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Hypoosmotic Stimulation of Ornithine Decarboxylase Activity in the Brine Shrimp *Artemia franciscana*

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ABSTRACT In the brine shrimp, long-term (several days) osmotic and ionic regulation is controlled principally by the activity of $Na^{+}K^{+}$ATPase. However, short-term cell volume regulation in *Artemia* and most euryhaline crustaceans is not understood. In this study, *Artemia* stage 1 nauplii reared at 32 ppt salinity and exposed to hypoosmotic conditions (12 or 4 ppt) exhibited an immediate increase in the activity of ornithine decarboxylase, the rate-limiting enzyme in polyamine synthesis. ODC activity increased within 15 min relative to control and reached a peak within 2 hr of hypoosmotic exposure. The level of increased enzyme activity was directly related to the degree of hypoosmotic exposure (a three- and sevenfold increase in activity at 12 and 4 ppt salinity, respectively). This increase in activity was apparently due to the synthesis of a new enzyme, based on cycloheximide administration. Activity decreased significantly by 4 hr but remained above that of the 32 ppt control throughout the 24-hr exposure period. Levels of putrescine increased significantly within 2 hr in organisms exposed to 4 ppt; spermidine and spermine levels were not affected. When nauplii were exposed to altered ion and/or osmotic concentrations it was determined that decreased total osmotic concentration was the proximate factor responsible for ODC induction. However, reduced ion concentration resulted in the induction/activation of DFMO-insensitive ODC activity, suggesting the production of a novel enzyme in polyamine metabolism during hypotonic exposure. Changes in ODC activity and polyamines preceded acclimatory changes normally associated with salinity acclimation, further suggesting that modulation of ODC activity and subsequent polyamines synthesis, degradation, and/or transport are mechanistically related to short-term regulation of osmotic and ionic balance. © 1996 Wiley-Liss, Inc.

Euryhaline organisms that experience large fluctuations in environmental salinity must deal with the problem of readjusting the osmotic and ionic concentrations of both the extra- and intracellular fluid compartments. Extracellular fluid (i.e., hemolymph) ion regulation is accomplished via the active uptake of the major ions (Na^{+} and Cl^{-}) from the medium, usually by the gill (for reviews, see Kirschner, '79; Mantel and Farmer, '83; Henry, '84); the intracellular osmotic concentrations of tissues (and therefore cell volume) is controlled by the adjustment of both inorganic ions and small organic compounds such as free amino acids and quaternary ammonium compounds (for reviews, see Gilles, '87; Pierce, '82). These physiological processes involve a number of changes that occur at the cellular level such as alterations in membrane permeability, activation and/or induction of enzymes, and cellular differentiation. While these cellular events have been well described, the mechanism(s) by which they are initiated and regulated are largely unknown.

One potential class of regulatory agents could be the biogenic amines, in particular the polyamines. The polyamines spermidine and spermine and their diamine precursor putrescine are the most abundant organic cations in eukaryotic cells. Polyamine metabolism and function in vertebrate tissues has been the topic of several major reviews (Heby, '81; Pegg and McCann, '82; Tabor and Tabor, '84; Russell, '85; Slotkin and Bartolome, '86; Pegg, '86, '88). Specifically, polyamine synthesis has been linked to changes in external osmotic and ionic concentrations in bacteria (Munro et al.,

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'72; Peter et al., '78; Yamamoto et al., '86), plants (Tiburcio et al., '86; Altman et al., '88), and mammalian cells (Perry and Oka, '80; Viceps-Madore et al., '82; Lundgren and Prokay, '88; Poulin and Pegg, '90; Poulin et al., '91). Polyamines have been shown to alter membrane permeability and glucose and ion transport (Elgavish et al., '84), and they are known to be involved in cellular differentiation and proliferation during normal development and in neoplasms and tumors (Pegg, '86, '88). Thus, there is abundant precedent for the involvement of polyamines in the basic cellular processes that also underlie the physiological mechanisms of salt and water balance in euryhaline crustaceans.

The initial and rate limiting step in polyamine synthesis is the catalytic conversion of L-ornithine to putrescine by the enzyme ornithine decarboxylase (ODC, EC 4.1.1.17). Putrescine is converted to spermidine and spermine by the addition of propylamine groups via the activity of their respective synthases. ODC activity often reflects the rate of macromolecular synthesis (RNA and protein), and its rapid turnover rate ($t1/2 = 10–20$ min; Russell, '85) is indicative of its important regulatory function in the cell. It is therefore plausible that if polyamines are involved in salinity adaptation, then ODC activity should be sensitive to changes in environmental salinity.

