at 85 and 105 d (end of September 1991) following initial deployment. Protozoan infection intensity was diagnosed (see below) in each oyster sampled for FAA. Protozoan infection was also determined monthly in 25 oysters sampled at random from each tray.

FAA analysis

Gill tissue was excised and frozen quickly on dry ice in the field. The frozen tissues were transferred to the laboratory, freeze-dried, and weighed. The remaining tissues of each oyster were used for protozoan infection diagnosis (see below). Each tissue sample was homogenized in 40% ethanol, boiled to precipitate protein, and centrifuged (20000×*g*) for 20 min. The supernatant was freeze-dried and the residue dissolved in an appropriate volume of lithium citrate buffer (pH 2.2). The amino acid composition of this solution was determined with an automatic amino acid analyzer (Beckman, System Gold). Amino acid concentrations were calculated as μmol g^{-1} dry wt. Most of the oysters at the high salinity site were parasitized by *Perkinsus marinus* in the latter stages of the study. In order to be sure that the amino acid concentrations were those of the oyster gill tissue alone, we tested the effect of the extraction procedure on samples of isolated *P. marinus* cells. Briefly, *P. marinus* cells (either from infected tissues or from cultures) were freeze-dried and extracted in ethanol as described above. No amino acids were detected in these extracts, establishing that the amino acid concentrations measured were from the oyster tissue only.

Protozoan infection diagnosis

The valves of each oyster were opened and small sections of gill, mantle and rectum were removed and each placed separately in tubes of thioglycollate medium containing antibiotics (Burreson 1991). Tissue was incubated for 4 d at 27 °C then removed, macerated on a glass slide, stained with Lugol's iodine solution and examined at 40 to 100× for the presence of *Perkinsus marinus* cells. Infection intensity was rated on the basis of the number of parasite cells per microscope field as light, moderate or heavy (Burreson 1991). *P. marinus* infections were expressed both as prevalence (the percentage of oysters infected) and intensity. Infection intensity was calculated as the average intensity of a sample based on the following assignments: negative = 0; light = 1; moderate = 3; and heavy = 5. Weighted prevalence of infection was calculated as the average intensity of infection within a sample of oysters. We also examined the oysters for the presence of *Haplosporidium nelsoni* (MSX) using standard histological techniques (Ford and Figueras 1988). MSX was not detected during the study.

Data analysis

Until *Perkinsus marinus* infections appeared, mean amino acid concentrations were calculated for each group of five oysters per sample date. In August, *P. marinus* infections were first detected. Infections were light initially, but intensified in September and October, concomitant with changes in the levels of several amino acids. Therefore, we have grouped the data into four phases based on both the period of salinity acclimation and disease prevalence and intensity [Phase 1: uninfected, acclimating to field salinities (Days 1 to 20); Phase 2: uninfected, salinity acclimation completed (Days 21, 35 and 49); Phase 3: acclimated, lightly infected (Day 85); and Phase 4: acclimated, heavily infected (Day 105)]. Data from collections within these phases were pooled and analysis of variance (ANOVA) was used to determine significant relationships among phases, disease intensity, and mean amino acid levels. In addition, data from samples within phases were analyzed to determine the relationships between infection level and FAA content in individual oysters.

Results

Growth and disease prevalence

The salinities at both sites were stable (±3‰) except for an increase in salinity from 8 to 12‰ between Days 10 and 20 at the low salinity site. Initially, the oysters at the high salinity site grew faster than those held at low salinity (see Fig. 1), but in August (Day 85) the oysters at high salinity became infected with *Perkinsus marinus* and growth was subsequently halted, as we have seen before (Paynter and

