An Early Enlargement of the Putrescine Pool Is Required for Growth in L1210 Mouse Leukemia Cells under Hypoosmotic Stress*

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Hypoosmotic stress is a potent inducer of ornithine decarboxylase (ODC) activity in a variety of mammalian cells, but the physiological relevance of this response has not been determined. To test whether an increased putrescine content confers a growth advantage at lower osmolarities, we compared the ability of L1210 mouse leukemia cells and of ODC-overproducing variants obtained from this cell line (D-R cells) to proliferate after a hypotonic shock (325 → 130 mosmol/kg). The growth rate of D-R cells at 130 mosmol/kg was ≥ 5-fold higher than in L1210 cells; and unlike the ODC-overproducing strain, L1210 cells underwent up to a 90% loss of viability over time as seen after restoration of normosmotic growth conditions and by trypan blue exclusion tests. The addition of putrescine or L-ornithine stimulated the proliferation of both cell sublines up to 5-fold in a concentration-dependent manner, with a maximal effect observed at about 10 and 100 μM, respectively. Putrescine restored virtually normal growth rates in both sublines at osmolarities as low as 190 mosmol/kg. No other α,ω-diamine was active in that respect whereas spermidine was markedly inhibitory. Furthermore, D-R cells incubated at 130 mosmol/kg showed a marked growth inhibition by 1-aminooxy-3-aminopropane (potent ODC inhibitor to which they are resistant in isotonic media) as a result of putrescine but not spermidine depletion. Whereas ODC was strongly and rapidly induced by hypotonic shock there was a precipitous decline in S-adenosylmethionine decarboxylase activity. Putrescine synthesis and accumulation were nevertheless reduced in D-R cells incubated at 130 mosmol/kg because of a decreased availability of L-ornithine. When either putrescine or L-ornithine was added to hypotonic media, D-R cells accumulated putrescine massively for extended periods together with a reduction in spermidine and spermine contents. Putrescine transport patterns were altered by hypotonic shock, net excretion of the diamine being reduced by about 80%, with a concurrent enlargement of the intracellular pool. Finally, parental L1210 cells incubated with an irreversible inhibitor of S-adenosylmethionine decarboxylase for 24 h until hypotonic shock and supplemented with putrescine in the presence of the drug thereafter exhibited a greatly exaggerated growth stimulation by the diamine. These results demonstrate an essential role for an early increase in putrescine content in the growth adaptation of a mammalian cell line to a lower osmolarity.

Although the naturally occurring polyamines, spermidine and spermine, and the diamine, putrescine, are now recognized as essential for cell growth in eukaryotes (1–3), their exact biological sites of action remain elusive. Spermidine and spermine, because of their higher positive charge at physiological pH, are likely to be bound to macromolecular anions in the cell, and most of their putative roles (e.g. nucleic acid structure modifiers, membrane stabilizers) are related directly to that property. On the other hand, putrescine is believed to serve mainly as a precursor of spermidine, together with decarboxylated S-adenosylmethionine, through the reaction catalyzed by spermidine synthase. Accordingly, the intracellular pool of putrescine is usually lower than that of spermidine and spermine in eukaryotes (1–3). Furthermore, only spermidine is able to restore maximal proliferative activity in mammalian cells depleted of polyamines through a blockade of both putrescine and decarboxylated S-adenosylmethionine biosynthesis (4, 5).

Osmoregulatory processes are important to most, if not all, organisms for the stabilization of the intracellular milieu against environmental fluctuations of water and ion activities. It is also recognized increasingly that volume or turgor regulation is an especially important function in cells involved in elevated solute transport, such as rapidly proliferating cells or various epithelial and endothelial cells (6–9). It is therefore not surprising that despite the divergence of biochemical pathways leading to the maintenance of volume (or thermodynamic water activity) both prokaryotic and eukaryotic organisms share several physiological responses to osmotic stress. One example is the almost universal use of amino acids and quaternary amines for osmoregulatory purposes in living cells (10). As the main osmolyte in most nonhalophilic species, K^+ content is modulated by ambient osmolality in both bacterial and animal cells through highly regulated transport systems (9). Very little is known about the regulatory aspects of cell functions which are narrowly dependent on K^+ in the presence of fluctuations of that ion.

Considerable evidence points to a specific role of putrescine under osmotic or ionic stress situations in both prokaryotes and eukaryotes. In several bacterial species there is a striking increase in putrescine content related inversely to medium osmolality whereas the spermidine level remains relatively unaffected (11–14). Polyamine auxotrophs have considerably more stringent growth requirements for putrescine in media

of low osmotic strength (15, 16). Furthermore, the expression of ornithine decarboxylase (EC 4.1.1.17; ODC)[1], the enzyme responsible for the biosynthesis of putrescine in mammalian cells, is enhanced strongly and rapidly upon lowering medium osmolality in various mammalian cell types (17–22). Although an increase in putrescine content is indeed observed after transfer to hypotonic conditions (17, 19, 20) the significance of this apparently widespread phenomenon in animal tissues is not known.

Recently, we have studied the mechanism responsible for ODC induction by hypotonicity in variant L1210 mouse leukemia cells (D-R) selected for resistance to the irreversible ODC inhibitor α-difluoromethylornithine (DFMO) through stable overexpression of the enzyme (4). Hypoosmotic shock led to the accumulation of ODC molecules through a severalfold increase in the rate of synthesis of the enzyme, in conjunction with a decreased rate of proteolysis, without any change in the ODC mRNA content (22). In the present work we assessed the possible adaptive function of an increased production of putrescine which would be expected to accompany the hypoosmotic stimulation of ODC expression. To compare the effect of hypoosmotic stress on growth in parallel with differences in the intracellular pool size of the diamine we have exploited the availability of two L1210 cell strains that have widely different putrescine contents as a result of a more than 100-fold difference in basal expression of ODC. Several lines of evidence are presented which show that an early increase in putrescine content is a major determinant of the ability of L1210 cells to grow in media of reduced osmolality.

EXPERIMENTAL PROCEDURES

Materials—L-[1-^{14}C]Ornithine (50–54 mCi/mmol) and S-[*carboxyl*-^{14}C]adenosyl-L-methionine (56.9 mCi/mmol) were obtained from Du Pont-New England Nuclear. 1-Aminooxy-3-aminopropane (AOAP) was kindly provided by Dr. J. A. Secrist (23). DFMO and 5'-(((Z)-4-amino-2-butenyl)methylamino)-5'-deoxyadenosine (MDL 73811) (24) were generous gifts from the Merrell Dow Research Institute (Cincinnati, OH). Amino acids and vitamins used for the fabrication of modified RPMI 1640 media were from Sigma and GIBCO/BRL Life Technologies, Inc. Other biochemical reagents were of analytical grade and were obtained from Sigma, Fluka, ICN BioMedicals, J. T. Baker Inc., and Fisher.

Cell Culture Conditions—L1210 mouse leukemia cells and their ODC-overproducing variants (D-R cells) were grown as suspension cultures as described previously (22). D-R cells were grown from 48 to 72 h in the absence of DFMO prior to osmotic shock experiments in order to relieve the inhibition of ODC activity.