This investigation reports on the sensitivity of ODC activity to a hypoosmotic salinity shock in the brine shrimp, *Artemia fransiscana,* a euryhaline crustacean. In addition, physiological concentrations of polyamines are presented in relation to ambient salinity, and the induction of a novel enzyme in polyamine metabolism is reported for the first time.

MATERIALS AND METHODS

Cysts of *Artemia* sp. were obtained commercially from the San Francisco Bay Brine Shrimp Company (lot SFBB 1879). These cysts have been used in preliminary studies with successful hatching in excess of 90%. Cysts were prepared according to procedures outlined by Clegg ('86).

Hypoosmotic stimulation of ODC activity and polyamines

Cyst were cleaned (in the absence of hypochlorite) and hydrated overnight in the refrigerator at 4°C. Approximately 40 g of hydrated cysts were incubated in a 20-liter cylinder containing 32 ppt salinity artificial seawater (ASW; Tropic Marin) at 28°C with light and aeration. At 22 hr nauplii were collected by siphoning phototaxic nauplii and transferring them into clean ASW. At 24 hr the nauplii were transferred randomly into 20-litre cylinders containing either 32, 12, or 4 ppt salinity. At times 0, 0.25, 0.5, 1, 2, 3, 4, 6, and 24 hr post-transfer triplicate subsamples of nauplii were removed from each treatment, rinsed briefly with cold distilled water three times, vacuum filtered, weighed, and assayed for DFMO-sensitive ODC activity. Additionally, triplicate subsamples were removed from each treatment at 0, 1, 2, 3, 4, 6, and 24 hr and rinsed briefly with cold distilled water three times, vacuum filtered, weighed, and stored at –70°C for determination of polyamine levels.

Effects of cycloheximide on induction of ODC activity

Nauplii were exposed to the protein synthesis inhibitor cycloheximide to determine whether increases in ODC activity during hypoosmotic exposure was the result of increased protein synthesis (enzyme induction) or activation of pre-existing enzyme. Stage 1 nauplii (24 hr) reared in 32 ppt salinity were transferred to two cylinders containing 4 ppt salinity ASW. One cylinder contained 1 mM cycloheximide dissolved in ethanol (final concentration of ethanol in ASW less than 0.1%), and the other contained ASW and the same concentration of ethanol (control). This concentration of cycloheximide is sufficient to inhibit 95% of protein synthesis in developing nauplii and does not result in mortality of the organisms (Conte et al., '73). Duplicate subsamples were removed from each cylinder, and ODC activity was determined at times 0, 0.45, 1.5, and 3 hr after transfer. Additionally, a subsample was removed from the control treatment at time 0.45 hr and transferred to a third cylinder containing cycloheximide as described above.

Effects of ionic or osmotic induction of ODC activity

Nauplii were exposed to either reduced total osmotic concentration (i.e., reduced salinity) or reduced NaCl concentration at constant osmotic concentration in order to determine which was the critical factor for the induction of ODC. Stage 1 nauplii (24 hr) were reared in 32 ppt ASW and transferred to four cylinders containing either 32 ppt ASW (controls), or 32 ppt ASW diluted to 22, 12, or 4 ppt with distilled water (producing reductions in both total osmotic and ionic concentrations). A second set of nauplii were transferred

to four additional cylinders containing either 32 ppt ASW (controls), or 32 ppt ASW diluted to 22, 12, or 4 ppt with distilled water, and then restored to 32 ppt (ca. 1,000 MOsm as determined by a Wescor 5500 vapor pressure osmometer) with sucrose to produce treatment conditions of constant osmotic concentration but reduced concentrations of NaCl. Nauplii were exposed to these treatments for 2 hr and were then removed and assayed for DFMO-sensitive ODC activity and DFMO-insensitive decarboxylase activity.

