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# Sequestered End Products and Enzyme Regulation: The Case of Ornithine Decarboxylase

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## INTRODUCTION

Ornithine decarboxylase (ODC), an initial enzyme of polyamine synthesis, is one of the most highly regulated enzymes in eucaryotic organisms (62). In the absence of clear knowledge of the function of the polyamines (putrescine, spermidine, and spermine), biochemists inferred that polyamines had special roles and that cells maintained polyamine concentrations within narrow limits. Here, we assemble evidence contradicting this view. In no organism is ODC feedback inhibited by an allosteric mechanism, and the sizes of polyamine pools may vary radically without having a profound effect on growth. We suggest that the apparent stability of polyamine pools in unstressed cells is due to their being largely bound to cellular polyanions. We further speculate that allosteric feedback inhibition, if it existed, would be inappropriately responsive to changes in the small, freely diffusible polyamine pool. Instead, slower mechanisms that control the amount of the ODC protein have evolved, and even these may be triggered inappropriately by irrelevant binding and release of polyamines from ionic binding sites. The existing control mechanisms cannot be rationalized in terms of a need for stabilizing the cellular polyamine content, and in fact, large, natural variations in the size of polyamine pools during the life cycle or the cell cycle might contribute significantly to the fitness of most organisms. We review these matters as they have been investigated in mammals, fungi, and bacteria. Information from plants is more complex and as yet inadequate to make generalizations, but appears to conform to the principles drawn here from studies with other organisms.

## FORMATION AND ROLES OF POLYAMINES

### Polyamine Metabolism

Polyamine biosynthesis begins with two decarboxylation reactions working in parallel (Fig. 1A). Ornithine is decarboxylated to putrescine by ODC, and *S*-adenosylmethionine (SAM) is transformed to decarboxylated SAM by SAM decarboxylase. Decarboxylated SAM is used to make spermidine by transfer of its aminopropyl moiety to putrescine and to make spermine by another aminopropyl transfer to

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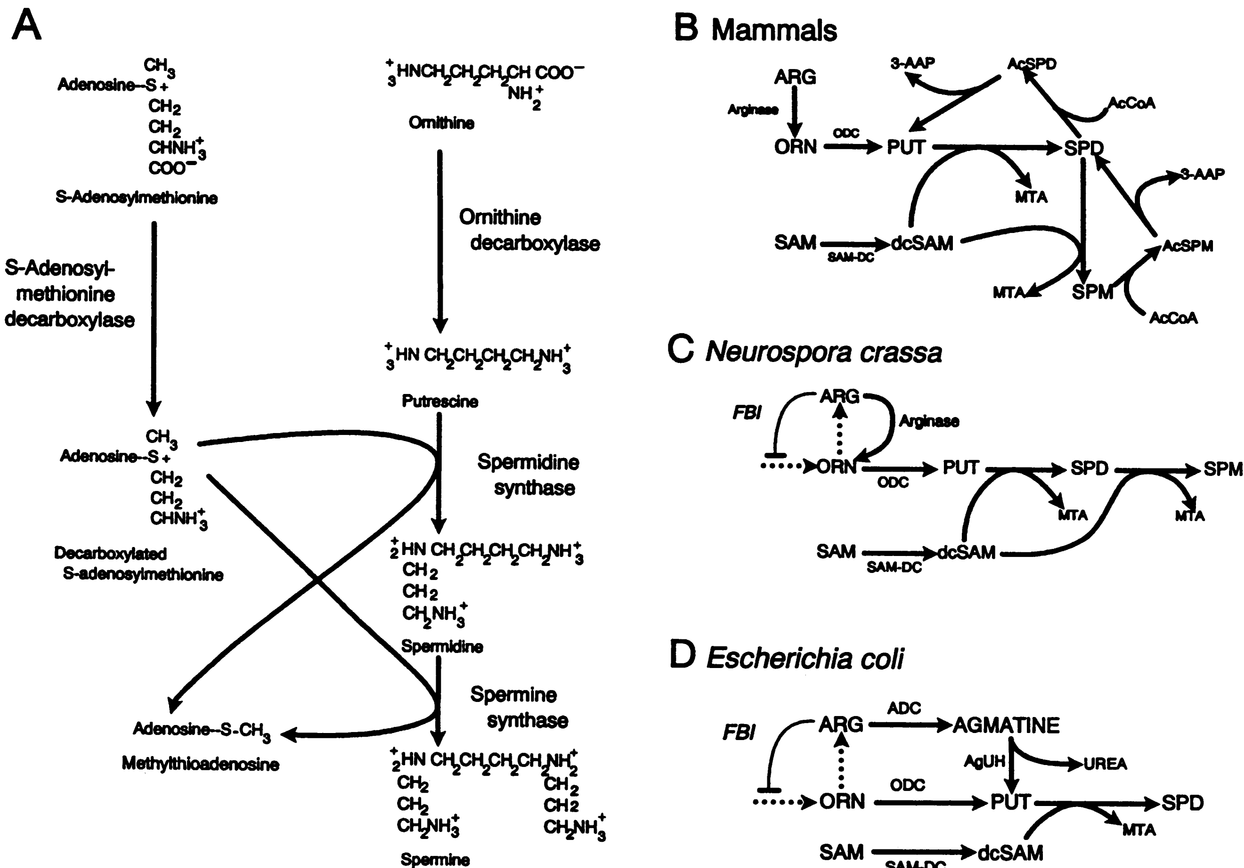


FIG. 1. The polyamine synthetic pathway (A) and its variants in mammals (B), *N. crassa* (C), and *E. coli* (D). In panels C and D, the dotted lines represent the endogenous biosynthesis of ornithine and arginine; in mammals (panel B), the ornithine used for polyamine synthesis comes largely from the diet. Abbreviations: ARG, arginine; 3-AAP, 3-acetamidopropanal; AcCoA, acetyl coenzyme A; AcSPD, acetylspermidine; AcSPM, acetylspermine; *FBI*, feedback inhibition (of arginine on ornithine biosynthesis); MTA, methylthioadenosine; ORN, ornithine; SAM-DC, SAM decarboxylase.

spermidine. Spermidine and spermine are both prominent in mammals as end products of the pathway, and the putrescine pool is usually low (41). Higher eucaryotes usually have a set of reactions which, with acetylation and oxidation (Fig. 1B), convert spermine to spermidine and spermidine to putrescine (41, 42). The acetylpolyamine pathway permits cells to adjust polyamine levels and to dispose of excess spermidine and spermine. In mammals, arginase participates in polyamine biosynthesis by making ornithine available from dietary arginine (Fig. 1B).