Hypoosmotic Stress Experiments—Cells in late exponential growth phase were harvested by low speed centrifugation and resuspended at 5 × 10^5 cells/ml in RPMI 1640 medium with the standard NaCl concentration (103 mM) and 14 mM sodium Hepes, 6.9 mM Mops (pH 7.4), 2 mM L-glutamine, antibiotics, and 10% (v/v) Nu-Serum (Collaborative Research Inc., Lexington, MA) as supplements. Aminoguanidine (1 mM) was added to all experimental media to inhibit the production of toxic products by amine oxidases present in the serum supplement. (The addition of aminoguanidine had no effect on polyamine contents or ODC activity under any osmotic condition tested.) Hypoosmotic shock was performed by transferring 1 volume of cell suspension to flasks or dishes containing 9 volumes of medium (preequilibrated at 37 °C and 5% CO$_2$) with the NaCl concentration adjusted to yield the indicated final osmolality, all other medium components being kept constant. The total volume of medium used was 10 ml for Falcon 100-mm dishes and 50–60 ml for T$_{150}$ flasks (Corning). Osmolality was measured using a freezing-point depression

[1] The abbreviations used are: ODC, ornithine decarboxylase; DFMO, α-difluoromethylornithine; AdoMetDC, S-adenosylmethionine decarboxylase; AOAP, 1-aminooxy-3-aminopropane; MDL 73811, 5'-(((Z)-4-amino-2-butenyl)methylamino)-5'-deoxyadenosine; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Mops, 4-morpholinepropanesulfonic acid.

osmometer (Advanced Instruments). Under those conditions, osmolality, total Na$^+$ (contributed from NaCl and Na$_2$HPO$_4$, NaHCO$_3$, and sodium Hepes/Mops buffers), and total Cl$^-$ (from NaCl and KCl) concentrations were varied from 130 to 325 mosm/kg (40–100% of control), 52 to 144 mM (36–100% of control), and 15 to 108 mM (14–100% of control), respectively. Cells were incubated for the indicated period at 37 °C in a 5% CO$_2$, 95% air atmosphere at 95% relative humidity. The cell number was determined with a Coulter Counter (model ZF; Coulter Electronics, Hialeah, FL) from aliquots taken at given times from triplicate dishes or flasks. Cell viability was assessed by trypan blue exclusion under a light microscope.

Polyamine Analysis and Enzyme Assays—Cells from osmotic shock experiments were harvested by centrifugation (1,930 × g for 90 s at 4 °C), washed once with 10 ml of serum-free RPMI 1640 salts/glucose solution made isosmotic to growth medium with NaCl (*i.e.* containing only inorganic salts, glucose, and Hepes/Mops buffer, pH 7.4, at the same concentration as in complete RPMI 1640 medium), and extracted with 10% (w/v) trichloroacetic acid. In order to measure extracellular polyamines, 0.5 ml of 100% (w/v) trichloroacetic acid was added to 4.5-ml aliquots of suspension media. After centrifugation (15,000 × g for 15 min at 4 °C) and extraction of trichloroacetic acid with 2 × 20 ml of diethyl ether, the extract was lyophilized and reconstituted with 300 μl of H$_2$O. The polyamine concentration was determined by ion-pairing high performance liquid chromatography (25). ODC and S-adenosylmethionine decarboxylase (AdoMetDC) activities were assayed by measuring the enzymatic release of ^{14}CO$_2$ from L-[1-^{14}C]ornithine and S-[*carboxyl*-^{14}C]adenosyl-L-methionine, respectively (26, 27). Protein content was measured by the method of Bradford (28) using bovine serum albumin (fraction V) as standard.

RESULTS

Comparison of the Ability of Normal L1210 Cells and Their DFMO-resistant Counterparts to Grow under Hypoosmotic Stress—D-R cells have a 5–10-fold larger basal putrescine content than parental L1210 cells when grown in the absence of DFMO (4, 23). Both cell lines were subjected to a 60% decrease in medium osmolality (325 → 130 mosmol/kg) by selectively decreasing the NaCl concentration of RPMI 1640 medium, conditions that had been found previously to induce the accumulation of enzymatically active ODC maximally (22). L1210 and D-R cell lines had very similar growth rates under normosmotic conditions (Fig. 1). However, only D-R cells grew exponentially at 130 mosmol/kg albeit with a much longer generation time than under normosmotic conditions (12 ± 3 and 66 ± 14 h at 325 and 130 mosmol/kg; mean ± S.D. from 7 and 13 separate experiments, respectively). Parental L1210 cells showed an extremely slow increase in cell number when incubated at 130 mosmol/kg (Fig. 1). Neither L1210 nor D-R cells underwent a general decrease in their viability for up to 48 h upon exposure to hypotonic shocks of that magnitude, as shown by the rapid resumption of normal growth rates after restoring normosmotic growth conditions (Fig. 1). ODC-overproducing L1210 cells thus possess a striking advantage over the parental cell line in their ability to proliferate at a low osmolality.

If a requirement for putrescine develops upon transfer to a hypotonic medium, addition of the diamine should stimulate growth under the new osmotic conditions. Indeed, putrescine (10 μM) strongly stimulated the growth rate of both cell sublines in hypotonic media (Fig. 2). In D-R cells, addition of the diamine decreased the generation time from 66 ± 14 to 32 ± 7 h (*n* = 12) while having no significant effect on cell growth at 325 mosmol/kg. Putrescine addition also exerted a major stimulation on L1210 cell proliferation exposed to hypotonic shock although their growth rate was still much inferior to that of their ODC-overproducing counterparts at the lower osmolality. When reversal to normosmotic conditions was delayed to 6 days after hypotonic shock, the onset of exponential growth required about 4 days in L1210 cells whereas D-R cells were rescued without any detectable lag
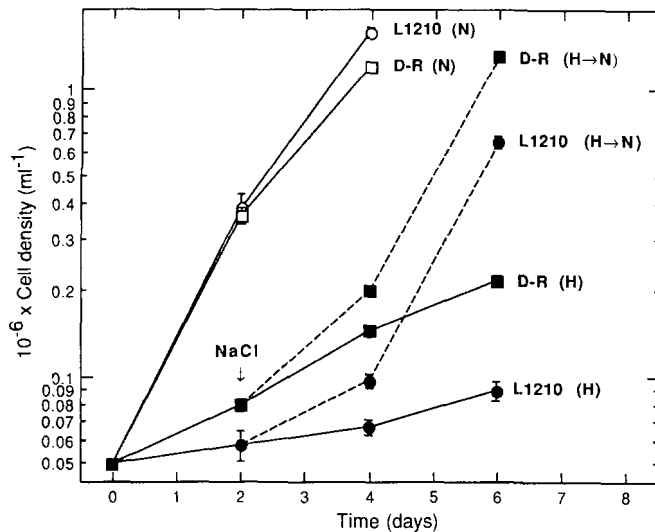
Putrescine and Response to Hypoosmotic Stress