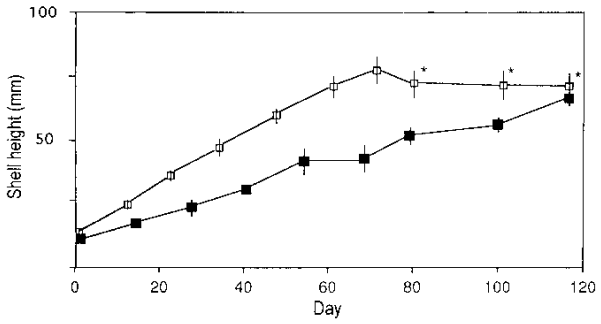

Fig. 1 *Crassostrea virginica*. Increase in shell height (growth) of oyster groups deployed at high salinity (20‰, open squares) and low salinity (8 to 12‰, filled squares) at Chesapeake Bay field sites. Infections first detected in oysters at high salinity on Day 85 as denoted by the asterisks

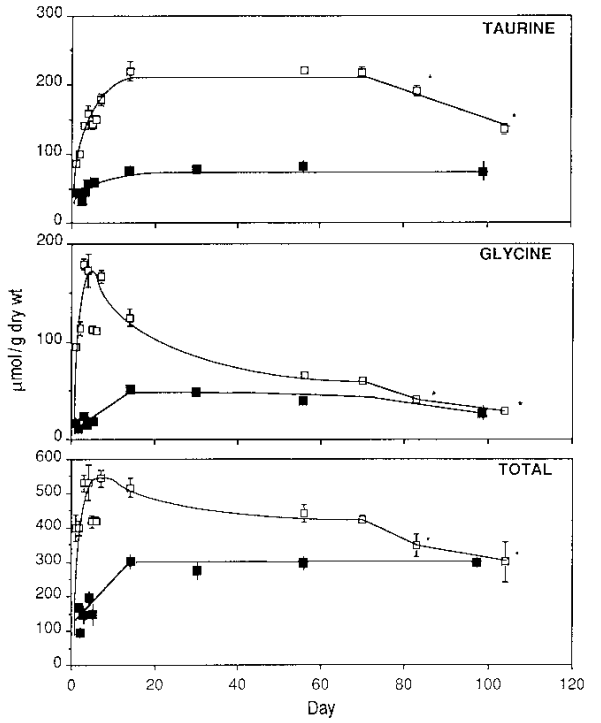

Fig. 2 *Crassostrea virginica*. Concentration of taurine, glycine and total free amino acid in gill tissues from oysters held at high (20‰, open squares) and low (8 to 12‰, filled squares) salinity sites. Transfer of oysters from low to high salinity occurred on Day 0. An increase from 8 to 12‰ occurred at the low salinity site between Days 10 and 20. Asterisks denote infection of oyster groups at high salinity by *Perkinsus marinus*

Burreson 1991). Infections at the high salinity site became more prevalent and more intense in September and October. The oysters held at low salinity did not contract the disease and continued to grow until November. At that point, the size of the low salinity oysters approximated that of the high salinity oysters. Mortality was relative low at both sites (<20%) as expected since the lethal effects of *P. marinus* usually occur during the second summer of infection (Andrews 1988).

Effect of salinity on intracellular amino acids

The FAA levels changed in gill tissues after transfer to the high salinity site (Fig. 2), essentially as predicted by earlier laboratory studies on other bivalve species. Within 24 h, glycine concentrations increased 8-fold and taurine concentrations doubled. The total FAA pool increased from approximately 150 to 400 µmol g^{-1} dry wt during the same time period. Glycine concentrations peaked at (180 µmol g^{-1} dry wt) between Days 10 and 15 then declined slowly after Day 20. Taurine concentrations increased more slowly, reaching a plateau around 225 µmol g^{-1} dry wt by Day 20, and remained at this level until around Day 85 when *Perkinsus marinus* infections were first detected. Overall, the total FAA increased to a peak around 500 µmol g^{-1} dry wt within 3 d and remained constant until the Day 85 sample when protozoan infections were first detected and total FAA levels declined. Taurine concentrations at Days 85 and 105 were significantly lower than the levels exhibited before infection. Proline concentrations rose quickly from undetectable levels to a peak 1 d after transfer [34.7 (±4.5) µmol g^{-1} dry wt] and declined to a level between 3 and 9 µmol g^{-1} dry wt until July (Day 56). Between Days 56 and 70 the proline concentrations ranged from 25.6 to 22.3 µmol g^{-1} dry wt, but after the first infection was detected on Day 85, the proline concentrations declined and ranged from 3 to 12 µmol g^{-1} dry wt throughout the remainder of the study period. Remarkably, glycine and total FAA declined to levels that are not different from those of the low salinity oysters.

