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ANAEROBIOSIS: A POSSIBLE SOURCE OF OSMOTIC SOLUTE FOR HIGH-SALINITY ACCLIMATION IN MARINE MOLLUSCS*

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SUMMARY

1. When stressed with high-salinity exposure, cell volume is restored in ventricles of *Modiolus demissus demissus* by a rapid accumulation of intracellular free amino acids.

2. Although the total amino acid pool increases and remains at a constant high level thereafter, the pattern and time course of accumulation is different for each major amino acid (glycine, alanine, taurine, and proline).

3. Initially, cell volume is restored by a rapid accumulation of alanine, but later its concentration decreases while glycine and taurine accumulate. Although at first not detected, the proline concentration increases, peaks and subsequently disappears again.

4. Isolated ventricles recover normal activity after large environmental salinity increases.

5. During recovery the intracellular free amino acid changes in isolated ventricles are similar to the initial pattern of accumulation in whole animals, i.e., alanine, and to a lesser extent, proline and glycine accumulate.

6. Finally, isolated ventricles undergo a period of decreased oxygen consumption on exposure to an increased salinity.

7. These results suggest that the initial stages of high-salinity acclimation in molluscs depends upon the synthesis of amino acids via a known anaerobic biochemical pathway.

INTRODUCTION

Molluscs, in company with other osmoconforming invertebrates, regulate cell volume during variation in external salinity by using intracellular free amino acids as osmotic solutes. While the mechanism of free amino acid regulation in response to dilute salinities has been studied in detail in many species (reviewed by Pierce, 1971*b*), very little is known about the mechanism of intracellular free amino acid accumulation which occurs in response to high salinity exposure.

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Based on data from a few species of euryhaline Crustacea, Florkin & Schoffeniels (1969) have proposed that the regulation of intracellular free amino acid concentration depends upon the effects of inorganic ions on the activity of glutamate dehydrogenase (GDH). In lowered salinities, amino acid concentrations decrease due to GDH inactivation, whereas in concentrated salinities amino acid accumulation results from an increased rate of amino acid synthesis brought about by higher GDH activity. In contrast, protein catabolism has been postulated as the mechanism responsible for free amino acid concentration increases in the gastropod *Melanopsis trifasciata* during high-salinity acclimation (Bedford, 1971). While both these mechanisms have been dismissed as the explanation of hypo-osmotic cell volume regulation in molluscs (Pierce & Greenberg, 1972, 1973), insufficient information is available to distinguish the actual mechanism of amino acid accumulation during high-salinity acclimation.

Both proposed mechanisms predict different sequences of amino acid accumulation. Glutamic acid should accumulate rapidly following an external salinity increase in the Florkin–Schoffeniels model, followed by later increases of the other amino acids as they are formed by transamination from glutamate. On the other hand, if protein catabolism provides the source of free amino acids, a simultaneous increase in all amino acids is likely. Therefore, in order to distinguish the mechanism involved, we examined the time course of intracellular free amino acid accumulation in an intertidal bivalve, *Modiolus demissus demissus* (Dillwyn), during high-salinity acclimation. This mussel was chosen for study for two reasons. First, the animal is a euryhaline osmoconformer that regulates cell volume by using the free amino acids taurine, alanine and glycine (Pierce, 1970, 1971*a*, *b*). Secondly, the isolated ventricle of *M. d. demissus* responds to salinity change in a manner similar to that of the whole animal, while presenting an easily measurable response – spontaneous beat – which is modified by salinity change (Pierce & Greenberg, 1972, 1973). Our results fail to support either theory of intracellular free amino acid accumulation. Rather, the data suggest that high salinity acclimation involves synthesis of amino acids utilizing an anaerobic biochemical pathway.

MATERIALS AND METHODS

Mussels and sea water

Ribbed mussels, *Modiolus demissus demissus*, were collected in a salt marsh on Assateague Island, Maryland. Animals were maintained in large aquaria containing aerated artificial sea water (ASW) (Instant Ocean, salinity = 34‰ = 1000 mOsm/-Kg H_2O) at constant temperature (15 °C) and under constant lighting.