FIG. 1. **Effect of hypoosmotic stress on the proliferation of L1210 and D-R cells.** At zero time exponentially growing L1210 (O, ●) or D-R cells (□, ■) were transferred to either normosmotic (N, 325 mosmol/kg) or hypoosmotic medium (H, 130 mosmol/kg). The cell number was measured at the indicated times. *Arrow*, normal osmolality was restored by the addition of NaCl to some cell cultures (H → N, *dashed lines*) 2 days after hypotonic shock. Each point is the mean ± S.D. of triplicate determinations from one representative experiment. Where not shown, the error was smaller than the symbol utilized.
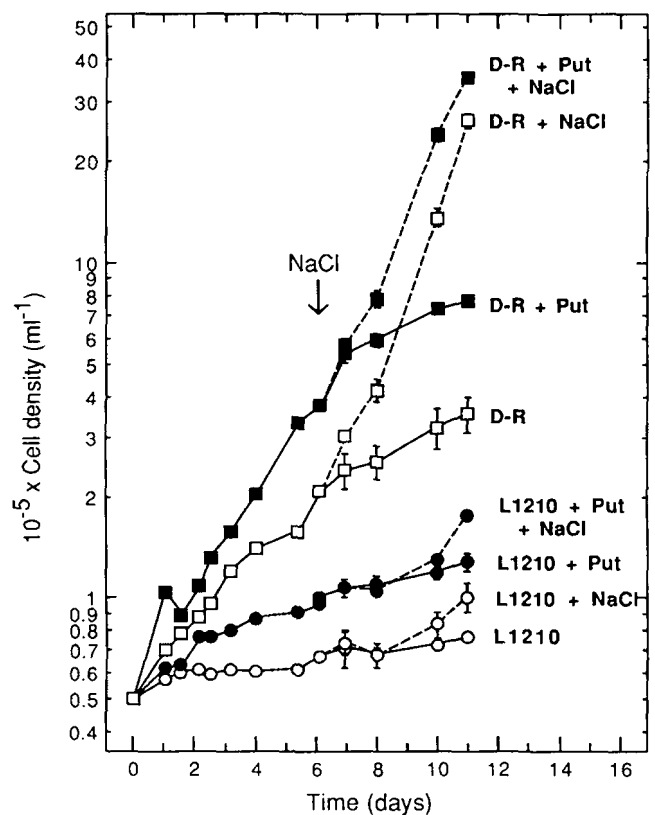

FIG. 2. **Effect of putrescine on cell proliferation in L1210 and D-R cells under hypoosmotic stress and reversibility of growth inhibition by late restoration of normosmotic conditions.** At zero time, exponentially growing L1210 (O, ●) or D-R (□, ■) cells were subjected to a hypoosmotic shock (325 → 130 mosmol/kg) and incubated in the presence (*solid symbols*) or absence (*open symbols*) of putrescine (10 μM) for the indicated period in T_{150} flasks at a seeding density of 5×10^4 cells/ml. After 6.3 days (*arrow*), triplicate 10-ml aliquots of cell suspension from each flask were transferred to 100-mm dishes, and cell growth was monitored for an additional 4.7-day period. Normosmotic conditions (325 mosmol/kg; *dashed lines*) were restored to half of the dishes by the addition of NaCl. Each point is the mean ± S.D. of triplicate determinations from one representative experiment.
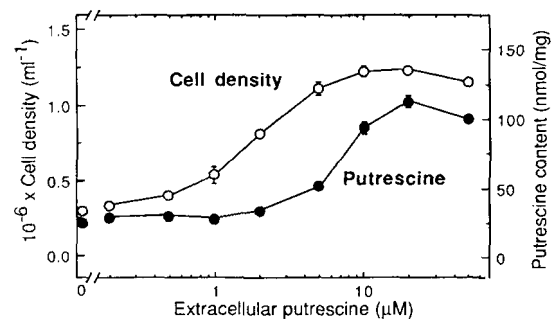
(Fig. 2). This difference was consistent with the viability of the respective subline at that stage (< 10% and 80% for L1210 and D-R cells, respectively), as estimated by trypan blue exclusion (results not shown). Putrescine supplementation did not affect the rate of growth recovery upon restoration of normotonicity in either L1210 or D-R cells (Fig. 2). Thus, although putrescine is a major limiting factor in the ability of L1210 cells to grow under hypoosmotic stress conditions their viability cannot be improved solely by the mere addition of putrescine at the time of hypotonic shock.

As little as 0.5 μM putrescine had a significant effect on the proliferation of D-R cells after 6 days although a maximal effect required diamine concentrations ≥ 10 μM (Fig. 3). Such treatment was accompanied by an increase in putrescine content. Putrescine concentration rose to as much as 7–8 mM, based on a median volume of 0.75 pl (as measured by electronic cell sizing and a protein content of 75 g·liter^{-1} (25)). On the other hand, spermidine content was unaffected by the addition of up to 50 μM putrescine whereas spermine level decreased by 30–40% (results not shown), presumably as a result of the massive increase in intracellular putrescine, a known inhibitor of spermine synthase at mM concentrations (2). Cadaverine decreased to undetectable levels in a concentration-dependent fashion, concomitantly with the accumulation of putrescine (data not shown). The addition of L-ornithine at concentrations up to 100 μM stimulated the proliferation of osmotically stressed D-R cells to the same extent as putrescine, in close parallel with an increase in putrescine content (data not shown). Neither putrescine nor L-ornithine (up to 500 and 300 μM, respectively) had any effect on D-R cell growth under normosmotic conditions. Thus, supplementation of D-R cells with putrescine or with its precursor promotes their proliferation at low osmolality and is associated with an intracellular accumulation of the diamine.

Spermidine as well as a series of α,ω-diamines that can share the putrescine transport system (29, 30) were evaluated

FIG. 3. **Concentration dependence of the effect of putrescine on growth and putrescine content in D-R cells under hypoosmotic stress.** D-R cells were grown for 6 days after transfer to hypoosmotic growth medium (325 → 130 mosmol/kg) from a starting density of 5×10^4 cells/ml, and cell number (O) as well as putrescine content (●) were determined. Putrescine was added at zero time at the concentration indicated. Each point is the mean ± S.D. of triplicate determinations from one representative experiment.

for their ability to affect the growth of D-R cells under hypoosmotic stress (Table I). No compound tested had any effect on cell proliferation at 325 mosmol/kg. Only putrescine stimulated cell growth under hypoosmotic conditions. Other

TABLE I

Effect of spermidine and various α,ω-diamines on cell proliferation in D-R cells under iso- and hypoosmotic growth conditions

At zero time, exponentially growing D-R cells were transferred to 60-mm dishes containing 5 ml of growth medium adjusted at 325 or 130 mosmol/kg with NaCl and containing the compound indicated (5 μM). The initial cell density was 5×10^4 cells·ml^{-1}. Cell density was then determined after a 3-day growth period. The values represent the mean $\pm$ S.D. ($n = 3$).

Compound	325 mosmol/kg		130 mosmol/kg	
	$10^{-5} \times$ Cell density	% of control	$10^{-5} \times$ Cell density	% of control
	ml^{-1}		*ml^{-1}*	
No addition	11.3 ± 1.2	100 ± 12	2.86 ± 0.20	100 ± 7
1,3-Diaminopropane	10.8 ± 0.3	95 ± 12	2.88 ± 0.09	101 ± 7
1,4-Diaminobutane (putrescine)	11.0 ± 0.9	97 ± 12	5.93 ± 0.33^a	207 ± 17
1,5-Diaminopentane (cadaverine)	10.1 ± 0.2	89 ± 2	2.88 ± 0.09	101 ± 2
1,6-Diaminohexane	10.8 ± 0.2	95 ± 2	2.88 ± 0.09	101 ± 2
Spermidine	10.1 ± 0.5	89 ± 2	1.56 ± 0.12^a	54 ± 2

^{a} $p < 0.01$ (Student's t test).