Determination of ornithine decarboxylase activity

ODC activity was determined according to the modified procedure of Landy-Otsuka and Scheffler ('78). Cysts and embryos were homogenized on ice (1:4, wet weight: reaction buffer) with a Brinkmann Polytron and then sonified with a Branson Sonifier Model 350. Cyst disruption was verified by microscopic analysis. Reaction buffer consisted of 50 mM K_2HPO_4 buffer, pH 7.5, 5 mM dithiothreitol, 0.2 mM EDTA, and 0.05 mM pyridoxal 5-phosphate. The crude homogenate was centrifuged for 20 min at 0°C and 20,000 RCF in a Sorvall RC-5 superspeed centrifuge, and the supernatant was retained for ODC analysis.

The specific activity of ODC was determined by measuring the release of $^{14}CO_2$ from DL-[1-^{14}C] ornithine hydrochloride (CFA.423, Amersham, specific activity 58 mCi/mmol). The enzyme reaction was performed in a 16-mm (ID) × 100-mm borosilicate test tube capped with a double-seal rubber stopper penetrated by a plastic centerwell (Kontes). The centerwell contained a 2 × 3-cm square of Whatman #1 filter paper saturated with 100 µl NCS tissue solubilizer (Amersham). First, 150 µl of the above supernatant was added to each tube and the reaction was initiated by addition of 30 µl of 0.5 µCi DL-[1-^{14}C] ornithine hydrochloride and cold L-ornithine (Sigma, final concentration of total L-ornithine, approximately 1.2 mM). The reaction was stopped after incubation for 90 min at 25°C by injecting 0.5 ml 5% trichloroacetic acid into all tubes. These tubes were allowed to stand overnight to permit maximal absorption of CO_2. Control tubes used to determine endogenous $^{14}CO_2$ release were prepared by adding first 0.5 ml TCA followed by 150 µl of ODC supernatant and 30 µl L-ornithine. Additional controls were performed by adding difluoromethyl ornithine (inhibitor of ODC, final concentration, 1 mM, gift from Merrell Dow) to the reaction mixture during incubation. Filter papers from all tubes were removed and placed in scintillation vials containing 4.0 ml Packard Scint A-XF scintillation fluid. Vials were allowed to stand in the dark overnight to reduce chemiluminescence before measuring radioactivity in a Beckmann LS5801 liquid scintillation counter. Specific activity of ODC was expressed as nmoles ornithine converted to putrescine in 1 hr per g wet weight tissue, per g dry weight tissue, per mg DNA, and per mg protein.

Determination of polyamines

Putrescine, spermidine and spermine were extracted and derivatized using a procedure modified from Watts et al. ('87). Cysts or embryos were homogenized in distilled water (1:4, w:v) using a Brinkmann Polytron and then sonified with a Branson Sonifier Model 350. Polyamines were extracted in the presence of perchloric acid (final concentration 0.45 N) at 0°C for 30 min. The acidified homogenate was centrifuged at 20,000 RCF and 0°C for 20 min, and the clear supernatant was retained for dansylation.

Forty milligrams sodium bicarbonate was placed in 8-ml capped glass tubes. Either 200 µl of the extracted supernatant or 200 µl of each standard (Sigma) was added to each tube. All tubes received 400 µl of dansyl chloride (20 mg/ml acetone, Pierce Biochemical) and were capped, vortexed, and incubated at 60°C for 1 hr in the dark. Samples were continuously mixed on an orbital shaker. At the end of 1 hr, 100 µl of proline (100 mg/ml H_2O, Sigma) was added to bind excess dansyl chloride. Tubes were incubated as above for 1 hr. Excess acetone was removed by vacuum evaporation. One milliliter of toluene was added to each tube; tubes were recapped, vortexed for 30 sec, and centrifuged at 200 RCF (Beckmann J-6) for 10 min at room temperature. Toluene was removed by pipet and the aqueous layer was extracted with another 1 ml of toluene. The two toluene extractions were pooled, dried, and resuspended in high-performance liquid chromatography (HPLC) grade methanol. The methanol extract was filtered through a 0.2-µm Teflon filter and used for HPLC.

HPLC was performed using a Perkin-Elmer Series 410 BioLC pump equipped with a Shimadzu RF 535 fluorescence detector interphased with a Nelson 760 series integrator. Samples were injected into a Rheodyne injector, and polyamines were separated on a 3-cm, reverse-phase C_{18} column with 5-µm pore size. Separation was accomplished using a gradient of acetonitrile and heptane sulfonic acid (5 mM, pH 3.5)/triethylamine (0.1%).