The amino acid levels in tissues from the oysters held at low salinity stayed constant after the period between Days 10 and 20 when the total FAA level went up, presumably in response to a 4‰ salinity increase at the site. The mean levels of glycine, taurine and total FAA remained constant at the low salinity site for the remainder of the study period (Fig. 2).

Association of *Perkinsus marinus* infection with changes in the amino acid pool in high salinity oysters

The levels of FAA in the oysters transferred to high salinity were significantly different among the three phases following salinity acclimation (Phase 1 through Day 20, Table 1). Mean levels of the major amino acids of the FAA pool, taurine, glutamate and glycine, and the minor components, threonine, serine and β-alanine, were all lower in the infected groups compared to acclimated, uninfected (Phase 2) values. All of the FAA, except taurine and glutamine, were significantly lower even with light infection intensity (42% infected; Phase 3), producing a 24% decline in the total FAA pool. Glycine and β-alanine levels de-

Table 1 *Crassostrea virginica*. Free amino acid (*FAA*) concentrations in gill tissues taken from oysters at high salinity during the acclimated and infected phases of the study. FAA values expressed as $\mu mol\ g^{-1}$ dry wt (±SEM). Phases represent different time periods in which the oysters sampled were all uninfected (Phase 2), partially infected (Phase 3), or all infected (Phase 4). (*N* number of oysters sampled during a given phase.) Weighted prevalence represents average intensity of infections found in the oysters sampled during that phase. Values connected by underline for a given FAA are not different from one another

FAA	Phase 2	Phase 3	Phase 4
N	9	24	19
Weighted prevalence	0	0.8	3.0
Taurine	218.9 (9.4)	190.2 (6.0)	135.9 (8.1)[ab]
Aspartate	23.2 (1.7)	23.5 (2.7)	24.6 (1.8)
Threonine	8.2 (1.4)	3.7 (0.3)[a]	3.1 (0.2)[a]
Serine	22.3 (1.5)	5.3 (0.3)[a]	8.7 (0.7)[ab]
Glutamate	43.3 (3.7)	26.7 (1.2)[a]	30.2 (1.2)[a]
Alanine	64.4 (4.3)	34.7 (1.4)[a]	55.1 (2.5)[b]
Glutamine	0.6 (0.6)	0.3 (0.3)	4.6 (2.0)[ab]
Glycine	59.1 (2.1)	37.3 (1.7)[a]	27.9 (1.1)[a]
β-alanine	19.5 (1.3)	12.1 (2.6)[a]	10.6 (0.6)[a]
Proline	25.6 (0.8)	5.1 (0.6)[a]	10.2 (0.7)[ab]
Total	435.6 (12.6)	315.4 (8.9)[a]	286.3 (9.5)[a]

[a] Significantly different from Phase 2 values ($P<0.05$)
[b] Significantly different from Phase 3 values ($P<0.05$)

clined further as infection intensity increased and taurine levels were substantially reduced in the acclimated, heavily infected (100% infected; Phase 4) oysters. Both glutamine and alanine levels increased in the Phase 4 group. Overall, the total FAA pool in the oysters at the high salinity field site declined by 33% between Phases 2 and 4 as infection intensified. These values at Day 105 were similar to those in the oysters kept at low salinity.