In fungi, in particular the ascomycetes *Saccharomyces cerevisiae*, *Neurospora crassa*, and *Aspergillus nidulans*, spermidine is the major polyamine (Fig. 1C; Table 1) (84). Putrescine and spermine are usually present at 1/10 or less the level of the spermidine pool. Moreover, some fungi, such as *N. crassa*, have almost no detectable spermine/spermidine acetyltransferase or oxidase activities, which simplifies analysis of polyamine metabolism (17, 18). Although the ornithine required for polyamine synthesis is made de novo, arginase permits cells to use arginine as an alternate source of ornithine when arginine feedback inhibits ornithine biosynthesis (13, 16) (Fig. 1C).

ODC is a dimer of 52- to 55-kDa subunits and is quite well conserved in sequence among eucaryotes from fungi to humans (11, 94). Putrescine is an obligatory activator of SAM decarboxylase in mammals and fungi. Therefore, ODC is a dominant controlling factor of the entire pathway (41). However, both decarboxylase activities are potential or actual rate-controlling steps of the pathway although the relative impact of changes of the two enzymes upon pathway flux varies greatly with the organism, cell type, or circum-

TABLE 1. Representative polyamine contents of *E. coli*, *N. crassa*, and mammalian cells

| Polyamine | Polyamine content of: | | | | | | |
|---|---|---|---|---|---|---|---|
| | *E. coli* DR112 cells[a] | | *N. crassa* (exponential growth[b]) | | | Mammals (BHK-21/C13 cells[c]) | |
| | nmol/mg of protein | mM[d] | nmol/mg of protein | mM | nmol/mg (dry wt) | nmol/mg of protein | mM |
| Putrescine | 95 | 28.4 | 2.6 | 0.32 | 0.8 | 0.7 | 0.13 |
| Spermidine | 14 | 4.2 | 60.0 | 7.2 | 18.0 | 11.4 | 2.07 |
| Spermine | 0 | 0 | 1.3 | 0.16 | 0.4 | 6.0 | 1.09 |

[a] Reference 40. Parameter: 3.35 μl of $H_2O$ per mg of protein.
[b] Reference 24. Parameters: 0.3 mg of protein per mg (dry weight); 2.5 μl of $H_2O$ per mg (dry weight) (82).
[c] Reference 7. Parameter: 5.5 μl of $H_2O$ per mg of protein (90).
[d] Figures are nominal concentrations, assuming homogeneous distribution in cell water.

stances. Information about the control of SAM decarboxylase is instructive for our argument and is discussed later in this review.

Bacteria differ significantly from fungi and mammals in the metabolism of polyamines (Fig. 1D) and, in fact, resemble plants in some respects (85). First, in *Escherichia coli*, two pathways lead to putrescine formation (54). One is the ODC reaction; the other begins with decarboxylation of arginine by a periplasmic (8) enzyme, arginine decarboxylase. The product, agmatine, is taken into the cell and converted to putrescine by agamatine ureohydrolase, with the elimination of urea. The net effect is to offer, as arginase does in higher forms, an alternative route of putrescine synthesis when abundant arginine feedback inhibits de novo ornithine biosynthesis (53).

The bacterial system differs in a second way: $Mg^{2+}$, rather than putrescine, is the activator of SAM decarboxylase, thereby uncoupling the synthesis of putrescine and decarboxylated SAM (85). Finally, ODCs of *E. coli* and many other bacteria are larger than the ODCs of fungi and mammals, being dimers of ca. 80-kDa subunits (85) with little or no similarity to the eucaryotic polypeptide (5) (the *E. coli* ODC sequence has GenBank accession no. M33766).

### Roles of Polyamines

Polyamines are essential for normal growth, as many studies with mutants and pathway inhibitors have shown (47, 83, 86, 93). The effects of polyamine starvation, however, do not yield satisfying information about the cellular roles of polyamines. Upon severe polyamine deprivation, protein and nucleic acid elongation rates diminish, the fidelity of translation is impaired (47), and chromosomes may disintegrate in later stages of starvation (73). Precise molecular mechanisms underlying these effects have not been defined.

Spermidine and spermine, bearing three and four net positive charges, respectively, are the most cationic small molecules of the cell. They therefore bind polyanionic macromolecules such as DNA, RNA, and phospholipids (37). The polyamines are different from other multivalent cations such as $Mg^{2+}$ in having a distributed charge, whose spacing may allow them to interact more flexibly with the phosphates of DNA and RNA (47). In vitro, spermidine and spermine have profound and beneficial effects upon macromolecular transactions in DNA replication (see, e.g., reference 45), transcription, and translation. In many cases polyamines simply stabilize interactions between macromolecules rather than occupy specific binding sites. One of the few specific roles that is known for a polyamine in macromolecular synthesis is in the synthesis of hypusine, a posttranslationally modified lysyl residue in eucaryotic initiation factor 5A (58). This modification arises by the oxidation of the aminobutyl group of spermidine after its transfer to a specific lysine residue (Lys-50 in the human protein [80]). Gene disruption studies with *S. cerevisiae* show eucaryotic initiation factor 5A to be essential to viability (80), and in vitro studies show the modification to be essential to function (58).

Putrescine, although often considered simply an intermediate of spermidine and spermine synthesis, actually has a vital role in some circumstances. In restricted studies of both bacteria (55) and mammalian cell (75), an expanded putrescine pool is required for adaptation to growth under hypoosmotic conditions.

The structural attributes of polyamines required in many reactions and in the growth of intact cells are not absolutely specific. Polyamine-dependent or polyamine-stimulated reactions continue in vitro, and cell growth progresses, although sometimes in a compromised fashion, with polyamine analogs derived from lysine, which is one carbon longer than ornithine (35, 36, 61). The relaxed structural specificity of cells for polyamines and the multiple roles of the polyamines suggest that even their exact concentrations within cells might not be critical.

