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POLYAMINES IN *Acanthamoeba castellanii*: PRESENCE OF AN UNUSUALLY HIGH, OSMOTICALLY SENSITIVE POOL OF 1,3-DIAMINOPROPANE

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High (15-25 mM) concentrations of 1,3-diaminopropane, a normally minor derivative of polyamine metabolism, have been observed in vegetative cells of *Acanthamoeba castellanii*. Trace amounts of a putative polyamine, which chromatographically behaved like norspermidine, were also found. The size of the intracellular pool of 1,3-diaminopropane was inversely related to the ambient osmolality and to the free amino acid levels during osmotic shock experiments. Due to its high concentration in *A. castellanii*, this diamine may be operative in ionic regulation during environmental stress. 1,3-diaminopropane may substitute for putrescine, a common diamine which was undetectable in *A. castellanii*.

Polyamines have been found in most organisms studied and have been widely recognized as regulatory factors in processes related to cell growth and differentiation (1,2). They have also been associated with osmotic (3) and thermal (4) adaptation, intracellular pH regulation (5), and cell membrane stabilization (6). However, their precise functions and mechanisms of action remain poorly understood.

In microorganisms and some plants, degradation of the polyamine spermidine occurs via dehydrogenation by a polyamine oxidase, yielding 4-aminobutyraldehyde, DAP[2] and a reduced acceptor as products (7). While 4-aminobutyraldehyde can be oxidized to 4-aminobutyric acid (GABA) (7), little is known about the metabolism of DAP. It is usually a very minor component, except in the cellular slime mold *Dictyostelium discoideum*, in which DAP concentrations can attain 8.5 mM (8,9). We report the presence of high levels

[2]Abbreviations: DAP, 1,3-diaminopropane; NPS, ninhydrin-positive substances; OGM, optimal growth medium; Tes, 2-{[2-hydroxy-1,1-bis-(hydroxymethyl)ethyl]-amino}ethanesulphonic acid; TLC, thin-layer chromatography.

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of DAP in another sarconid protozoan, Acanthamoeba castellanii. Interestingly, the more common diamine putrescine was undetectable in these cells in any of the growth conditions studied. Moreover, the intracellular pool of DAP was found to be negatively correlated with the osmolality of the suspending medium.

MATERIALS AND METHODS

Cell culture: Acanthamoeba castellanii, Neff strain, was grown in a proteose peptone/yeast extract medium supplemented with glucose (OGM) at 30°C in the dark (10). Some cultures were also obtained through serial transfers in a chemically defined medium (DGM-250) containing the 5 essential amino acids, as well as Gly, Lys, Thr, Trp, salts, vitamins and glucose as in (11), plus 22 mM mannitol and 10 mM CH_3 COOK. Initial pH was adjusted to 7.0. Osmolality was 240 and 250 mosmol/kg for OGM and DGM-250 respectively, as determined by cryoscopy.

Osmotic stress experiments: After 96 h growth in OGM, cells were harvested by centrifugation (1930 g, 90 s), pooled and washed 3 times in DGM-250 (buffered with 10 mM Tes, pH 7.0 and readjusted to 250 mosmol/kg by decreasing mannitol to 12 mM). Amoebae were resuspended in buffered DGM-250 at a final population density of 4-6 x 10^7 cells/ml and preincubated for 150 min at 30°C in 125 ml conical flasks with constant shaking. Osmolality was then either increased to 500 mosmol/kg (DGM-500) with buffered DGM-250 added with 306 mM mannitol, or decreased to 40 mosmol/kg (DGM-40) with a solution containing only the salts, vitamins and buffer of the preincubation medium. Final cell concentration was set to 3-5 x 10^6 cells/ml. Aliquots were withdrawn at indicated times, and washed 3 times with isosmotic incubation medium in which amino acids had been replaced with osmotically equivalent amounts of mannitol. Cells were sonicated in ice-cold 0.5 M $HClO_4$. The homogenate was centrifuged (15 min, 20 000 g, 0-4°C) and the supernatant kept at -20°C until analysis.