While mean FAA content of the experimental oyster population during a given phase was significantly associated with mean infection intensity of the population, an analysis of the FAA concentrations in gill tissues from oysters grouped by diagnostic level (N, L, M, H) indicates that the effect of the parasite infection is not graded to diagnostic level (Table 2). Gills from oysters in Phases 3 or 4 (including Phase 3 negatives) all had very similar reductions in the intracellular FAA pool.

Discussion

The pattern of amino acid accumulation by *Crassostrea virginica* gill cells following exposure to high salinity stress in the field is very similar to laboratory results obtained in both intact ribbed mussels (*Geukensia demissus*) and isolated tissues (Baginski and Pierce 1977, 1978; Bishop et al. 1994). Rapid increases in the total FAA pool of the gills occurred in oysters after placement at the high

Table 2 *Crassostrea virginica*. Free amino acid (*FAA*) concentrations in gill tissues taken from oysters of various infection levels at high salinity during Phases 2, 3 and 4 of study. See "Results" for an explanation of diagnostic levels. FAA values expressed as $\mu mol\ g^{-1}$ dry wt (±SEM). Infection intensities were determined by the no. of parasite cells per microscope field observed in tissue samples: (*0* no cells found; *1* light infection, *3/5* moderate and heavy infections.) Uninfected oysters from Phase 2 and 3 presented in separate columns because in many cases they are significantly different. (*N* number of oysters with a given infection intensity.) Weighted prevalence represents average intensity of infections found in the oysters within a group. Values connected by underline for a given FAA are not different from one another

FAA	Infection intensity			
	0 (Phase 2)	0 (Phase 3)	1 (Phases 3 and 4)	3/5 (Phases 3 and 4)
N	9	14	17	13
Weighted prevalence	0	0	1	4.4
Taurine	218.9 (9.4)	179.0 (7.2)[a]	163.2 (13.2)[a]	151.3 (9.9)[a]
Aspartate	23.2 (1.7)	21.9 (2.5)	25.4 (1.5)	21.5 (1.6)
Threonine	8.2 (1.4)	3.6 (0.3)[a]	3.2 (0.3)[a]	3.5 (0.3)[a]
Serine	22.3 (1.5)	4.8 (0.3)[a]	7.3 (0.6)[ab]	8.2 (1.0)[ab]
Glutamate	43.3 (3.7)	25.9 (2.0)[a]	30.4 (1.1)[a]	29.2 (1.4)[a]
Alanine	64.4 (4.3)	36.3 (4.0)[a]	46.9 (3.6)[ab]	50.8 (2.7)[ab]
Glutamine	0.6 (0.6)	1.9 (1.3)	5.0 (2.2)[a]	1.5 (1.2)
Glycine	59.1 (2.1)	40.2 (5.3)[a]	37.4 (3.1)[a]	30.9 (2.8)[a]
β-alanine	19.5 (1.3)	16.0 (4.7)	10.7 (0.9)[a]	9.6 (0.5)[ab]
Proline	25.6 (0.8)	3.0 (0.8)[a]	9.7 (2.2)[ab]	8.6 (1.7)[ab]
Total	435.6 (12.6)	310.7 (9.0)[a]	313.8 (8.5)[a]	315.1 (7.3)[a]

[a] Significantly different from Phase 2 values ($P<0.05$)
[b] Significantly different from infection intensity 0 (Phase 3) values ($P<0.05$)

salinity site, but individual amino acids contributed to the elevation with very different time courses. Glycine, proline and taurine all rose in sequence, with the metabolically active amino acids gradually declining toward control levels while taurine concentrations remained high. Unlike previous studies on *G. demissus*, an early alanine rise did not appear in the oyster gills, perhaps because it occurred within the first 24 h and our first sampling interval was too late to find it. In other bivalve species, the high salinity-induced rise in glycine begins as alanine concentrations fall (Baginski and Pierce 1977, 1978; Bishop et al. 1994). After 24 h at the high salinity site, the glycine rise in the gill tissue was already well underway, so it is possible that an alanine rise and decline had already occurred by the time of our first sample. Clearly, the oysters respond to salinity changes in the field by rapid and coordinated syntheses of several amino acids.