## ODC REGULATION

### Mammalian Cells

**ODC regulation and growth.** Early observations on ODC showed that regenerating tissues (78), hormonally stimulated tissues, and mitogenically activated cells in culture displayed rapid, 10- to 100-fold augmentation of ODC activity (2), followed by a decline even before the cells initiate DNA synthesis (21). The transient expression of ODC activity suggested a dynamic balance between synthesis and inactivation, consistent with the short half-life of the enzyme (78). The impression that ODC had a special relationship to cell growth was reinforced by the demonstration that activation of the ODC gene was in many cases a primary response to mitogenic activation, not requiring prior protein synthesis. In this respect, ODC resembled the expression of certain families of nuclear proto-oncogenes during the $G_0$-to-$G_1$ transition (22, 77). Indeed, the DNA sequences surrounding the promoter of the mammalian ODC gene may regulate its responsiveness to a variety of growth factors (2). The onset of growth may involve as much as a 10-fold increased transcription of ODC mRNA (1, 42) and, in some cases, recruitment of preexisting ODC mRNA into polysomes (92). The 100-fold augmentation of activity often seen in the first cell cycle after stimulation of untransformed cells by serum or growth factors, however, is not maintained in subsequent cycles (47). ODC activity persists at about a 10-fold higher level than that seen in growth-arrested cells, and variations are not related to the cell cycle.

Normal progression through the cell cycle requires polyamines (47). Later work with cultured cells showed that some polyamine synthesis was necessary for growth but that the regulation of ODC could be greatly disturbed by molecular manipulations without a serious effect on growth rate (31, 88). The data force us to doubt that cellular levels of polyamines have to be closely controlled and make us wonder why mammalian ODC displays such a wide amplitude of regulation.

**ODC regulation in response to polyamines.** In mammals, augmentation of the synthesis of ODC follows interruption of polyamine synthesis, and rapid loss of ODC usually follows replenishment of polyamines (63). The speed of these regulatory responses is unusual for a biosynthetic enzyme. Often activity increases within minutes, and in most organisms the half-life of ODC upon polyamine replenishment is reduced to 30 ± 15 min.

In contrast to growth-related control of ODC, changes in ODC activity in vivo in response to polyamine depletion or repletion are not accompanied by changes in ODC mRNA levels (39, 48, 69, 70). Two classes of posttranscriptional mechanism have been proposed to explain this. The first is a control of ODC mRNA translation by the polyamines. ODC mRNA has a long leader, and in some organisms its secondary structure clearly impedes translation (28, 38, 46). It is not clear, however, whether polyamines modulate this impediment, and in several in vitro (66) and in vivo (89) studies with

leaderless mRNA constructs, polyamines still depress the production of ODC. This does not alone exclude the possibility that the remaining mRNA is translationally controlled. The second posttranscriptional hypothesis is that polyamines cause a posttranslational degradation of enzyme subunits before they assemble into active dimers. Supporting this view are observations in mammals (89), similar to those in *S. cerevisiae* (25), that neither the fraction of ODC mRNA in polysomes nor the size distribution of polysomes engaged in its translation is altered in the presence of polyamines. Only the coding region of the ODC mRNA in engineered mRNA constructs was required to display control by polyamines. Finally, the magnitude of the effect of polyamines on in vivo labeling of the enzyme during synthesis in the presence of [$^{35}$S]methionine decreased as labeling times became shorter, as it would if rapid degradation were opposing the accumulation of the product (89). Our purpose is not to resolve this controversy, but to emphasize the rapid and unusual control of the rate of accumulation of active ODC in mammals at the posttranscriptional level.

Turning to enzyme inactivation, the polyamine-induced loss of activity generally coincides with enzyme degradation (23, 81). In many systems, the rate of enzyme turnover is modulated by the polyamines. Most ODCs have PEST sequences, i.e., amino acid sequences rich in proline, glutamate, aspartate, serine, and threonine, which are found in most enzymes that turn over rapidly (76). Results with engineered ODC proteins partially confirm the importance of these sequences in turnover (27). Enzyme degradation appears to take place by a nonlysosomal, nonubiquitin, ATP-requiring process (4). Furthermore, a putrescine-induced, ODC-binding "antizyme" that blocks activity has been described (56). According to the model, this protein facilitates degradation of the ODC protein.

The speed and amplitude of ODC regulation led many investigators to assume that control was directed to maintaining specific concentrations of intracellular polyamines. In the course of continuing work, however, three striking anomalies surfaced. One was that ODC responded dramatically to proportionately very small (less than 5%) increases or depletions of the cellular polyamine pool (34, 49). The second anomaly contradicted the implication of the first: the cellular concentration of polyamines could vary greatly without having serious effects upon growth, a point we expand upon below. The third anomaly was entirely overlooked until recently: no ODC—even those of procaryotes—was allosterically inhibited by spermidine and/or spermine at physiological concentrations. In view of these observations, we face two paradoxes. If the dramatic regulation of ODC activity finely controls the cellular concentrations of polyamines, why has allosteric feedback inhibition of ODC activity, the most fluent known means of enzyme control, never evolved? If the cell is indifferent to polyamine concentrations beyond a minimal amount, why is ODC regulated so radically?

### *N. crassa*

The overall phenomenology of ODC regulation in *N. crassa* (3, 94), like the enzyme itself (23, 94), is similar to that in mammals. In *N. crassa*, the ODC mRNA level, the ODC activity, and the concentration of polyamines rise within a few hours of germination of asexual spores to levels characteristic of the rapid mycelial growth in minimal medium (95). The major polyamine of this fungus, as in most fungi, is spermidine (61). ODC of *N. crassa* is not controlled allosterically by the polyamines (23). Instead, a combination of mechanisms involving polyamine-mediated turnover and augmentation of ODC synthesis combine to regulate ODC activity over a 50- to 100-fold range (3). The ODC mRNA of *N. crassa*, like those of mammals, has an extremely long, untranslated leader (94). The ODC protein, again like those of mammals, contains two PEST sequences (94). In contrast to the situation in mammals (and in the fungus *S. cerevisiae* [25]), spermidine starvation in *N. crassa* leads to an increase in the amount of ODC mRNA. The changes can, in most circumstances, account for the increase of ODC synthetic rates (94). Polyamines exert no negative posttranscriptional control on ODC synthesis.