Analytical methods: Cell numbers were determined with a Coulter counter (10). Free amino nitrogen (NPS) was determined with a ninhydrin method (12) using glycine as standard, following addition of CH_3COOK and centrifugation of the precipitated $KClO_4$ at 0°C. Free proline, alanine and glutamate were assayed by dilution of the radioactivity of their ^3H-labelled dansyl/^{14}C-labelled amino acid derivatives (13). Polyamines were analyzed using either qualitative TLC or quantitative HPLC of their dansyl and benzoyl derivatives, respectively (14). Micropolyamide 5 x 5 cm sheets (Schleicher and Schuell, Keene, NH) were used for TLC. Chromatography was either one-dimensional with benzene:acetic acid (9:1 v/v) or two-dimensional using this solvent in the second direction, and 88% (w/v) formic acid:H_2O (2:100 v/v) in the first direction. HPLC was performed using a Waters system (Model 680 controller, Model 510 pumps, Model UGK injector and Model 440 UV detector). Separations were obtained on a μ-Bondapak C_{18} reverse phase column (3.9 x 300 mm; 10 μm) at a flow rate of 1 ml/min using 64% methanol (HPLC, Fisher Scientific) in water. Mass spectra were obtained with a Hewlett-Packard 5992 GC-mass spectrograph (direct inlet probe; electron beam energy, 8 eV).

RESULTS AND DISCUSSION

Analysis of polyamines in Acanthamoeba castellanii grown under different conditions consistently revealed the presence of high levels of DAP (Table 1). Confirmation of DAP identity came from its cochromatography with authentic

Table 1. Intracellular contents in DAP in A. castellanii grown under various conditions

Condition (Medium / time)	DAP content (fmol/cell)
OGM / 27 h	20.1
OGM / 96 h	24.3
OGM / 96 h + DGM-250 / 2.5 h	45.1
DGM-250 / 96 h	37.1

didansyl- or dibenzoyl-DAP applied together with cell extracts, and from mass spectrometry of its benzoyl derivative (molecular ion peak, m/e = 282.20). The cellular concentration of DAP, calculated using previous determinations of cell water contents under similar conditions (15), was estimated at 15-25 mM. A putative polyamine present in trace amounts in the cells cochromatographed with norspermidine (3,3'-iminobispropylamine) in both TLC and HPLC. Putrescine, as well as cadaverine, agmatine, spermidine and spermine were undetectable even after overloading the chromatograms. Cellular levels in the micromolar range would have been detected with the dansylation procedure used.

Only in Dictyostelium discoideum have comparably high levels of DAP (8.5 mM) been reported (8,9). However, in this organism putrescine and spermidine were also found in unusually high amounts (14 and 1.6 mM, respectively) (8,9). Physarum polycephalum also contains high levels of putrescine along with lower spermidine contents, though no DAP has been reported (16). It thus appears that the presence of a high diamine pool is a common feature among sarconid protozoans. Nevertheless, the predominance of DAP in A. castellanii indicates a unique pattern of polyamine metabolism.

We observed an inverse correlation between ambient osmolality and cellular contents in DAP when A. castellanii was exposed to an osmotic shock (Fig. 1a). A sudden, two-fold increase in osmolality induced a rapid reduction of DAP level to approx. 25% of its initial value. Conversely, a decrease from 250 to 40 mosmol/kg slowed its depletion from the onset of stress. The slow but steady decrease of DAP observed under isosmotic conditions could result