Intracellular FAA levels were much lower in the gills of the groups of high salinity adapted oysters infected by *Perkinsus marinus*. As the infection intensified in the group, the amino acid pool declined by 40% of its original level largely due to taurine decreases in all the oysters, including those scored as uninfected by our diagnostic method. Since taurine levels in oysters or mussels acclimated to a particular salinity remain at the acclimated level unless a subsequent salinity change occurs (Baginski and Pierce 1977; Soniat and Koenig 1982; Bishop et al. 1983) and given the lack of a similar decline in taurine in the low salinity oysters, the reductions in taurine and other FAA in the Phase 3 oysters are likely related to protozoan infection rather than some type of seasonal change. Since the infection is thought to initiate in the intestine, the rectal, gill and mantle assays for parasites may miss oysters with intestinal infections which have not yet spread beyond the intestinal area. Most likely, the uninfected oysters that showed declines were actually infected, but misdiagnosed. In addition, growth in the field is affected in much the same way as the FAA. Growth ceased after Day 70, but the infection was not detected until Day 85. The reduction in growth was precipitous during the early phases of infection and, in a previous study, remained low until the oysters either died or until cold weather arrived (Paynter and Burreson 1991). Together, these observations on the FAA pool and growth suggest that *P. marinus* infection has significant impacts on the physiology and biochemistry of the oyster soon after infection.

Our study did not determine the physiological consequences of the FAA pool reduction. However, since oysters are osmoconformers, a reduction of 33% in intracellular FAA (i.e., between Phases 2 and 4) must be compensated for by the elevation of other intracellular solutes. At present, these solutes are not known but inorganic ions such as Na^+, K^+, and Cl^- are obvious possibilities. An increase in intracellular ion concentration of this magnitude is likely to produce negative physiological effects (Yancey et al. 1982). The decreased FAA concentration might be caused either by perturbations in the synthesis of FAA important for salinity tolerance or in the membrane characteristics which keep the FAA inside the cell once they are synthesized. The intracellular amino acids that are utilized for salinity tolerance are synthesized largely by the mitochondria (Paynter et al. 1984; Pierce et al. 1991). Once synthesized, the amino acids are transported to the cytosol where their intracellular concentration is regulated by the permeability control mechanisms of the cell membrane (Pierce and Politis 1990). Therefore, the presence of *Perkinsus marinus* might affect the permeability control mechanisms of the cell membrane, or synthetic mechanisms in the mitochondria. In addition to the effects on the osmolytes, impaired mitochondrial function could result in a reduction in ATP production, which could account for the reduction of growth observed in the presence of the parasite. We are currently testing these possibilities, but in any case, the present study suggests that *P. marinus* infection might be altering cell membrane permeability properties or intracellular synthetic pathways or both.

Finally, the initial accumulation of FAA in response to hyperosmotic shock is the result of a complex biochemical regulatory process that allows oyster cells to route carbon and nitrogen in a very specific way (Bishop et al. 1983). Since the biochemical pathways used to respond to hyperosmotic stress may be the same, or very similar, to the pathways involved in hypoxic tolerance (Baginski and Pierce 1975), it is possible that the ability of oysters to tolerate hypoxia, which is common in Chesapeake Bay (MacKiernan 1987), might also be diminished by *Perkinsus marinus* infection. A reduced tolerance of hypoxia could lead to the large-scale mortalities that occur in Chesapeake Bay oysters during exposure to low oxygen water, which occurs frequently during the summer months over many oyster bars. We are presently examining the interactions between hypoxia and *P. marinus* infection on the physiology of oysters.

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