The anomalies mentioned above, namely the responsiveness of the regulatory system to small changes of polyamine content, the indifference of the growth rate to large changes in polyamine content, and the lack of feedback inhibition of ODC by polyamines, all prevail in *N. crassa* (15, 23).

### *E. coli*

*E. coli* has biosynthetic ODC and arginine decarboxylase (ADC) activities. Under semianaerobic conditions at low pH, "inducible" ODC and ADC enzymes appear, distinct from the biosynthetic enzymes, if excess amino acids are present (85). These activities are involved in pH control and are not relevant to the discussion below. In minimal medium, biosynthetic ODC and ADC both contribute to putrescine formation (Fig. 1D). When arginine is added, feedback inhibition of ornithine synthesis deprives ODC of a substrate and putrescine forms through the activity of ADC and agmatine ureohydrolase (40, 53). Unlike eucaryotic organisms, the *E. coli* strains studied normally contain 5 to 10 times as much putrescine as spermidine (40, 53) (Table 1).

Neither ODC (40) nor ADC (54) is inhibited by physiological concentrations of polyamines. The $K_i$ values for putrescine and spermidine are in the millimolar range (54). Moreover, the periplasmic location of ADC (8) puts it beyond the reach of feedback inhibition by intracellular polyamines. However, the rates of synthesis of the two decarboxylases are geared to the growth rate, and formation of all of the putrescine-forming enzymes is under indirect negative control of cyclic AMP (cAMP) and cAMP-binding protein. Polyamines also weakly repress the synthesis of ODC and ADC (50, 87). Other studies show a fourfold derepression of ODC following addition of arginine to an ADC-less mutant (40, 52). (In such mutants, arginine blocks ornithine biosynthesis, but cannot, as in the wild type, serve as an alternate source of putrescine [Fig. 1D].) The enzymes appear to be relatively stable. When excess putrescine and spermidine are added to cells growing with limiting amounts of ornithine, the ODC and ADC activities of the cells are adjusted at a rate corresponding to dilution of a partially repressed, stable enzyme by growth (87).

## POLYAMINE POOLS

### Mammals

**Effects of variation of polyamine pools.** We may now explore a common postulate about intracellular polyamine pools: must their intracellular levels be stringently controlled?

Studies with first-generation (33) or more sophisticated (10, 67) inhibitors of polyamine biosynthesis have shown that mammalian cells grow well despite large changes in the

size of the polyamine pools and in the relative amounts of putrescine, spermidine, and spermine. One experiment (44), done with L1210 cells, illustrates this point. A SAM decarboxylase inhibitor, *S*-(5′-deoxyadenosyl)methylthioethylhydroxylamine (AMA), at 0.1 mM, causes a 60% reduction in growth and a 50% reduction in colony-forming efficiency after 5 days. At that point, the putrescine, spermidine, and spermine pools are 19-, 0.14-, and 0.10-fold normal levels, respectively. The inhibition of growth and colony-forming ability was prevented entirely by addition of spermidine at the same time as AMA. (Spermine cannot be made from spermidine under these conditions, because SAM decarboxylase is inhibited.) After 5 days, the polyamine pools are, respectively, 19-, 0.40-, and 0.04-fold normal levels (44). The authors conclude that spermidine is the most important polyamine in sustaining growth.

Previous studies with L1210 and SV-3T3 cells treated with an irreversible inhibitor of ODC, α-difluoromethylornithine, and with nonmetabolizable analogs of spermidine and spermine (e.g., 1,1,12,12-tetramethylspermine) suggested that either spermidine or spermine, and many of the analogs, could support growth but that spermine was not essential if spermidine was present (57, 64, 65). Even earlier work (74) demonstrated that cells treated with α-difluoromethylornithine could grow well with as little as 15% of the normal spermidine pool and that two analogs (having extensions of one and two methylene groups in the aminobutyl moiety of spermidine) would reverse the growth inhibitory effects substantially. The particulars of these experiments are less important than the observation that radical differences in polyamine structure, intracellular concentration, and ratios are compatible with viability.

Normal growth requires a minimum amount of polyamines, as these studies also imply. This point was rigorously proven with mutants lacking ODC. Although growth of ODC-less cells was indeed wholly dependent on the addition of polyamines (72, 83), about 6 days of growth was required to deplete polyamine pools to the point that growth ceased.

These findings directly contradict the casual assumption that the sophisticated control of ODC reflects the need to control polyamine pools within critical limits.

An alternative reason for responsive control of the amount of ODC protein is that excess polyamines might be toxic. Indeed, the addition of spermine to many cell types causes inhibition of growth. Brunton et al. (6) showed that if 2 mM spermine is added to hamster kidney fibroblast cells, it inhibits growth, even if care is taken to prevent extracellular formation, by serum oxidases, of toxic oxidation products, especially acrolein. Spermidine is not particularly inhibitory under the same conditions. In a later study, investigators in the same laboratory obtained evidence suggesting that much of the toxic effect of spermine was due to its intracellular metabolism via copper-containing amine oxidases (7). Therefore one must be careful in interpreting inhibition of growth by polyamines (7, 51). Small amounts of toxic derivatives might escape detection, and intracellular levels of free, as opposed to bound, polyamines have not been rigorously monitored under these (or, in fact, any) conditions. Therefore, we are not certain in any case whether spermine itself is toxic. The issue is rendered moot by the finding that toxic derivatives may form fairly readily from high concentrations of spermine in mammalian cells or serum.

As noted above, ODC is not inhibited to any great extent by the polyamines. If toxicity lies in accumulation of spermidine and spermine, however, rather than putrescine, one might expect feedback inhibition to have evolved in the SAM decarboxylase or spermidine synthase reactions. Significantly, the purified SAM decarboxylase of the liver and mammary gland is inhibited by 150 μM spermine, but is not significantly inhibited by spermidine (79). As explained below, spermine is unlikely ever to reach this concentration within cells normally. Inhibition of SAM decarboxylase by spermine may therefore represent a safety mechanism by which excess spermine synthesis can be prevented, rather than a means of controlling the pool around normal levels.