TABLE II

Effect of the ODC inhibitor AOAP on cell proliferation and polyamine contents in D-R cells grown under iso- and hypoosmotic conditions

D-R cells were incubated for 3 days (325 mosmol/kg) or 7 days (130 mosmol/kg) in 100-mm dishes containing 10 ml of medium at the indicated osmolality in the presence or absence of the drug. Cell cultures were harvested at the end of the experimental period for the determination of cell density and polyamine contents. Values represent the mean $\pm$ S.D. of three to six measurements from one representative experiment.

Treatment	Osmolality	$10^{-6} \times$ Cell density	Diamine and polyamine contents		
			Putrescine	Spermidine	Spermine
	mosmol/kg	*ml^{-1}*		*nmol/mg*	
Control	325	1.27 ± 0.02	18.1 ± 3.2	15.8 ± 1.5	4.8 ± 0.5
Control	130	0.61 ± 0.02	51.9 ± 5.4	13.0 ± 0.6	2.7 ± 0.1
+500 μM AOAP	325	1.19 ± 0.01	1.9 ± 0.2	8.1 ± 1.4	6.7 ± 1.1
+500 μM AOAP	130	0.21 ± 0.01	11.1 ± 2.4	10.7 ± 1.8	2.4 ± 0.4

diamines were completely ineffective whereas spermidine inhibited cell growth by 46%. Taken together, these results suggest that putrescine, or its synthesis from L-ornithine, is acutely limiting for an optimal growth rate in D-R cells subjected to hypoosmotic stress. Spermidine cannot substitute for putrescine, thus indicating that putrescine is not merely acting to compensate for a deficiency in spermidine synthesis which might arise from a reduced supply of decarboxylated S-adenosylmethionine, as a result of the repression of AdoMetDC activity by hypotonic shock (see Fig. 6A).

A direct consequence of a specific requirement for putrescine in D-R cells growing at low osmotic strength should be the development of a sensitivity to concentrations of ODC inhibitors to which they are resistant under normosmotic conditions. We therefore measured the effect of AOAP, an extremely potent reversible inhibitor of ODC (23, 31), on the proliferation of D-R cells as a function of medium tonicity. Although D-R cells are equally resistant to DFMO and AOAP (23) the latter was chosen for these studies because much lower concentrations are needed for inhibition, and the osmotic effect of the ODC inhibitor itself is therefore negligible. The D-R cells became markedly sensitive to AOAP (500 μM) at 130 mosmol/kg (Table II). This inhibition was associated with an 80% decrease of putrescine content in osmotically stressed cells with no significant effect on spermidine or spermine contents. On the other hand, although putrescine and spermidine were depleted by 89 $\pm$ 17 and 51 $\pm$ 10%, respectively, in drug-treated cells at 325 mosmol/kg, growth was totally unaffected by AOAP, as observed previously (23). Putrescine (10 μM) was able to reverse completely the AOAP-induced inhibition of cell growth (Fig. 4), indicating the specificity of the effect. Spermidine (up to 10 μM) did not prevent to any extent the growth inhibition induced by
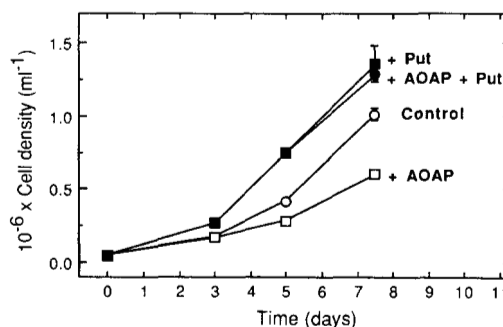

FIG. 4. **Effect of the ODC inhibitor AOAP on cell proliferation in D-R cells under hypoosmotic stress.** At zero time D-R cells were transferred to hypoosmotic medium (130 mosmol/kg) in the presence of AOAP (200 μM), putrescine (*Put*, 10 μM), or both compounds. The cell number was determined at the indicated times. Each point is the mean $\pm$ S.D. of triplicate determinations from one representative experiment.

the drug but rather decreased cell proliferation by itself (see Table I).

The apparent requirement for putrescine for growth under hypoosmotic conditions should also make L1210 cells more dependent on putrescine supplementation than D-R cells. This prediction was confirmed by measuring the relative stimulation of cell proliferation by the addition of 10 μM putrescine as a function of osmolality in the two sublines (Fig. 5). L1210 cells grew much more poorly and were differentially more sensitive to exogenous putrescine compared with the ODC-overproducing variants at osmolalities ranging from 140 to 270 mosmol/kg, with a maximal stimulation observed around 150 mosmol/kg. On the other hand, D-R cells became
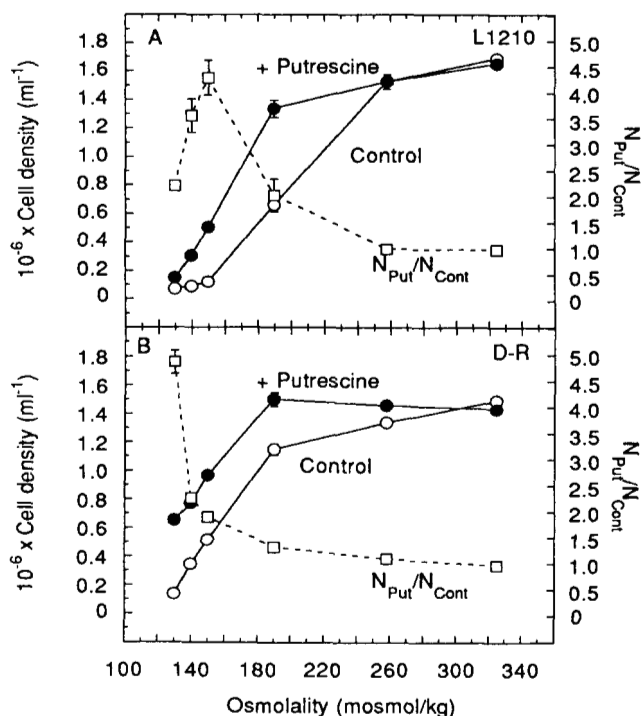
FIG. 5. **Effect of osmolality on growth stimulation by putrescine in L1210 and D-R cells.** L1210 (*A*) or D-R cells (*B*) were transferred to growth medium at the indicated osmolality obtained by varying the NaCl concentration, in the presence or absence of 10 μM putrescine. The cell number was determined after a 4-day incubation. *Left ordinate*, each point is the mean ($\pm$ S.D.) of triplicate determinations of cell number from one representative experiment. *Right ordinate*, the relative growth stimulation by putrescine (N_{put}/N_{cont}) is expressed as the ratio ($\pm$ S.D.) of the cell number observed in the presence of putrescine (N_{put}) to that measured in its absence (N_{cont}).

FIG. 6. **Time course of the effect of a hypoosmotic shock (325 $\rightarrow$ 130 mosmol/kg) on ODC and AdoMetDC activities in D-R cells.** *A*, at zero time, exponentially growing D-R cells were transferred to normosmotic (*N*, 325 mosmol/kg) or hypoosmotic (*H*, 130 mosmol/kg) growth medium at a density of 3.5×10^5 cells/ml. At the indicated times, AdoMetDC (*dashed lines*) and ODC (*solid lines*) activities were measured as described under "Experimental Procedures." *B*, cells were plated at the usual seeding density (5×10^4 cells/ml) in the presence (*solid symbols*) or absence (*open symbols*) of putrescine (*Put*), and ODC was measured at the indicated times. Each point is the mean $\pm$ S.D. of triplicate determinations from one representative experiment.

acutely dependent on putrescine addition only when osmolality was decreased to 130 mosmol/kg. Putrescine conferred a virtually complete independence of the growth rate on medium osmolality at a value as low as 190 mosmol/kg (*i.e.* 42% lower than at normotonicity) in D-R cells and to a slightly lesser degree in L1210 cells. It is apparent that putrescine is a major factor limiting potential for cell proliferation under hypotonic conditions and that D-R cells are likely to be better adapted in that respect because of their higher basal ODC activity and putrescine content.