Statistical significance between treatments was determined by Student's t-test. Values were considered significantly different if $P < .05$.

RESULTS

Hypoosmotic stimulation of ODC activity and polyamines

During hypoosmotic exposure, ODC activity increased with a decrease in environmental salinity in stage 1 nauplii (Fig. 1A). These increases in ODC activity were correlated inversely with the exposure salinity. Maximal increases in activity were seen within 2 to 3 hr. The increase in activity occurred rapidly; ODC activity was 40% higher in organisms exposed to 4 ppt salinity than the controls (32 ppt) within 15 min of exposure, and almost doubled within 30 min (Fig. 1B). Similar time-course activation of ODC activity has been reported in mouse mammary tissues (Perry and Oka, '80).

Under control conditions (32 ppt) putrescine levels remained constant for approximately 3 hr and then steadily declined thereafter (Fig. 2A). These decreases correspond to normal developmental changes in putrescine content (Watts, unpubl. data). Superimposed on this pattern was a salinity-dependent increase in putrescine concentra-

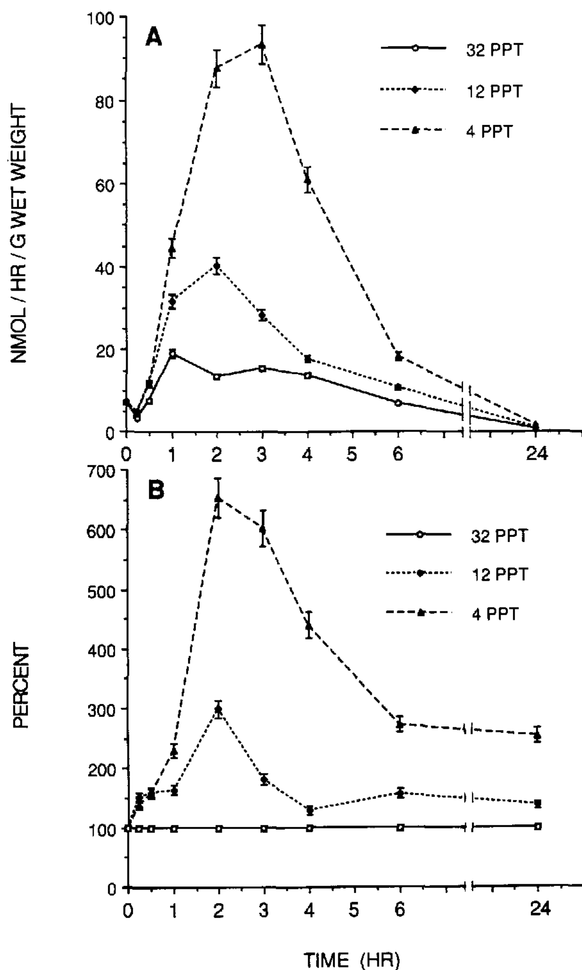

Fig. 1. Changes in DFMO-sensitive ornithine decarboxylase activity, expressed as **(A)** nmoles ornithine converted to putrescine per gram wet weight nauplii and **(B)** percent of control, during acute exposure to hypoosmotic conditions. Stage 1 *Artemia* nauplii were reared from cysts at 32 ppt salinity and transferred to either 32 (control), 12, or 4 ppt salinity. Values represent the mean and standard error of triplicate batches of nauplii (ODC was assayed in duplicate for each batch) removed at each time period post-transfer.