All experimental salinities were prepared by appropriate dilutions of ASW with glass-distilled water. The concentration of each test salinity was determined before use with an osmometer (Osmette, Precision Systems). Osmotic concentrations were converted to parts per thousand (‰) using the formula:

$$\text{test solution salinity} = \frac{\text{test solution osmolarity (mOsm/Kg } H_2O) \times 36‰}{1054 \text{ mOsm/Kg } H_2O}.$$

Intracellular free amino acid accumulation in ventricles removed from mussels during high-salinity acclimation

Two groups of mussels were acclimated for at least 3 weeks to 12‰ and 36‰ ASW. *M. d. demissus* is fully acclimated to any non-lethal salinity after this time period (Pierce, 1970). Following the acclimation period, ventricles were removed from the group of animals in 12‰ and the intracellular free amino acid concentrations determined (see below). The remaining mussels in 12‰ ASW were then returned to 36‰ ASW. Thereafter, ventricles were periodically removed from these reacclimating animals and intracellular amino acid concentrations determined. The experiment was terminated when the concentrations of intracellular free amino acids of the reacclimating animals approached the ventricular amino acid concentrations in mussels acclimated to 36‰ (Pierce & Greenberg, 1972). In addition, as a control, amino acid concentrations were determined in ventricles from mussels maintained in 36‰ ASW for the duration of the experiment.

Intracellular free amino acid concentrations were determined according to previously described methods (Pierce, 1971*b*). After removal from the mussels the ventricles were frozen, lypholysed, weighed and homogenized in 80% ethanol. The homogenate was heated to boiling and subsequently centrifuged for 10 min at 20,000 *g*. Finally, the supernatant was lypholysed and the residue dissolved in sodium citrate buffer (pH 2·2). The amino acid content of this solution was determined with an amino acid analyser (JEOL, model JLC–6AH). Data were analysed in this and subsequent experiments using Wilcoxon (Mann–Whitney) non-parametric methods.

Effect of high salinity on mechanical activity of isolated ventricles

M. d. demissus were acclimated to either 12 or 24‰ ASW as previously described. Ventricles were then isolated from four animals and each suspended from a force-displacement transducer (Grass Model FT.03-C) in an aerated organ bath containing the acclimation salinity according to the method of Pierce & Greenberg (1972). The mechanical activities of the hearts were monitored by recording the output of the transducers with a 4-channel oscillograph (Grass Model 7).

After all hearts were beating rhythmically, a higher-salinity ASW was introduced into two baths, while the remaining two hearts were washed with the acclimation salinity ASW and served as controls. All baths were washed at 30 min intervals with 5 bath volumes of the appropriate salinity until normal mechanical activity of the test hearts returned.

Intracellular free amino acid concentrations in isolated ventricles exposed to different salinities

Whole animals were acclimated to 12‰ ASW as previously described. Then, several hearts were isolated and divided into two groups. One group was placed in aerated organ baths containing 12‰ ASW; the other group was placed directly into 36‰ ASW. After 9 h (double the longest time to recovery of normal mechanical activity in the previous experiment), the hearts were removed, frozen, lypholysed, weighed and the intracellular free amino acid concentrations determined as previously described.

Effect of salinity on rate of oxygen consumption of isolated ventricles

Ventricles were removed from whole animals previously acclimated to either 12 or 36‰ ASW as usual and placed directly into chambers containing test solutions of either 12 or 36‰ ASW. After a 45 min equilibration period in the test solution, ventricular oxygen consumption rates were measured with an oxygen electrode (Yellow Springs Instrument Company, Model 53 Oxygen monitor) at constant temperature (25 °C). The change in percentage oxygen saturation of the solution was continuously monitored with a strip chart recorder (Varian Techtron, Model 135-1) which displayed the output from the oxygen electrode. The electrode was standardized against air-saturated ASW of the same salinity as each test solution and an internal zero point. In addition, a standardized mixture of oxygen and nitrogen gas (Instrument Laboratory Standardized Gas) was used to check instrument calibration. Following the measurement, the hearts were frozen, lypholysed and weighed. The decrease in oxygen saturation of the test solution was converted to oxygen consumption in μl O_2 consumed/mg dry wt/hr by using standard tables (Krogh, 1959).