Time Course of ODC and AdoMetDC Activities in D-R Cells under Hypoosmotic Stress—In order to examine in more detail the osmotic regulation of putrescine content, we next performed time course studies on the changes in ODC and AdoMetDC activities in D-R cells. As shown in Fig. 6A, hypotonic shock elevated ODC activity up to 20-fold, with maximal values at 24 h being 40 times higher than in parallel incubations in normosmotic medium. The increase observed was almost linear with time for the first 24 h, and a slow decline in ODC activity was observed thereafter although values remained higher than during growth at normotonicity throughout the time course (Fig. 6B). The absolute value of the peak of ODC activity induced by hypoosmotic shock was up to 2.5-fold higher than that reported in serum-free media (22) and is equivalent to a maximum of 3.3% of soluble proteins being represented by ODC, as calculated from the catalytic activity of the homogeneous enzyme from mouse kidney (26). The expression of ODC is still under negative control by putrescine (32, 33) under hypoosmotic conditions, as shown by the partial inhibition of enzyme induction ob-
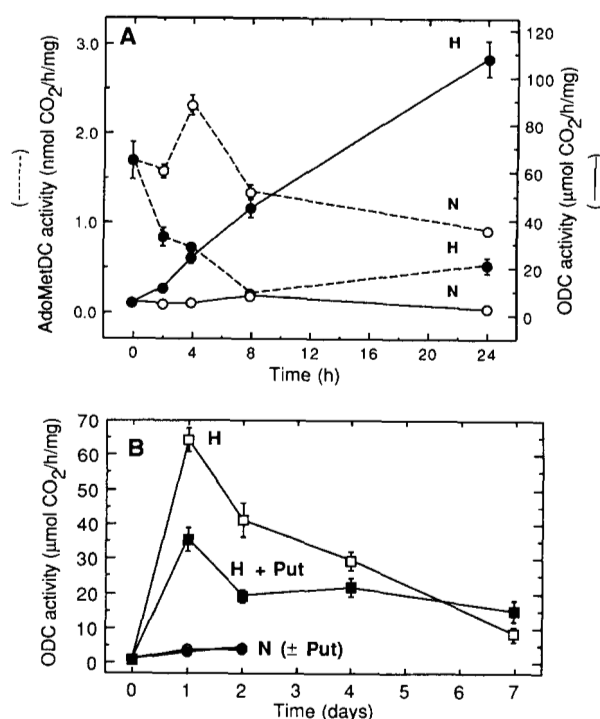
served in cells treated with putrescine at 130 mosmol/kg (Fig. 6B). The repression of ODC induction at 24 h after L-ornithine or putrescine addition was proportional to the extent of intracellular putrescine accumulation consecutive to these treatments (Table III). In marked contrast with the effect on ODC activity hypotonic shock elicited an exponential decline of AdoMetDC activity during the first 8 h, with a half-life of 2.8 h (Fig. 6A). On the other hand, AdoMetDC activity was relatively stable during the initial 8-h interval under isotonic conditions and decreased by about 50% only between 8 and 24 h of incubation. These data thus indicate that the expression of ODC and AdoMetDC is affected in a diametrically opposed fashion by hypoosmotic stress.

Time Course of Putrescine and Polyamine Content in D-R Cells under Hypoosmotic Stress—Intracellular putrescine content did not increase upon hypoosmotic shock despite the simultaneous dramatic induction of ODC and decrease in AdoMetDC activities (Fig. 7). Nevertheless, although putrescine content had decreased by about 50% after 24 h of incubation under either osmotic condition, hypotonicity delayed the onset of the decrease in putrescine levels to about 4 h after the osmotic shock (Fig. 7, *A* and *B*). Moreover, although cells incubated at 325 mosmol/kg excreted putrescine in a linear fashion between 2 and 24 h, there was a sharp reduction in extracellular putrescine at 130 mosmol/kg. Thus, net putrescine production, as measured by the total amount of the diamine present in cell extracts and growth media, was reduced by about 80% over a 24-h incubation under hypoosmotic shock, and the low amounts of the diamine released initially

TABLE III

Effect of putrescine (Put) and L-ornithine (Orn) addition on ODC activity and intracellular polyamine content in D-R cells exposed to hypoosmotic stress

Additions were made at time of transfer to the indicated osmotic condition. Other experimental conditions are described in the legend to Figs. 6A and 7, A and B.

Time	Osmolality	Addition	ODC activity	Diamine and polyamine content[a]			
				Putrescine		Spermidine	Spermine
				Intracellular	Extracellular		
h	*mosmol/kg*		*μmol CO$_2$/h/mg*	*nmol/mg*			
0	325	None	5.2 ± 0.2[b]	53.1 ± 0.7	<2	28.7 ± 0.2	5.2 ± 0.3
4	325	None	5.1 ± 0.5	29.6 ± 1.8	88.1 ± 7.1	26.3 ± 2.1	4.5 ± 0.3
4	130	None	24.1 ± 2.3	52.4 ± 2.9	36.8 ± 8.0	27.5 ± 1.5	4.7 ± 0.3
4	325	+10 μM Put	5.3 ± 0.4	52.3 ± 2.7	[8.3 ± 0.5]	27.4 ± 2.1	5.0 ± 0.9
4	130	+10 μM Put	26.5 ± 1.2	105.6 ± 3.8	[5.7 ± 1.2]	32.1 ± 2.0	4.4 ± 0.5
4	325	+100 μM Orn	3.6 ± 0.3	61.5 ± 8.4	116.9 ± 7.1	24.6 ± 2.1	4.3 ± 0.4
4	130	+100 μM Orn	23.6 ± 1.0	120.1 ± 1.7	24.0 ± 2.9	35.3 ± 1.2	4.1 ± 0.2
24	325	None	2.8 ± 0.1	29.4 ± 2.7	153.6 ± 12.0	25.3 ± 1.7	5.0 ± 0.8
24	130	None	108.4 ± 7.3	28.6 ± 0.3[c]	6.1 ± 0.5	27.6 ± 0.6	4.3 ± 0.9
24	325	+10 μM Put	2.6 ± 0.1	31.0 ± 0.6	[38.7 ± 0.7]	27.3 ± 0.7	4.7 ± 0.3
24	130	+10 μM Put	41.3 ± 1.3	55.1 ± 2.5	[3.1 ± 0.6]	11.1 ± 0.4	3.7 ± 0.3
24	325	+100 μM Orn	0.5 ± 0.1	42.1 ± 2.0	333.7 ± 45.9	27.7 ± 1.2	4.3 ± 0.2
24	130	+100 μM Orn	14.1 ± 0.5	146.9 ± 11.3	91.0 ± 7.8	10.4 ± 1.7	3.3 ± 0.9