TABLE 2. Fractions of the spermidine and spermine pools bound to various cellular constituents, and the remaining ("free") pool[a]

| Cell type and polyamine[b] | % of cellular polyamine | | | | | Concn (μM) of free polyamine |
|---|---|---|---|---|---|---|
| | Bound to: | | | | Free | |
| | DNA | RNA | P-lipid | ATP | | |
| Bovine lymphocytes | | | | | | |
| Spermine (1.6 mM) | 18 | 66 | 2.4 | 8.7 | 4.8 | 77 |
| Spermidine (1.3 mM) | 13 | 57 | 2.9 | 12 | 15 | 195 |
| Rat hepatocytes | | | | | | |
| Spermine (0.88 mM) | 6 | 85 | 4.4 | 2.7 | 1.9 | 16 |
| Spermidine (1.15 mM) | 4.6 | 78 | 6.2 | 4.3 | 6.7 | 77 |

[a] From Watanabe et al. (90).
[b] Concentrations of polyamines in parentheses are nominal values, based on total cellular polyamine and intracellular water.

An important recent study on transgenic mice (31, 32), carrying an aberrantly regulated human ODC, is consistent with the view that control of ODC is not geared to stabilizing polyamine pools. Certain transgenic animals had as much as 24-fold (testis, liver) to 80-fold (brain) higher ODC activity than normal in some tissues, although not all tissues were affected equally. The only abnormality found in these transgenic mice was male gametic dysgenesis. The pools of putrescine in the tissues were highly elevated, demonstrating that the ODC transgene was functional (31). However, the pools of spermine and spermidine were almost normal, well within the range tolerated by cultured cells in studies cited above (31). This suggests again that stringent defense against excessive pools of spermine and/or spermidine (by excretion, acetylation, or both; by a low $V_{max}$ of the spermidine and spermine synthases; or by feedback inhibition of SAM decarboxylase) prevails in this mammalian species. The significance of the study is that a radical elevation of ODC activity, leading to high levels of putrescine in vivo, is compatible with the viability of a whole mammal (32; see also Addendum in Proof).

**Pool sequestration.** Many observations strongly suggest that polyamines are bound to cell constituents in vivo (see citations in reference 59). Although no rigorous test of this proposition has been made by using mammals, findings in *N. crassa* (described below) can be applied to mammalian cells. A recent set of calculations, drawn from biochemical parameters of bovine lymphocytes or rat liver, are instructive (90) (Table 2). The numbers are based on the $K_d$ values of polyamines from polyanionic macromolecules and ATP, the ionic conditions, the cellular polyamine concentrations, and the polyanion concentrations. Using the conservative assumption that the concentrations of $K^+$ and $Mg^{2+}$, major relevant cations of the cell, were 150 and 2 mM, respectively, Watanabe et al. (90) calculated (i) that most of the spermidine and spermine would be bound to rRNAs, with a modest amount of both bound to DNA, and (ii) that the

concentration of free polyamine of any kind ranges from 7 to 15% of total spermidine (77 to 195 μM in cell water) and from 2 to 5% of total spermine (16 to 77 μM). Reduction of the $K^+$ concentration to 100 mM would diminish the estimated free spermidine and spermine concentrations about twofold. Thus the levels of free polyamines that might serve as regulatory effectors are quite low and, in fact, might be quite variable if the ionic composition (or ionic strength) of the cellular interior was itself variable. With the obligatory caution that intracellular conditions may not resemble those assumed in these speculations, it would be hard to reconcile the $K_i$ of SAM decarboxylase for spermine (150 μM) with the likely ambient spermine concentration (16 to 77 μM) if feedback sensitivity had evolved to maintain constant spermidine and spermine concentrations.

### *N. crassa*

Because *N. crassa* embodies the paradoxes of control and response to polyamines noted in mammals, it is a suitable, simple organism for resolving them. It is particularly useful because spermidine represents over 90% of the polyamine pool and because little turnover of polyamines takes place during growth. Relating enzyme-regulatory phenomena to the actual polyamine status of the organism may clarify the physiological role of the control mechanisms.

**Effects of variation of polyamine pools.** In *N. crassa*, the normal spermidine content is about 18 nmol/mg (dry weight); the putrescine and spermine contents are 0.8 and 0.4 nmol/mg, respectively (Table 1). None of the polyamines are significantly turned over or derivatized except as intermediates of the biosynthetic pathway (18, 60).

Mutants of *N. crassa* blocked in the enzymes arginase (*aga*), ODC (*spe-1*), and SAM decarboxylase (*spe-2*) reveal the effects of manipulation of polyamine pools on growth (16, 24, 71). Arginase is the normal route of ornithine formation when arginine is added to the medium, because the added arginine feedback inhibits ornithine synthesis (Fig. 1C). Therefore, in the *aga* mutant, addition of arginine causes ornithine and polyamine starvation. Growth of the *aga* mutant is normal for two doublings after arginine addition, during which ODC derepresses 70- to 100-fold (15). Thereafter, growth diminishes to half the normal rate and continues indefinitely (16). The growth rate becomes lower only when the cellular spermidine level decreases to 20% of normal. After a number of generations, the cells contain no putrescine or spermidine. In their place, small amounts of two analogs are found: cadaverine (1,5-diaminopentane) and aminopropylcadaverine, the former being made by the weak lysine decarboxylase activity of the highly derepressed ODC (61).

The ODC-less *spe-1* mutant has an absolute requirement for spermidine, but can actually grow at a low rate as long as the level of spermidine carried over from the inoculum remains above 2 nmol/mg (dry weight) (ca. 12% of normal) (71). The SAM decarboxylase-deficient *spe-2* mutant also has an absolute spermidine requirement, but unlike the *spe-1* mutant, it accumulates high levels of putrescine (71). The *spe-2* mutant grows extensively after inoculation, until its spermidine pool declines to 0.3 nmol/mg (dry weight) (2% of normal), at which point growth stops (71).

These observations lead to several conclusions. First, spermidine is an essential metabolite. However, the organism grows well even after extreme reduction of the spermidine content. Second, structurally altered polyamines support indefinite growth. Third, in the *spe-2* mutant, excess putrescine fulfills some of the functions of spermidine, or renders the small residual spermidine pool more useful, perhaps by promoting spermidine exchange among higher-affinity binding sites.