RESULTS

The time course of intracellular free amino acid accumulation in ventricles of acclimating mussels

During the first 8 days of exposure of 12‰ ASW acclimated mussels to 36‰ ASW, the total intracellular free amino acid pool of ventricles rapidly increased and remained constant thereafter. Although the pool size remained constant during acclimation,

Table 1. *The intracellular free amino acid concentration of ventricles of* M. d. demissus *taken from animals during acclimation to* 36‰ *sea water*

(The animals were previously acclimated to 12‰ sea water. N = 5 samples of 20 ventricles each per time interval.)

Time exposed to 36‰ sea water (days)	Amino acid concentration (μmole/g dry wt.)*								
	Ala	Asp	Glu	Gly	Pro	Ser	Tau	Thr	Total amino acid pool
0	43 (6·8)	54 (6·9)	35 (3·4)	64 (5·4)	0	6 (0·4)	77 (2·8)	4 (0·3)	283 (19·2)
0·33	128 (6·6)	17 (1·3)	57 (2·6)	76 (3·6)	0	4 (0·5)	83 (7·2)	2 (0·4)	367 (11·6)
2	341 (40·6)	34 (3·2)	41 (2·5)	111 (7·7)	26 (8·9)	8 (1·4)	81 (2·4)	8 (1·7)	650 (49·3)
5	358 (13·3)	46 (7·3)	30 (1·7)	191 (8·5)	48 (6·6)	12 (3·1)	107 (9·5)	9 (0·3)	803 (37·6)
8	377 (34·0)	37 (4·4)	34 (2·5)	233 (17·6)	74 (5·1)	9 (1·4)	101 (1·9)	9 (1·0)	876 (20·0)
11	282 (36·6)	30 (6·1)	28 (3·9)	278 (28·8)	72 (5·1)	12 (1·8)	108 (3·7)	9 (1·1)	821 (22·1)
14	263 (23·4)	38 (2·2)	32 (3·5)	303 (15·8)	84 (4·1)	10 (1·1)	108 (4·6)	9 (0·6)	847 (30·5)
17	234 (22·4)	36 (4·1)	28 (3·6)	341 (20·1)	83 (11·2)	10 (0·9)	121 (10·0)	8 (0·6)	859 (42·8)
20	260 (14·6)	40 (5·6)	29 (6·1)	332 (7·6)	79 (7·2)	12 (2·5)	109 (9·5)	8 (0·6)	870 (15·3)
23	204 (20·1)	40 (7·1)	24 (2·6)	377 (12·2)	75 (5·0)	10 (1·4)	110 (6·6)	8 (0·5)	848 (21·5)
29	208 (10·4)	35 (5·4)	30 (3·4)	361 (12·4)	62 (2·5)	7 (0·7)	135 (10·3)	6 (0·2)	829 (13·7)
35	197 (31·4)	32 (9·1)	33 (2·9)	361 (19·7)	57 (4·8)	7 (1·1)	130 (0·7)	7 (1·0)	825 (47·8)
41	194 (15·0)	40 (8·6)	32 (5·0)	408 (4·0)	47 (3·5)	8 (0·6)	173 (6·0)	7 (0·4)	910 (11·3)
53	167 (23·3)	46 (3·0)	23 (2·6)	396 (10·4)	9 (5·3)	6 (0·7)	162 (7·5)	6 (1·0)	873 (18·5)
65	209 (20·6)	36 (6·2)	33 (3·8)	300 (19·0)	19 (1·2)	7 (2·3)	178 (13·0)	6 (1·0)	786 (34·8)
77	201 (4·4)	32 (2·5)	39 (2·3)	295 (24·5)	0	5 (0·9)	201 (17·2)	4 (0·2)	759 (21·2)
89	121 (8·8)	50 (3·4)	27 (1·9)	282 (11·8)	0	3 (0·4)	211 (10·2)	4 (0·3)	698 (21·2)
101	175 (27·0)	42 (5·5)	33 (2·8)	250 (6·0)	0	2 (0·1)	241 (2·8)	3 (0·2)	753 (28·7)

* S.E.M. Shown in parentheses.