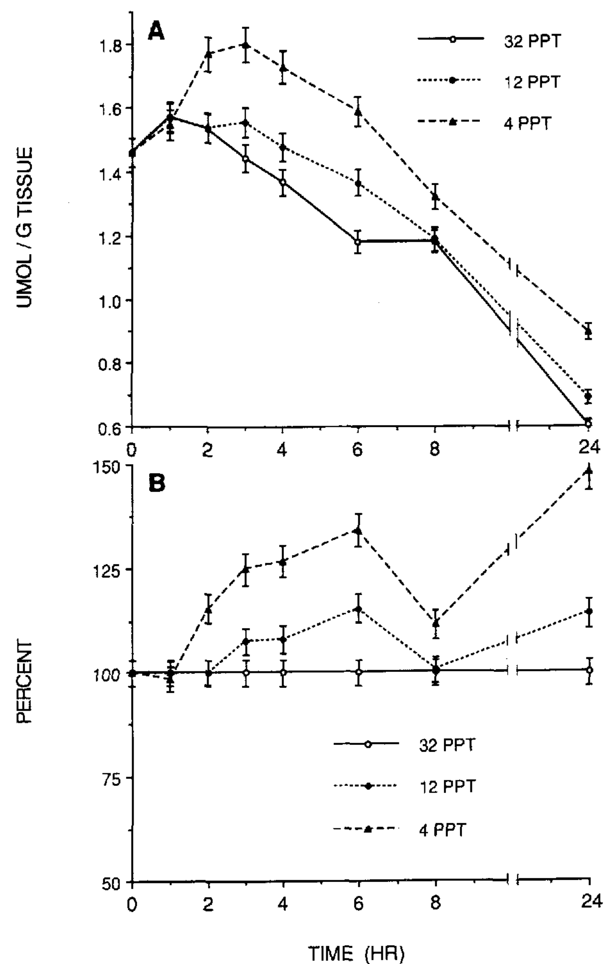

Fig. 2. Changes in putrescine levels, expressed as **(A)** µmoles per gram wet weight nauplii and **(B)** percent of control, during acute exposure to hypoosmotic conditions. Stage 1 *Artemia* nauplii were reared at 32 ppt salinity and transferred to either 32 (control), 12, or 4 ppt salinity. Values represent the mean and standard error of putrescine levels obtained from triplicate batches of nauplii (putrescine was assayed in duplicate for each batch and averaged) removed at each time period post-transfer.

tions. Putrescine concentrations were between 0.1 and 0.3 μmol/g higher in nauplii transferred to 12 ppt between 3 and 6 hr post-transfer. This difference increased to between 0.2 and 0.5 μmol/g for the 4 ppt treatment and was again significant (Fig. 2). The metabolic turnover of putrescine was not determined. The lack of differences among the treatments after 8 hr may be related to molting events (population transition from a stage 1 to a stage 2 nauplii). Spermidine and spermine levels were not affected by salinity (data not shown).

Effects of cycloheximide on induction of ODC activity

The addition of cycloheximide to the culture medium resulted in a decrease in ODC activity (Fig. 3). This decrease in activity was seen in batches of nauplii exposed at time 0 and assayed 0.75 and 1.5 hr later, and in batches exposed at time 0.75 hr after exposure to reduced salinity and assayed 0.75 and 2.25 hr later. The apparent half-life of this enzyme at these conditions was 40–50 min.

Effects of ionic or osmotic induction of ODC activity

The effects of decreased total osmotic concentration vs. decreased NaCl concentrations (at constant osmolality) on the activity of ODC are shown in Figure 4. DFMO-sensitive ODC activity was induced by a reduction of the total osmotic concentration of the ambient medium. The induction displayed a salinity-dependent response, with the largest increase in activity occurring at 4 ppt. At constant osmolality (1,000 mOsm) there were no differences in ODC activity for any of the three media with lowered NaCl concentrations. Remarkably, the presence of a DFMO-insensitive decarboxylase was also detected. This previously unreported enzyme showed induction in response to altered NaCl concentrations and not osmolality per se (Fig. 4).

DISCUSSION

Artemia, one of the most euryhaline crustaceans known, maintains its hemolymph hypoosmotic and hypo-ionic to the ambient medium at salinities above about 8 ppt, its isosmotic/iso-ionic salinity (Russler and Mangos, '78). Therefore, when transferred from 32 ppt to lower salinities, *Artemia* faces a reduced salt load but a greater water load from the medium. The presumptive physiological