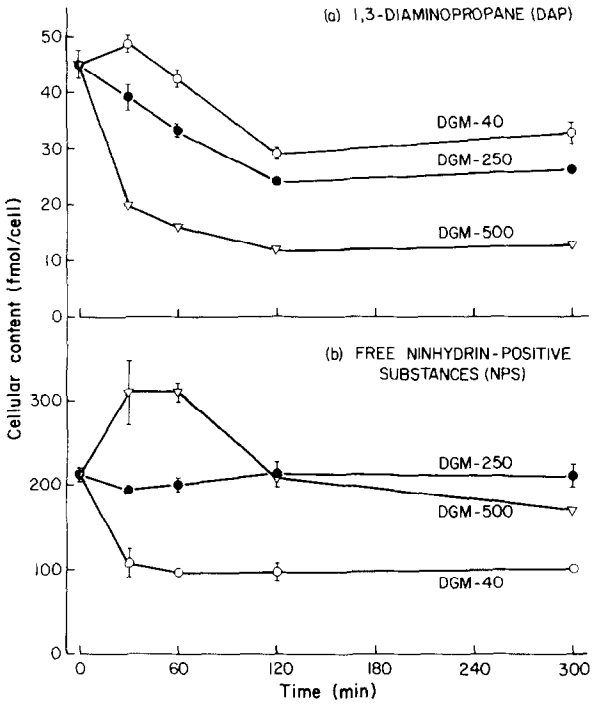

Fig. 1. Effect of osmotic shocks on cellular contents in (a) DAP and (b) free NPS in A. castellanii. Free NPS do not include proline. (○ , ● , ▽ : 40, 250 and 500 mosmol/kg, respectively). Results are mean ± S.E.M. (vertical bars).

from the unavoidable drop of population density at zero time of measurements, and from continuing adjustment to DGM-250.

Free amino acids are preferentially used as part of a regulatory response in A. castellanii undergoing acclimation to osmotic stress (10,17). The sudden lowering of intracellular levels of DAP under hyperosmotic conditions coincides with the early accumulation of free NPS (fig. 1b). It is also concomitant with accumulation of alanine and precedes marked increases in glutamate and proline contents (results not shown). A sharp decrease in free NPS (Fig. 1b) and free amino acids (17) occurs early after a hypo-osmotic shock, at a time when DAP contents remain stable (Fig. 1a). We were unable to detect significant amounts of DAP in the suspension medium from any osmotic condition. An amount of DAP corresponding to a 10% decrease in the initial cellular level would have easily been detected had the diamine been released into the medium. These observations suggest that the rate of metabolic interconversions between

DAP and amino acids may depend on osmolality, leading to opposite rates of accumulation in the two pools with changing osmotic conditions. This is supported by preliminary experiments indicating that aminooxyacetate, a broad spectrum inhibitor of pyridoxal-5'-phosphate-dependent enzymes, delays the changes in both DAP and free amino acid pools (unpublished results).

However, pathways connecting DAP to amino acid metabolism are far from clear. With the exception of the enzyme from one bacterial species, diamine oxidases from various sources do not use DAP as a substrate *in vitro* (18). Furthermore, most aminotransferases are inactive towards DAP (19), and may even be inhibited by this diamine (20). Spermidine, and to a lesser extent, spermine, are the usual precursors of DAP (7). Both are derived from putrescine (1,2), making the origin of DAP in *A. castellanii* uncertain in view of the absence of known precursors in our strain. It is known that 2-difluoromethylornithine, a potent irreversible inhibitor of eukaryotic ornithine decarboxylase (EC 4.1.1.17), the key enzyme in polyamine biosynthesis (1,2), does not affect growth in *A. castellanii* (21). Further information on DAP metabolism in this species is thus needed to evaluate its relation with the regulation of amino acid metabolism.

The four-carbon homolog putrescine is also regulated by external osmolality in *E. coli* (22), mammalian cell lines (23,24) and higher plants (3). A specific role for putrescine is indicated by the fact that spermidine and spermine are little affected under the same conditions (3,22-24). It has been suggested (5,8,24) that diamines, being divalent cations under physiological conditions ($pK_1 > 9$) and having a high affinity for anionic macromolecules (25), could play many as yet undefined roles in the preservation of macromolecular integrity, especially under conditions of inorganic ion deficiency (25). It is of interest that in *D. discoideum* grown in the presence of DAP, putrescine is largely displaced by the expanding pool of DAP (8). This raises the possibility that both diamines are at least partially interchangeable for this organism, as growth is little affected (8).

Although the presence of norspermidine in A. castellanii still awaits confirmation by chemical analysis, it is worth noting that this unusual polyamine is synthesized from DAP in several organisms living under extreme environmental conditions (26).

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