[a] Values in brackets are given in μmol/liter.
[b] Mean ± S.D. of three or more replicate samples.
[c] Cadaverine was also detected intra- and extracellularly (9.4 ± 0.4 and 0.53 ± 0.13 nmol/mg, respectively) under these conditions.
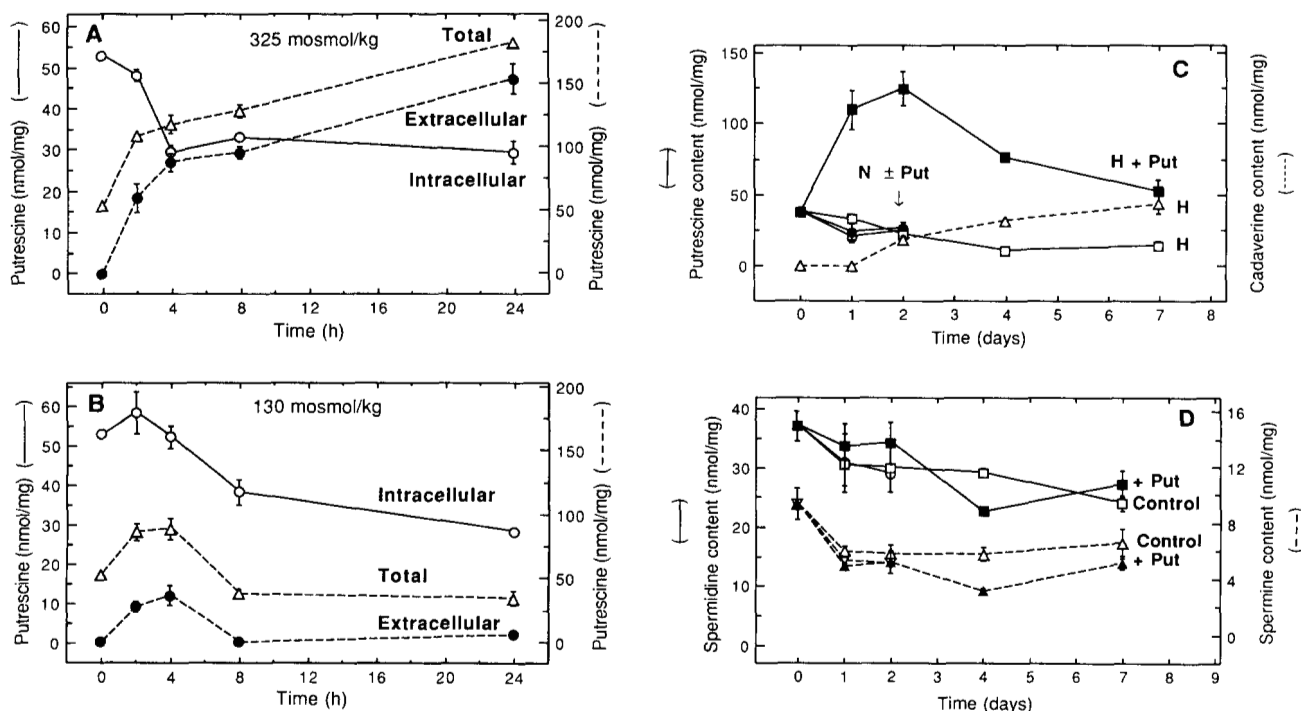

FIG. 7. **Time course of diamine and polyamine contents in D-R cells incubated under hypoosmotic stress.** *A* and *B*, at zero time exponentially growing D-R cells were transferred to normosmotic (*A*, 325 mosmol/kg) or hypoosmotic (*B*, 130 mosmol/kg) growth medium at a density of 3.5 × 10⁵ cells/ml. The putrescine content was measured in both cell extracts (*intracellular*) and in the medium (*extracellular*) at the indicated times. The *total* content is the sum of intra- and extracellular contents of the diamine. *Solid lines* indicate the intracellular putrescine content; *dashed lines* illustrate extracellular and total putrescine contents of the culture. Each point represents the mean (± S.D.) of ≥ three analyses of replicate dishes from one representative experiment. *C*, cells were plated at the normal seeding density (5 × 10⁴ cells/ml) in the presence (*solid symbols*) or absence (*open symbols*) of putrescine (*Put*), and intracellular diamine content was measured at the indicated times in cells incubated at 130 (*H*) or 325 mosmol/kg (*N*). Cadaverine was detected only in cells incubated at 130 mosmol/kg in the absence of exogenous putrescine (△, *dashed line*). *D*, time course of spermidine (*solid lines*) and spermine contents (*dashed lines*) in cells incubated at 130 mosmol/kg (□, ■, △, ▲) or 325 mosmol/kg (○, ▽) in the presence (*Put, solid symbols*) or absence (*control, open symbols*) of putrescine, as described in *C*. Putrescine had no effect on polyamine contents at 325 mosmol/kg, and only values for untreated cells (○, ▽) are presented for the sake of clarity. Each point represents the mean ± S.D. (*n* ≥ 3).

under these conditions were subsequently taken up by the cells. Cadaverine (1,5-diaminopentane), a diamine formed through the low affinity of L-lysine as a substrate for ODC (34), was detected intracellularly only in extracts from D-R cells incubated under hypoosmotic stress for $\geq$ 24 h (Fig. 7C; Table III, Footnote c), most likely as a result of both the large increase in ODC levels and a more efficient competition of L-lysine as a substrate. There was an almost inverse relationship between the time courses of putrescine and cadaverine content (Fig. 7C). Thus, the relative inability of D-R cells to synthesize putrescine under such conditions could not be attributed to a secondary effect of low osmolality on the catalytic properties of ODC. Both spermidine and spermine contents decreased rapidly at the onset of exponential growth under either osmotic condition (Fig. 7D), and both polyamines were detected in trace amounts only ($\leq$ 50 nM) in the incubation medium under either osmotic condition (results not shown).

Several cell lines of murine origin, including L1210 cells, have no detectable arginase activity (23, 35) and are thus believed to depend strictly on exogenous ornithine and/or serum arginase for the *de novo* synthesis of putrescine. Thus, one possible explanation for the reduction of putrescine production in D-R cells exposed to a hypotonic shock, despite the massive increase in ODC activity, could be a reduced availability of the substrate, L-ornithine. The addition of exogenous L-ornithine (100 μM) to hypotonically treated D-R cells resulted in a total production of putrescine comparable or even slightly higher than that measured under isotonic conditions (Table III), thus confirming that the substrate for ODC did become more limiting upon exposure to hypotonic shock. Substrate availability was obviously somewhat limiting even under isotonic conditions, as seen by the 50–100% increase in net putrescine production upon the addition of ornithine to control cells. Upon the addition of ornithine, the amount of putrescine released under normosmotic conditions exceeded by about 2- and 8-fold the intracellular putrescine levels at 4 and 24 h, respectively, whereas there was a 2–5-fold excess of intracellular putrescine (relative to the medium) in parallel incubations at 130 mosmol/kg.