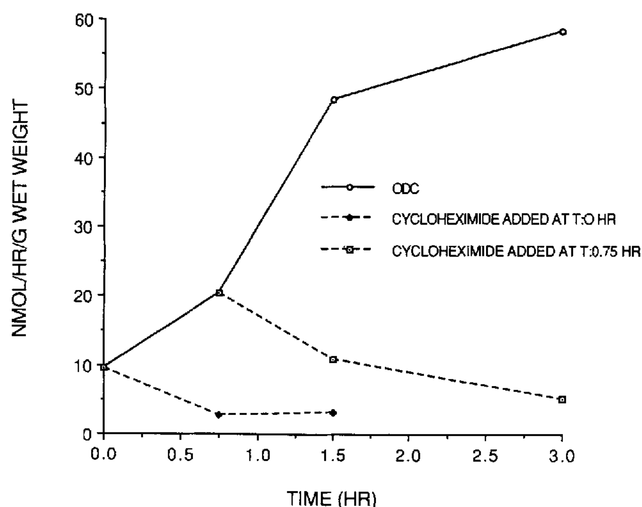

Fig. 3. Effects of cycloheximide (1 mM) on ornithine decarboxylase activity during exposure of nauplii to hypoosmotic conditions. Stage 1 nauplii were reared from cysts at 32 ppt salinity and transferred to either 32 ppt (control) or 4 ppt at time 0 hr or at time 0.75 hr. Values represent the means of duplicate determinations of ODC activity from two batches of nauplii removed at each time point post-transfer (historical variance around these means is <5%).

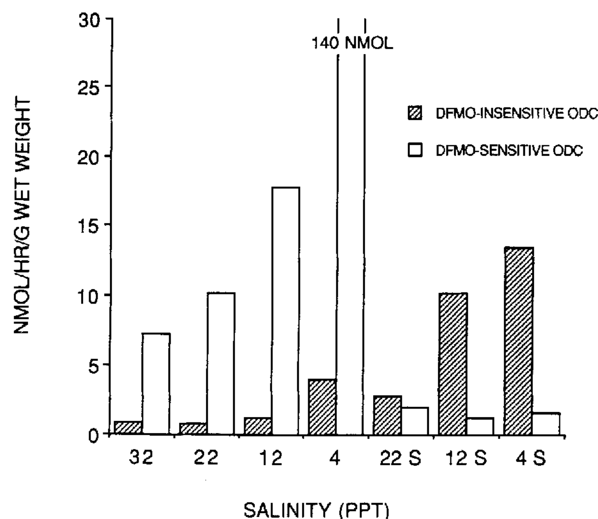

Fig. 4. Effects of variable ionic and osmotic exposure on DFMO-sensitive and DFMO-insensitive ornithine decarboxylase activity. Stage 1 nauplii reared at 32 ppt salinity from cysts were exposed to either 32 ppt (control) or 22, 12, or 4 ppt for 2 hr, representing reduced ionic and osmotic concentration. Similarly, nauplii were exposed to 22, 12, or 4 ppt salinity containing sucrose (S) which was added to adjust the osmotic conditions to that of the control (32 ppt, ca. 1,000 mOsm) for 2 hr, representing reduced ionic concentration only. At the end of 2-hr exposure, nauplii were harvested, rinsed, and assayed for ODC activity. Values represent the means of duplicate determinations (historical variance around these means is <5%).

responses would be a down-regulation of the salt secretion mechanisms and the initiation of an intracellular volume regulatory response.

Exposure of *Artemia* to reduced salinity conditions resulted in a dramatic increase in whole organismal ODC activity. The increase in ODC activity was inversely related to the level of salinity reduction, suggesting that the physiological induction of ODC activity was related to processes associated with acclimation to reduced environmental salinity. Although it was not done in this study, it would be of interest to examine tissue-specific changes in ODC activity in order to determine if the induction of activity was localized to ion-transporting epithelia, or whether it was a general tissue phenomenon.

Based on the studies using cycloheximide, the hypoosmotic-induced increase in enzyme activity appears to be the result of increased protein synthesis. Similar results were found using several mammalian tissues and cells (see Poulin and Pegg, '90, for references). The half-life of *Artemia* ODC was 40–50 min (based on our observed decreases in activity in the presence of cycloheximide) and is similar to that found in other cells (reviewed by Russell, '85). The speed of the induction of enzyme activity (occurring within minutes), coupled with the short apparent half-life, suggest that the modulation of ODC activity occurs very early in the cellular response to osmotic stress. The induction of ODC in *Artemia* represents one of the earliest activated enzyme systems that has been described in any euryhaline organism during hypoosmotic stress, as compared to hours or days for $Na^{+}K^{+}$ATPase and carbonic anhydrase in crustacean gills (Towle et al., '76; Neufeld et al., '80; Henry and Cameron, '82; Henry and Wheatly, '88). We have not attempted to determine if ODC induction is also regulated at the level of transcription; however, other investigators have found no effects when using transcription inhibitors such as actinomycin D (Perry and Oka, '80).