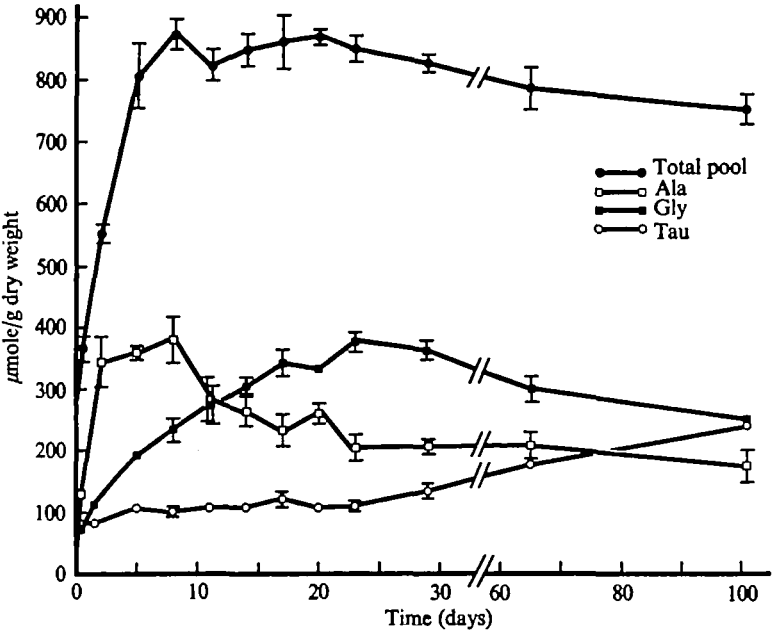

Fig. 1. Time course of intracellular amino acid concentration increases in ventricles removed from *M. d. demissus* during acclimation to high salinity. At time 0 animals were transferred from 12 to 36‰. Error bars are standard errors. For those points without error bars, standard errors are less than the size of the point.

both the pattern and the time course of accumulation was different for each major amino acid (alanine, glycine, taurine and proline) (Table 1, Fig. 1).

Initially, alanine concentration increased rapidly, peaked and levelled off by the end of 8 days following the salinity change. Subsequently, the alanine concentration slowly decreased and again levelled off to a net gain (final concentration – initial concentration) of 132 μmole/g dry tissue weight (Fig. 1).

Intracellular free glycine accumulated at a slower rate than alanine. The peak glycine concentration was not reached until 41 days following the salinity increase. After that period glycine decreased and finally levelled off to a net gain of 186 μmole/g dry tissue weight (Fig. 1).

The rate of taurine accumulation did not become substantial until after 23 days of exposure to increased salinity. In contrast to alanine and glycine, the taurine concentration continued to increase for the duration of the experiment. The net gain in taurine concentration was 164 μmole/g dry tissue weight (Fig. 1).

Intracellular free proline concentrations showed a fourth pattern of variation during the acclimation period (Table 1). Initially, proline was not detected. Proline first appeared 2 days after the salinity change and increased in concentration to a maximum of 84 μmole/g dry weight of tissue after 14 days. Thereafter, proline concentration declined until disappearing again after 77 days.

By the end of the acclimation period there were no significant differences ($P = 0{\cdot}05$) between the concentrations of alanine, glycine and proline in the acclimating animals and the respective concentrations in control mussels kept in 36‰ for the duration of the experiment (Table 2). On the other hand, taurine concentrations in the acclimating

Table 2. *Concentrations of the major intracellular free amino acids in ventricles removed from mussels following complete high-salinity acclimation, compared with amino acid concentrations in ventricles of the control group*

(Group A, 12‰ acclimated mussels transferred to 36‰ SW and maintained at 36‰ for 101 days. Group C, control mussels maintained at 36‰ throughout experiment.)

	Amino acid concentration (μmole/g dry wt.)*			
	Ala	Gly	Pro	Tau
Group A	175 (27·0)*	250 (6·0)	0	241 (2·8)
Group C	195 (18·5)	234 (8·0)	0	290 (16·6)

* S.E.M. shown in parentheses.

animals were significantly different ($P = 0{\cdot}05$) from control at the end of the experiment. Therefore, with the possible exception of taurine, the accumulation of intracellular free amino acids in *M. d. demissus* ventricles was completed 101 days after the external salinity increase.