Exogenous putrescine led to an initial intracellular accumulation of the diamine comparable to that obtained with L-ornithine at 130 mosmol/kg. This accumulation was more sustained than under isosmotic conditions (Table III). The extracellular putrescine concentration decreased by 65–75% over the experimental period in cells incubated with the diamine at 130 mosmol/kg whereas it almost quadrupled under isotonic conditions. This is consistent with a decline in putrescine production and an enhancement of its uptake induced by hypotonic shock. A common effect of exogenous L-ornithine and putrescine was to decrease spermidine content by up to 60% in cells incubated under hypoosmotic conditions whereas they had no effect on the polyamine content at 325 mosmol/kg (Table III). The time course of putrescine and polyamine contents under hypoosmotic conditions was influenced strongly by the initial cell density. When the seeding density was identical to that used for growth experiments, putrescine accumulation from the medium was maximal for the first 48 h of incubation and decreased slowly thereafter (Fig. 7C). No increase in putrescine content was detected after 24 or 48 h of growth under isotonic conditions in the presence of the exogenous diamine. Spermidine and spermine contents were decreased by hypoosmotic stress, but the effect was only apparent in the midexponential growth phase (Fig. 7D).

Taken together, these results thus suggest that a major effect of hypoosmotic stress in D-R cells is to inhibit the excretion of putrescine after its *de novo* synthesis from L-ornithine, resulting in a massive accumulation of the diamine. Thus, although ornithine is more limiting for putrescine synthesis at low osmolality, hypoosmotic stress clearly favors an enlargement of the putrescine pool even when putrescine synthesis is restored to control rates through ornithine supplementation or when the diamine is provided exogenously.

An Enlargement in the Putrescine Pool Is Required at an Early Stage in the Adaptive Response to Hypoosmotic Stress—Delaying the addition of putrescine or L-ornithine to increasing intervals after hypoosmotic shock decreased the ability of putrescine to stimulate growth in D-R cells, especially between 24 and 48 h (data not shown). These results suggest that an increase in putrescine pool size and/or flux is critically needed at early stages during acclimatization to hypoosmotic stress. We therefore speculated that the striking difference between D-R cells and parental L1210 cells in their ability to proliferate at low osmolality is related to the presence in the former subline of a much larger pool of putrescine. To test this hypothesis, we attempted to induce the accumulation of putrescine in parental L1210 cells through treatment with MDL 73811, an irreversible inhibitor of AdoMetDC (24). The sole addition of the inhibitor (10 μM) at the time of transfer to hypoosmotic conditions (130 mosm/kg) had no effect on cell growth or polyamine content measured after 8 days whereas it slightly potentiated the effect of putrescine (Fig. 8, Table IV). As described above, incubation of L1210 cells for such a period at 130 mosmol/kg resulted in a major loss in viability. However, when L1210 cells were preincubated with MDL 73811 for 24 h prior to hypotonic shock, the co-incubation with the AdoMetDC inhibitor and putrescine increased the initial growth rate (Fig. 8) and the size of the putrescine pool (Table IV) to values observed in osmotically stressed D-R cells incubated with putrescine (see Figs. 2, 3, and 7C). The latter effect was observed despite approximately 60 and 90% decreases in spermidine and spermine contents, respectively, which probably caused growth to slow down gradually. These data are consistent with a requirement for an early increase in putrescine content for optimal growth-related functions in hypotonically stressed cells. Thus, D-R cells appear to be better adapted to media of lower osmolality by virtue of their basal overexpression of ODC and their higher putrescine content at the time of transfer to hypotonic conditions.
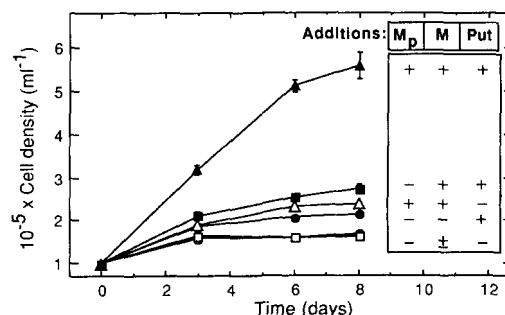

FIG. 8. **Effect of the AdoMetDC inhibitor MDL 73811 on the proliferation of L1210 cells under hypoosmotic stress.** Prior to the experiment, L1210 cells were preincubated for 24 h in the presence or absence of 10 μM MDL 73811 (M_p). At zero time, the cells were then transferred to hypoosmotic medium (325 $\rightarrow$ 130 mosmol/kg) in the presence or absence of the enzyme inhibitor (M), putrescine (*Put*, added at 10 μM), or both compounds. The cell number was determined at the indicated times following after shock. Each addition made is indicated by the corresponding plus (+) or minus (−) signs on the *right* portion of the graph.

TABLE IV

Effect of the AdoMetDC inhibitor MDL 73811 on polyamine content in L1210 cells grown for 8 days under hypoosmotic conditions (130 mosmol/kg)

Twenty-four h before osmotic shock, L1210 cells growing exponentially under standard conditions were either treated with 10 μM MDL 73811 or received no drug addition. The inhibitor exerted no significant growth inhibition during that preincubation period. Five-ml aliquots of cell suspension were transferred to T_{150} flasks containing 45 ml of hypotonic growth medium ($t = 0$) supplemented or not with 10 μM putrescine (Put) and/or MDL 73811. Cells were harvested for polyamine determination after an 8-day incubation, as indicated. Values represent the mean $\pm$ S.D. of three to six measurements from one representative experiment.

Addition(s)		Diamine and polyamine content		
Preincubation	Incubation	Putrescine	Spermidine	Spermine
			nmol/mg	
No addition	No addition	6.9 $\pm$ 0.1	4.9 $\pm$ 0.3	12.6 $\pm$ 0.5
No addition	Putrescine	9.1 $\pm$ 0.7	4.9 $\pm$ 0.3	7.7 $\pm$ 0.5
No addition	MDL 73811	5.1 $\pm$ 0.8	3.8 $\pm$ 0.4	9.6 $\pm$ 1.1
No addition	Putrescine + MDL 73811	13.9 $\pm$ 1.4	5.5 $\pm$ 0.6	6.4 $\pm$ 0.9
MDL 73811	MDL 73811	37.1 $\pm$ 4.2	7.2 $\pm$ 0.1	3.4 $\pm$ 0.4
MDL 73811	Putrescine + MDL 73811	109.3 $\pm$ 5.0	2.0 $\pm$ 0.2	1.4 $\pm$ 0.2

DISCUSSION

The present study establishes that putrescine plays a major role in the capacity for mouse leukemia cells to grow at low osmotic strength. Overproduction of ODC and the resulting higher basal putrescine content confer to D-R cells a striking advantage over the parental phenotype in its adaptation to hypoosmotic stress. Moreover, putrescine greatly extends the range of osmolalities under which L1210 cell growth proceeds at an approximately constant rate. Likewise, mimicking the high putrescine content of the D-R cells by treatment of parental L1210 cells with an AdoMetDC inhibitor results in a similar, albeit transient, ability to proliferate at a very low osmolality despite the impairment in spermidine biosynthesis. Finally, an enlargement of the putrescine pool appears to be an early cellular requirement for growth after hypotonic shock in that the capacity of the cells to respond to the addition of the diamine and to survive to a 60% decrease in osmolality is gradually lost. A few early reports described a putrescine requirement for proliferation at low osmolality in bacteria (15, 16). The present results demonstrate that the marked induction of ODC by hypotonic shock seen in a number of mammalian models (17–22) may have a physiological relevance.

Although hypotonic shock activates an acute accumulation of enzymatically active ODC in D-R cells and represses AdoMetDC activity net putrescine production was inhibited severely. Deficient synthesis of putrescine under hypoosmotic conditions arises from a decreased availability of L-ornithine, as shown by supplementation experiments with the amino acid. The site(s) at which substrate availability is decreased by hypotonic conditions (*e.g.* arginase activity, ornithine transport, competing enzyme activities) remain to be determined.