We must ask for *N. crassa*, as we did for mammalian cells, whether elaborate ODC regulation has evolved, not to adjust pools continuously, but instead to prevent synthesis of toxic levels of intracellular polyamine.

Addition of 5 mM putrescine or spermidine to the growth medium of *N. crassa* slightly inhibited the onset of mycelial growth, but growth rates thereafter were normal (19, 20). Analysis of intracellular polyamines indicates that the putrescine pool rises from 0.8 to about 12 nmol/mg (dry weight) when putrescine is added and that the spermidine pool rises from 18 to about 33 nmol/mg when spermidine is added (16, 18, 20). If the polyamines were distributed evenly in cell water (2.5 ml/g [dry weight] [82]), which, as we shall explain, they are not, the higher concentrations would be 4.8 mM putrescine and 13.2 mM spermidine.

A more extreme flooding of the polyamine pools can be brought about in the *puu-1* (putrescine uptake) mutant of *N. crassa*, which strongly concentrates polyamines within the cell (19, 20). After addition of 5 mM putrescine to the mutant, the intracellular pool of putrescine rises from 0.8 to over 200 nmol/mg (dry weight) (nominally 80 mM). Addition of 5 mM spermidine leads to an intracellular pool of 70 nmol/mg (ca. 28 mM). At these extremes, the *puu-1* strain is inhibited at the onset of growth, but once growth begins, cells with 200 nmol of intracellular putrescine per mg grow at about half the normal rate.

It is difficult to test the effects of excess polyamines in *N. crassa* because the organism sequesters much (but not all) of its excess polyamine within vacuoles (19). At higher levels, moreover, *N. crassa* excretes polyamines into the surrounding medium. However, the vacuolar uptake and the excretion themselves indicate that cells have at least moderately elevated polyamine pools and survive well. Therefore, although our lack of precise knowledge of the maximal polyamine pool size limits our conclusions regarding the toxicity of polyamines, it is clear that some excess can be tolerated.

Our information on polyamine pools, then, deprives our question about the elaborate regulation of ODC of a premise. Although polyamines are essential, minuscule amounts can support growth and a little more can support normal growth. At the other extreme, high concentrations of polyamines are detrimental to cells, but elevated intracellular concentrations of polyamines still appear to be compatible with growth. The elaborate, 70-fold regulation of ODC is as mysterious as ever, because it cannot reflect a need for continuous and precise adjustment of polyamine pools.

**Polyamine sequestration.** The binding of cellular polyamines by cellular anions in vivo has been impossible to prove by cell fractionation methods, precisely because polyamines bind so readily to polyanions in solution after extraction. However, reliable information on this point is essential to an understanding of polyamine-mediated control of ODC (14). The most definitive work in this area has been done with *N. crassa*.

Two experiments show that polyamines are sequestered in living cells of *N. crassa*. In the first (59), a tracer level of [$^{14}$C]ornithine, given to wild-type cells, was monitored during its metabolism to spermine. It was found that the large resident pool of spermidine diluted new, labeled molecules very little as they were used for spermine synthesis. The specific radioactivities of intermediates showed that 80 to 90% of the spermidine was bound, unable to serve as a

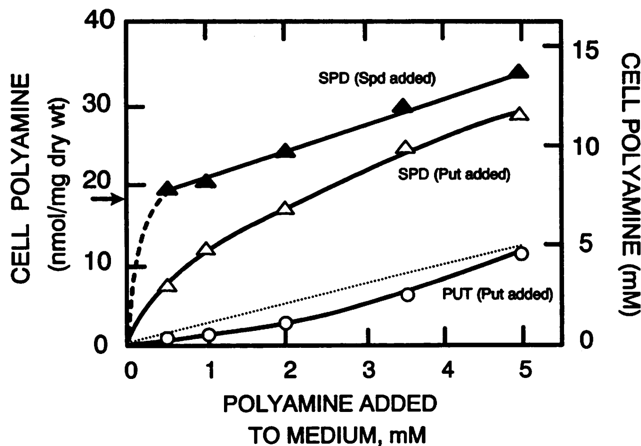


FIG. 2. Relation between external polyamine and intracellular polyamine concentrations during growth of the *spe-1* (ODC-less) mutant of *N. crassa*. Steady-state, exponential cultures, started in media containing different concentrations of polyamine, were grown for 18 h and sampled three times during the last 6 h. The intracellular pools of putrescine and spermidine were measured, averaged, and expressed as nanomoles per milligram (dry weight) (left ordinate) or as micromolar in cell water (2.5 ml/g [dry weight]) (right ordinate). Open symbols: polyamine pools of cultures grown in various concentrations of putrescine. Solid symbols: spermidine pools of cultures grown in various concentrations of spermidine (no putrescine was present in these ODC-less cultures). Dotted line: equal concentrations in medium and cell water. Arrow on left ordinate: normal concentration of spermidine in wild-type, unsupplemented cultures. Redrawn from reference 18 with permission from Academic Press.

metabolic intermediate. Other evidence showed that only 25% of the cellular spermidine was in the *N. crassa* vacuole, bound tightly to inorganic polyphosphate (59). Most of the remaining 75% of the cellular spermidine, therefore, lay in other cell compartments (cytosol, nucleus, perhaps mitochondria), bound to other polyanions, as suggested by Watanabe et al. (90) for mammalian cells.

An entirely different experiment confirms this conclusion. In wild-type *N. crassa* growing in the normal medium, the active transport of polyamines is inhibited and polyamines simply equilibrate slowly across the cell membrane (18, 20). We could therefore test for binding of polyamines within cells of the *spe-1* mutant, which must obtain all its polyamines from the medium (18). In exponential *spe-1* (or wild-type) cultures, supplemented with various amounts of putrescine, external and internal putrescine concentrations were similar (Fig. 2). (The intracellular putrescine concentration is actually lower at most points in the *spe-1* mutant because of its continuous use in spermidine synthesis.) Variation of the amount of spermidine yielded a different result (Fig. 2). Changes in internal spermidine concentrations were similar to changes in external concentrations, but the internal level was 18 nmol/mg higher at all external concentrations, even the lowest (0.5 mM). Thus, even when the active transport system was not working, spermidine appeared to be taken up quite efficiently at low external concentrations. We interpret the concentrative transport at low external spermidine concentrations to be a titration of fixed anionic groups within the cell. At higher concentrations, a free pool of spermidine, able to equilibrate with external spermidine, appears. In fact, permeabilized cells of *N. crassa* retain virtually all of their intracellular polyamines (18).