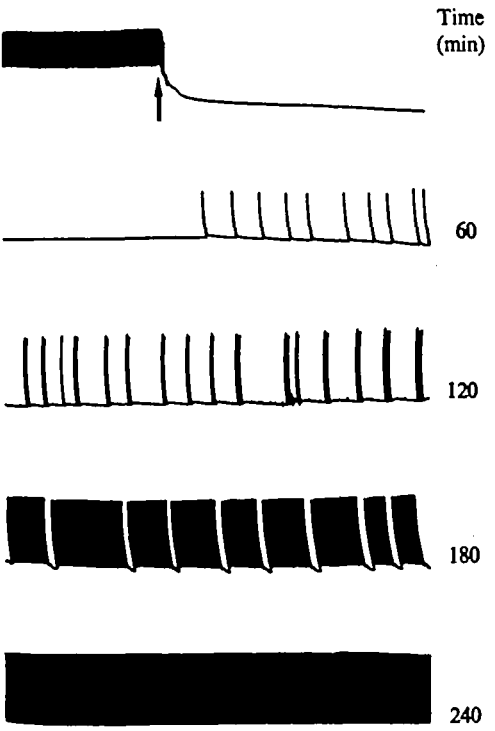

Fig. 2. Typical mechanical recording obtained from an isolated *M. d. demissus* ventricle taken from a mussel previously acclimated to 12‰ ASW. The records are 15 min segments which immediately precede the time indicated.

The mechanical response of isolated ventricles to increased salinity

When challenged with a non-lethal increase in external salinity, the immediate response characteristic of an isolated *M. d. demissus* ventricle was loss of spontaneous beat followed by a period of quiescence. After some time in the increased salinity

Table 3. *The time course of the recovery of the mechanical response of isolated* M. d. demissus *ventricles following exposure to high salinities* (N = 8 *experiments*)

Salinity change (‰)	Maximum time to recovery of normal activity (min)*
12–36	230 (30·7)
12–24	41 (12·0)
24–36	24 (3·5)

* S.E.M. shown in parentheses.

Table 4. *Changes in intracellular free amino acid concentrations in isolated ventricles of low salinity acclimated* M. d. demissus *following exposure to increased salinity*

Salinity change (‰)	Amino acid concentrations (μmole/g dry wt)*			
	Alanine	Glycine	Proline	Taurine
12–12	30 (7·5)	33 (4·3)	0	103 (5·1)
12–36	231 (14·3)	53 (1·9)	61 (0·9)	105 (8·1)

* S.E.M. shown in parentheses.

Table 5. *Comparative rates of oxygen consumption of isolated* M. d. demissus *ventricles before, during and after high salinity acclimation.* (N = 8 *experiments*)

Acclimation salinity-test salinity (‰)	Rate of O_2 consumption (μl/mg dry wt/hr)*
12–12	2·43 (0·13)
12–36	1·21 (0·02)
36–36	1·48 (0·05)

* S.E.M. shown in parentheses.

spontaneous activity reappeared, usually irregular at first, and gradually returned to control levels. A mechanical record typical of those obtained is shown in Fig. 2.

Furthermore, the time needed for ventricular recovery after salinity increases was directly dependent upon the magnitude of salinity change (Table 3). For instance, ventricles recovered from 12‰ salinity increases (12‰–24‰ and 24‰–36‰) significantly earlier (P = 0·01) than those exposed to a 24‰ increase (12‰ –36‰).

Isolated ventricles: amino acid levels and rates of oxygen consumption during high salinity adaptation

During a 9 h exposure to an increase in external salinity (12–36‰) isolated *M. d. demissus* ventricles accumulated intracellular free amino acids in a manner similar to the initial accumulation shown by ventricles removed from acclimating mussels (Table 4). The intracellular concentrations of alanine, glycine and proline were higher following exposure to the increased salinity. Intracellular free alanine increased more than either glycine or proline. The taurine concentration remained unchanged.

Oxygen consumption rates of isolated ventricles exposed to a salinity increase (12–36‰) were significantly lower (P = 0·01) than the rates of ventricles kept in the

salinity of acclimation (either 12‰ or 36‰) (Table 5). In comparison, isolated ventricles acclimating to 36‰ ASW showed a 50% lower oxygen consumption rate than hearts in 12‰ ASW and 18% lower than hearts already acclimated to 36‰ ASW.