The requirement for putrescine under hypoosmotic conditions does not arise merely from an insufficient supply of precursors for polyamine biosynthesis. First, spermidine could not replace putrescine for the stimulation of cell growth under hypoosmotic conditions. Second, a deficiency in putrescine *per se* was sufficient for growth retardation by AOAP in osmotically stressed D-R cells. This contrasts with the cor-

relation of the antiproliferative effect of ODC inhibitors with spermidine, rather than putrescine depletion, under standard osmot c conditions (3, 5, 23). Finally, the massive intracellular accumulation of putrescine induced by MDL 73811 in the presence of putrescine exerted a potent synergistic effect on the initial growth rate of parental L1210 cells at 130 mosmol/kg despite the resulting inhibition of polyamine biosynthesis. The continued proliferation rate of either unsupplemented or putrescine-treated D-R cell cultures was decreased after treatment with the AdoMetDC inhibitor at either 325 or 130 mosmol/kg (results not shown), stressing the fact that polyamines are still essential cell components under hypoosmotic conditions.

D-R cells accumulated putrescine to far higher levels and for much longer periods at 130 than at 325 mosmol/kg when provided with either L-ornithine or putrescine. Different features of the cellular response to hypoosmotic shock described in this study likely contribute to this massive increase in putrescine content. The induction of ODC by hypotonic shock certainly provides the most obvious mechanism to increase putrescine production rapidly when substrate is readily available. The mechanism responsible for the increases in the rate of translation and in the half-life of ODC consecutive to hypoosmotic shock (22) cannot be attributed to a relief of feedback inhibition by polyamines (36, 37) since the content of the latter was constant during the period of enzyme induction. Although osmotically induced changes in the microcompartmentation of polyamines cannot be ruled out, an intriguing possibility is that changes in intracellular ion activity and composition influence ODC translation selectively, possibly through regulation of the ODC mRNA secondary structure in the 5'- and/or 3'-untranslated regions (38–40). Our results also demonstrate that ODC expression is still under negative control by putrescine (32, 33) during its induction by hypoosmotic stress. A similar repression of the osmotic induction of ODC after putrescine addition was reported in other cells (17, 20). It is not yet clear why concentrations of L-ornithine greater than 100 μM were less effective in stimulating D-R cell proliferation at 130 mosmol/kg and extensively depleted spermidine and spermine contents. One possibility is that putrescine accumulation might reach a threshold level over which the diamine actually displaces polyamines from their intracellular binding sites, leading to their elimination from the cell, a mechanism suggested for the polyamine depletion induced by (bis)ethyl polyamine analogues (41).

A second mechanism that appears to play a major role in enlarging the steady-state pool of putrescine is an increased net uptake of the diamine. Since actual transport rates were not determined in this study, it is not known whether hypoosmotic stress mostly enhances putrescine uptake or decreases its efflux. Nevertheless, it is apparent that net excretion of putrescine synthesized in D-R cell cultures supplemented with L-ornithine was strongly reduced in a hypotonic medium, with a corresponding intracellular accumulation of the diamine. Furthermore, exogenous putrescine increased the intracellular content of the diamine to 2–5-fold higher values under hypoosmotic conditions than in putrescine-supplemented isotonic D-R cell cultures. Since the rate of polyamine transport is under negative regulation by the intracellular pool of polyamines (42–44) it may be suggested that the osmoregulatory response alleviates that control or that the thermodynamic activity of polyamines is actually reduced as a result of stronger binding to macromolecules because of the lower ionic strength. The speed of elevation of putrescine uptake consecutive to hypotonic shock observed in D-R cells as well as in other systems (19, 20) eliminates an effect of

hypoosmotic stress on the number of transporters because of the long apparent half-life of the protein(s) involved (45).

The rapid decline of AdoMetDC activity which follows hypotonic shock in both D-R and parental L1210 cells is a third, potentially important, factor favoring putrescine accumulation in the stress response of these cells. A similar effect of hypotonic treatment on this enzyme has been reported in Ehrlich ascites tumor cells, in which a decreased rate of spermidine synthesis from radiolabeled putrescine was also detected (20). The repression of AdoMetDC expression by low osmolality may be a specific adaptive phenomenon, but it could also be a consequence of the general inhibition of protein synthesis observed upon transfer to hypotonic medium (22). Because of the ability of the diamine to stimulate AdoMetDC activity and the processing of the proenzyme (46, 47) a higher putrescine content could stimulate the rate of catalysis by AdoMetDC and thereby compensate for the decrease in enzyme levels induced by low osmolality. However, hypotonicity by itself did not affect polyamine content in D-R cells, and therefore the rate of decarboxylated AdoMet synthesis appears to be sufficient for polyamine synthesis.

Since the sum of putrescine levels recovered in cell extracts and media decreased with time in D-R cells incubated under hypoosmotic stress, without a concomitant increase in spermidine nor spermine, it appears that putrescine was metabolized further through a catabolic pathway. The identity of this pathway is as yet uncertain since oxidase activities should have been completely inhibited by the presence of aminoguanidine, and there was no evidence of the presence of N^1-acetylputrescine (which would be presumably formed through the action of spermidine N^8-acetyltransferase on putrescine (48)) in medium extracts. There was no effect of 4-aminobutyric acid (up to 200 μM), an intermediate in putrescine catabolism, on cell proliferation or diamine and polyamine contents (results not shown). Thus, it is quite unlikely that the stimulation of cell growth by putrescine is mediated through its catabolism although an active pathway for putrescine degradation exists in L1210 cells exposed to hypoosmotic stress.

The mechanism through which putrescine affects cell functions related to adaptation to hypoosmotic stress is not known. The overwhelming majority of mammalian cell types can actively regulate their volume after a hypoosmotic shock (reviewed in 7, 9). The mechanism involved in most cases is an efflux of K^+ ions and accompanying counterions through various channels together with the exit of osmotically obliged water. Little is known, however, about the regulation of cellular functions in the new steady-state reached by cells after the osmotic shift. A number of mammalian cell lines are known to proliferate indefinitely, albeit more slowly, at osmolalities as low as 180–200 mosmol/kg, including L5178Y mouse lymphoma cells (49, 50). It was found that L5178Y cells adapted to grow for extended periods in hypoosmotic media had a normal volume and Na^+ content but a K^+ content reduced in close proportion with the osmolality (49). In view of its well known sensitivity to changes in the K^+/Na^+ ratio (51, 52) and its regulation at different stages by putrescine, spermidine, and spermine (1, 53), protein synthesis would be a likely candidate for one of the possible sites of action for putrescine in cells exposed to hypoosmotic stress. A strong induction of both ODC expression (21, 54, 55) and putrescine transport (41, 56) can also be brought about by amino acids transported via a Na^+-dependent transport system. These also stimulate K^+ efflux for osmoregulatory purposes and decrease the K^+/Na^+ ratio (6, 8). The rapid accumulation of putrescine generally induced by growth factors that produce

increases in Na^+-dependent amino acid transport (57, 58), putrescine transport (59, 60), and ODC expression (42, 61) as early responses could thus have a role in ion regulation analogous to that postulated for the adaptation to hypoosmotic stress conditions.

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