The most significant finding in this experiment (18) was that the amount of spermidine needed in vivo to titrate anionic constituents of the *spe-1* mutant was equal to the normal spermidine pool of wild-type cells, namely 18 nmol/mg (dry weight) (Fig. 2). This implies that the size of the wild-type polyamine pool is determined not by specific requirements for cell growth, but simply by the coulombic interaction between the cationic polyamines (spermidine and spermine) and cellular polyanions. Presumably most of the binding is adventitious; polyamines are therefore bound at many sites at which they are dispensable (9), with a variety of affinity constants. The existence of small, unbound pools of polyamines must depend on an appropriate balance between growth, as it adds more polyanions to the intracellular environment, and the synthesis of polyamines to titrate them.

In sum, only a small fraction of the cellular spermidine and putrescine is unbound, and thus metabolically active, in the cell. This correlates well with earlier observations. In both mammalian cells and *N. crassa*, regulatory effects of polyamine starvation and addition occur long before the cellular polyamine content changes significantly. This is expected, because changes in synthetic rate, or additions to the medium, cause immediate and proportionately large changes in the free polyamine pools, which behave as the metabolic signals. Both the lack of facile equilibration of isotopically labeled metabolites and the rapid response of ODC to small perturbations of polyamine metabolism indicate an important feature of the bound pools: most of them are not readily exchangeable, and thus they cannot easily buffer major changes in the size of the unbound pools by rapid dissociation.

### *E. coli*

**Effects of variation of polyamine pools.** A large amount of work has been done on polyamine metabolism in *E. coli* (for a review, see reference 85). A distinctive finding in this organism is that multiple mutants lacking arginine, ornithine, SAM, and lysine decarboxylases can grow, albeit very slowly, with no putrescine, spermidine, cadaverine, or aminopropylcadaverine (30, 86). This explains a common difficulty of most investigators (see reference 30 and references therein) in selecting mutants for ODC and arginine decarboxylase. Only the most severe mutants have a clear phenotype, because even a small amount of putrescine is efficiently converted to spermidine (52). The fact that polyamines cannot be found in multiple mutant strains still able to grow slowly (86) does not prove conclusively that polyamines are entirely dispensable; the lower limits of detection may have been too high for them to be detected. *E. coli* extends the pattern of behavior of eucaryotic cells: although polyamines (spermidine in *E. coli*) are needed for optimal growth, the growth rate is not severely affected by a mild depletion of the pool.

Excess polyamines have clear, deleterious effects upon ribosome structure and function in bacteria (29, 43, 91), and similar effects may underlie some of the toxicity of spermine seen in eucaryotes (51). A study of *E. coli* strains bearing multicopy plasmids carrying the biosynthetic ODC or SAM decarboxylase genes showed only mild elevations (less than twofold) in putrescine and spermidine levels, respectively (40). The control of intracellular polyamine levels reflects excretion of the bulk of the excess putrescine, and feedback inhibition of SAM decarboxylase by spermidine, which controls the spermidine concentration. (Spermidine was not

excreted.) The authors conclude that because ribosomes bind virtually all of the intracellular spermidine, the feedback sensitivity of SAM decarboxylase is relevant only in conditions in which "excess amounts of spermidine" are produced. The term "excess" appears to mean a pathological excess, not the slight excursions normally corrected by the feedback inhibition seen in other biosynthetic pathways of bacteria. If this is truly the case, the feedback sensitivity of *E. coli* SAM decarboxylase has the same metabolic role as it does in mammals (79), as suggested above.

**Polyamine sequestration.** In the work referred to above (40), Kashiwagi and Igarashi rely on previous work from their laboratory (41) on polyamine transport in *E. coli*. Their study showed that whole cells appeared to transport putrescine and spermidine unidirectionally and that little of the previously transported $^{14}C$-polyamine could be driven out of the cells by addition of unlabeled polyamine. However, membrane vesicles prepared from the same cells did not retain $^{14}C$-polyamine under these conditions; it readily exited the cells when unlabeled polyamine was added. The authors conclude that intracellular binding to macromolecules (37) accounts for the apparently unidirectional transport of polyamines in living cells of *E. coli*. The data are entirely consistent with the apparent intracellular binding of spermidine in *N. crassa* (18) described above and with studies of exchange transport in this organism (17). Other studies of polyamine binding to ribosomes or to nucleic acids in *E. coli* are consistent with these findings (12, 26).

## CHOICE OF ODC CONTROL MECHANISMS

### Why Is ODC Not Feedback Inhibited by Polyamines?

We have described paradoxical features of polyamine metabolism in three radically different types of organisms. First, one of the more extreme amplitudes of control of any enzyme is found in the ODC activity of fungi and animals, even though a wide variation of polyamine content is easily tolerated. Second, the unusual mechanisms of controlling this enzyme do not include the most immediate and effective metabolic control mechanisms, allosteric feedback inhibition. If one abandons the common assumption that pools must be maintained within narrow limits, the lack of feedback inhibition is entirely understandable. However, there are more subtle, perhaps even more important reasons that this mechanism might be lacking: the sequestration of polyamines within the cell, and the intermittent need for high intracellular levels of polyamines.

**Feedback inhibition and polyamine sequestration.** With feedback inhibition, changes in the concentration of a metabolic end product immediately and reversibly affect the rate of an early enzyme reaction by allosteric binding of the end product or related metabolites to the enzyme. Free polyamine pools, which would be effectors of feedback inhibition, are poor indicators of the polyamine status of the cell. Polyamines can be recruited by mass action from the bound state for some time before cell growth is limited by polyamine starvation. The reservoir of usable polyamines is substantial and may suffice for as much as two doublings of mass. As depletion continues, the polyamines remaining bound will be those bound with higher and higher effective affinities to their binding sites.