DISCUSSION

After exposure to an increase in external salinity, volume regulation by *M. d. demissus* is at first achieved by a rapid accumulation of intracellular free alanine. Thus, during the initial portion of high salinity acclimation, alanine alone provides an increasing source of intracellular osmotic solute for halting osmotic water loss to the more concentrated environment. As acclimation proceeds, alanine concentrations decline, while glycine, proline and taurine accumulate. However, in spite of the variation in individual amino acid accumulation rates, the total amino acid pool remains at the initial level established during the alanine peak. Isolated ventricles exposed to high salinity show an intracellular free amino acid accumulation pattern very similar to the initial pattern found in ventricles *in situ*; i.e. a large increase in alanine concentration occurs, accompanied by smaller increases in proline and glycine during the period of time before recovery of normal mechanical activity. Furthermore, in company with the alanine increase, oxygen consumption of isolated ventricles is depressed during exposure to high salinity.

These results, taken together with the extensive data on low salinity acclimation in bivalves (Pierce, 1971*b*; Pierce & Greenberg, 1972, 1973), strongly indicate that the Florkin–Schoffeniels theory of intracellular free amino acid accumulation does not apply to molluscs. That theory predicts that glutamic dehydrogenase activity should increase in high salinity resulting in the initial production of glutamic acid which, in turn, participates in transamination reactions. However, in *M. d. demissus* ventricles, the initial amino acid accumulated is alanine, not glutamic acid. Alanine could still be formed by transamination from glutamic acid under reaction conditions allowing for constant glutamate concentration. However, in several molluscan species glutamic dehydrogenase activity is either low or lacking (reviewed by Campbell & Bishop, 1970; De Zwaan & Van Marrewijk, 1973) and therefore glutamate is an unlikely source of alanine.

Another possible source of the intracellular free amino acids that accumulate during high-salinity acclimation is protein catabolism (Bedford, 1971). However, unless some unique proteins are involved, protein degradation would result in simultaneous accumulation of a variety of amino acids. Since our data clearly show unique time courses of accumulation for individual amino acids, protein catabolism also seems an unlikely source of free amino acids during high salinity acclimation. At present, a third explanation seem to satisfy all our results, namely that amino acid accumulation in molluscan tissues during exposure to high salinity is dependent upon anaerobic metabolism.

Under anaerobic conditions many organisms, including molluscs, utilize a biochemical pathway of glucose degradation which results in intracellular alanine accumulation (reviewed by Saz, 1971; Hochachka, Fields & Mustafa, 1973). In brief, glucose degradation is shifted away from the Krebs cycle at the level of phosphoenolpyruvate such that alanine and succinate are formed as end-products. Molluscs

known to utilize this pathway include *Crassostrea virginica* (Hammen, 1969), *Rangia cuneata* (Stokes & Awapara, 1968; Chen & Awapara, 1969*a*, *b*), *Mytilus edulis* (De Zwaan & Zandee, 1972; De Zwaan & Van Marrewijk, 1973) and *Modiolus demissus* (Malanga & Aiello, 1972).

The data presented in this paper are consistent with this hypothesis. First, the decreased rate of oxygen consumption by isolated ventricles during high-salinity acclimation suggests that glucose catabolism has in part been shunted away from the Krebs cycle to an anaerobic pathway. Indeed, the enzymes controlling the transition between aerobic and anaerobic metabolism are capable of simultaneous operation (Livingstone & Bayne, 1974). Secondly, ventricles also accumulate proline, along with alanine, during the initial steps of intracellular free amino acid accumulation. In this case, proline appears to be only a by-product of the initial volume regulation process, since during acclimation it disappears and thus can serve only transiently as an intracellular solute. However, the presence of proline is a further indicator of anaerobic metabolism. It accumulates along with alanine during anaerobiosis (Isserhoff, Tunis & Read, 1972) and, in fact, proline has been postulated to be an important end-product of energy production for this anaerobic pathway (Hochachka *et al.* 1973).

In summary, high-salinity adaptation in molluscs appears to depend upon an initial rapid increase in intracellular alanine concentration produced by an anaerobic biochemical pathway. With time in high salinity, the other osmotically important amino acids slowly increase in concentration while alanine declines, until complete physiological acclimation to the higher salinity is achieved.

We are currently testing the effects of salinity on both succinate concentration and anaerobic enzyme activities to establish the relationship between anaerobiosis and high salinity acclimation.

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