The increase in ODC activity during exposure to reduced salinities was the result of a reduction in the total osmotic concentration for the medium (i.e., reduced osmolarity) and not a reduction in the concentration of specific ions such as Na or Cl. Interestingly, it is also a reduction in osmotic concentration (osmotic pressure) that initiates the volume regulatory response in various invertebrate tissue and cell types (Pierce and Greenberg, '73; Amende and Pierce, '80). It is therefore plausible to suggest that the increase in cell volume (cell swelling) in response to media of lowered osmotic concentration is the initial signal for ODC induction.

Other studies have indicated that it is both the osmotic and ionic concentrations of the medium that regulate ODC activity in cells and tissues (Perry and Oka, '80; Viceps-Madore et al., '82; Lundgren and Prokay, '88). Sodium ion was an active regulator of ODC activity, being stimulatory in some tissues and inhibitory in others. Potassium has also been implicated in ODC regulation (Lundgren and Prokay, '88). While most of these results were obtained using tissues which generally exist in a stable osmotic and ionic environment, invertebrate tissues exist under conditions of fluctuating osmotic and ionic concentrations. Volume readjustment in some aquatic arthropod species is accomplished by an initial efflux of both Na^{+} and Cl^{-} and sometimes K^{+} (Kevers et al., '79, '81; Freel, '78; Warren and Pierce, '82). If this is the case in *Artemia,* then decreased intracellular Na^{+}, Cl^{-}, or K^{+} concentrations could also have been responsible for ODC induction. Alternatively, induction of ODC could be the first step in the physiological mechanisms responsible for the initial efflux of intracellular inorganic ions and/or intracellular organic compounds. Further research is necessary to elucidate the specific relationship between ODC activity and cell volume regulation.

Exposure of nauplii to reduced NaCl concentrations at constant osmolarity resulted in an unexpected increase in the activity of a DFMO-insensitive decarboxylase. Because this enzyme does not respond to DFMO (a known irreversible inhibitor of most ornithine decarboxylase molecules, including that in *Artemia*) (Bey et al., '87; Powell et al., '92), we presume it to be a non-specific decarboxylase (possibly a lysine decarboxylase) that has been induced or activated, and that is using ornithine as a substrate. The function of this enzyme is unclear. The appearance of this new enzyme may represent an alternative mechanism for polyamine synthesis, or it could have a separate role in cell volume regulation. The catabolism of free amino acids is known to increase in crustacean tissues exposed to hypoosmotic conditions (Haberfield et al., '75; Taylor et al., '77), and thus the DFMO-insensitive carboxylase may simply be involved in the general catabolic reduction of the intracellular amino acid pool that occurs at low salinity. Further studies are necessary to determine the nature and role of this enzyme.

As reported in other studies, putrescine is the only polyamine that shows an increase in concentration with exposure to reduced salinities. Levels of spermidine and spermine were not affected. The role of putrescine during hypoosmotic stress has not been determined in any study. However, the accumulation of putrescine reported in this and other studies, as well as the increase of transport and flux into cells during hypoosmotic stress, suggest that putrescine is involved in the maintenance of cellular homeostasis during hypoosmotic stress. Lee ('92) indicated that levels of polyamines, particularly putrescine, were inversely proportional to Na^+K^+ATPase activities in nauplii acclimated to various salinities. Furthermore, Lee determined that physiological levels of polyamines could inhibit Na^+K^+ATPase activity in vitro. It is therefore possible that polyamine production could influence the activity of Na^+K^+-ATPase in *Artemia,* in such a way that would effect the down-regulation of the processes involved in active NaCl secretion.

The mechanisms by which any molecule influences cell volume and ion regulation are not well understood (Mantel and Farmer, '83). Many studies have investigated the role of transport proteins and other proteins associated with these phenomena. Transport proteins such as the Na^+:H^+, Cl^-:HCO_3^-, and Na^+:K^+ transporters have been implicated in cell volume and ion regulation. However, mechanisms involved in short-term activation or inhibition, and long-term acclimation of these and other proteins are not known. The involvement of secondary cellular messengers has been postulated, but little is known of these or other molecules. We believe one or more of the biogenic amines may act as both short- and long-term activators or inhibitors of these proteins, either through direct interaction with the transport proteins or indirectly through changes in transcriptional and translational processes that influence the production and distribution of the proteins. The role of polyamines in the control and regulation of cell volume homeostasis is currently being investigated.

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