In unstressed cells, the size of the very tiny "free" polyamine pool (which in isotope experiments may comprise both unbound and freely exchangeable bound polyamines) may be quite vulnerable to perturbations unrelated to polyamine synthesis or demand, such as the onset of rRNA synthesis or variations in osmotic strength and cell water. If the free pool is so delicately balanced by synthesis and withdrawal to the bound state, allosteric responses with a short time constant would fluctuate radically, without significance. We emphasize that the scope of this argument applies to the polyamine pathway in all organisms.

Students of metabolism might argue with the implications of this thesis: there are examples of sequestered metabolic intermediates that are made by feedback-inhibitable enzyme pathways. For instance, in *S. cerevisiae* and in *N. crassa*, although 99% of the large arginine pool is in vacuoles and 1% is in the cytosol, arginine is nevertheless the effector of feedback inhibition of early steps of ornithine synthesis (13). The difference between arginine synthesis and polyamine synthesis is that in the former, end product sequestration in vacuoles is itself controlled adaptively by the cytosolic arginine concentration (13), and this mechanism collaborates with feedback inhibition and repression in regulating arginine biosynthesis. Polyamine sequestration, however, is a passive, chemical phenomenon that cannot be wholly overridden by biological mechanisms.

**Feedback inhibition and episodic polyamine synthesis.** Another argument can be made against the fitness of feedback inhibition of polyamine synthesis. At different times in the life of a cell or organism—times at which a large inventory of polyamines might have to be made in advance of need—the biologically appropriate rates of polyamine synthesis and the level of polyamine accumulation might be quite high. For example, stimuli that cause quiescent cells to grow might lead to substantial derepression of the ODC gene and thus to polyamine synthesis and might override opposing mechanisms, such as polyamine-mediated repression. Indeed, this may be why ODC derepression is a primary response to growth stimuli at the onset of growth of mammalian cells. Enzymes (ODC or SAM decarboxylase) calibrated to maintain a constant polyamine pool size would be clearly maladaptive.

A case in which a large expansion of the putrescine pool is essential for survival is known (75). Substantial amounts of putrescine are required for L1210 mouse leukemia cells to grow well under hypoosmotic conditions. This is consistent with the older observations that ODC was induced by hypoosmotic shock (68). Whether the inducing stimulus is the dilution or binding of polyamines upon the increase of cell water is not known. A large accumulation of putrescine is also required in some strains of *E. coli* under hypoosmotic conditions (55), and feedback inhibition of ODC by putrescine or spermidine in this organism might again be maladaptive.

### Alternate Control Mechanisms

How have cells evolved to deal with the problem of control by a chemically sequestered metabolite? A relevant metabolic signal for ODC regulation is the persistent depletion or excess of free polyamines that would occur upon sustained starvation or excess. Persistent trends in pool size may be integrated by responses with a longer time constant, such as alterations in the rates of synthesis and degradation of a key enzyme, in this case ODC. This will ensure that changes in the rate of polyamine synthesis are damped against more transient changes of the free pool.

Two unusual mechanisms of control of ODC levels in a variety of eucaryotic organisms—posttranscriptional control (including mRNA recruitment) and rapid enzyme turnover—

are able to cause more rapid changes in enzyme levels than transcriptional derepression of a stable enzyme. With these mechanisms, organisms seem to have evolved opposing means of controlling ODC, intermediate in speed between transcription (too slow) and allosteric feedback inhibition (too fast). Because the sizes of the free polyamine pools to which these mechanisms respond are labile, ODC appears to have unusual, and perhaps biologically trivial, responsiveness to a variety of stimuli. In effect, the problems postulated for an allosteric mechanism prevail to some extent in the mechanisms actually used by many eucaryotes.

Finally, some control of polyamine synthesis is vital, even if we disregard the issue of carbon and nitrogen economy. The polyamines are essential for normal growth, albeit at lower levels than actually exist in cells. In addition, the higher polyamines are, directly or indirectly, toxic, and control of the amount of ODC (and SAM decarboxylase) protein takes its place with polyamine excretion, derivatization, and interconversion in averting lethal excesses of these compounds.

## CONCLUSIONS

The complexity of ODC regulation has led to the notion that ODC and the polyamines have special significance to cell growth. Later work showed that most cells tolerate wide variations in the amount of cellular polyamines. The unusual mechanisms controlling ODC, therefore, cannot have evolved to fine-tune the concentrations of the pathway end products.

The data on polyamine sequestration, which suggest that most of the polyamines are bound to cell constituents, offer a major insight into the history of polyamine research. The rough constancy of the polyamine composition of unstressed cells was once thought to correlate well with the unusual mechanisms controlling ODC activity and to reflect the requirements of cells for a constant polyamine concentration. We now see that the polyamine content characteristic of various cell types might simply reflect the constancy of the macromolecules that are titrated by these basic amines. This is a chemical rather than a regulatory phenomenon, and it may owe nothing to a sophisticated control system.

The existence of unusual control mechanisms for ODC is correlated with a universal lack of allosteric feedback inhibition. We argue that feedback inhibition would be inappropriate, and possibly unworkable, in the face of end products so readily bound to cell constituents. Because the free pool does not immediately indicate the polyamine status of the cell, control mechanisms with responses slower than feedback inhibition have evolved to sense longer-term trends of intracellular polyamine concentration. These mechanisms, suitably modified, are compatible with episodes of rapid polyamine accumulation that may be needed during growth, development, or unusual environmental conditions. The peculiarities of ODC control highlight the complexity of the pathway and deepen our need to discover just what the polyamines actually contribute to cell growth and the fitness of organisms.

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## ADDENDUM IN PROOF

A recent article by Z.-P. and K. Y. Chen (Biochim. Biophys. Acta **1133**:1–8, 1991) describes a variant mammalian neuroblatsoma cell line with an amplified ODC gene. The cells grew at a normal rate despite a 90-fold greater ODC activity than that of normal cells and putrescine, spermidine, and spermine pools that were 215-fold, 30-fold, and 14-fold greater, respectively, than in the parental strain. Although the variant cell line did not differentiate normally in response to cAMP (with or without a polyamine inhibitor), it did so upon removal of serum. Although the authors note that the polyamine pools of the variant strain may be compartmentalized, the data are consistent with the view that greatly elevated levels of intracellular spermidine and spermine and not of themselves toxic to this cell line.

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