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Uptake, Intracellular Binding, and Excretion of Polyamines during Growth of *Neurospora crassa*¹

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In *Neurospora crassa* mycelia, the amounts of the main polyamines, putrescine and spermidine, are approximately 0.8 and 18 nmol/mg, dry weight. We wished to know what determines these pool sizes. In the growth medium, externally added polyamines enter cells largely by a nonsaturable, diffusional system. In a mutant unable to synthesize polyamines, internal and external spermidine appear to equilibrate across the cell membrane during growth. However, this was true only after an intracellular "sink," with a capacity equal to the amount of spermidine found in wild-type cells, had been saturated. We speculate that internal anionic binding sites, detectable in permeabilized cells, sequester virtually all of the spermidine normally found in exponentially growing *N. crassa*. Further evidence for this view was that in mature, stationary cultures, excess spermidine is excreted. Putrescine is also excreted if its concentration in the cell is abnormally high. The control of pool size by intracellular binding and excretion may be an advantage in this pathway, because feedback inhibition does not prevail, enzyme regulation is by comparison slow, and excessive polyamines are toxic. © 1989 Academic Press, Inc.

Polyamine biosynthesis in animals and fungi begins with the decarboxylation of ornithine by ornithine decarboxylase (ODC),³ followed by the conversion of the product, putrescine, to spermidine and spermine. These compounds are multivalent cations, with two, three, and four ionizable amine groups, respectively.

The polyamines have several peculiarities not shared by most biosynthetic intermediates. First, they are sequestered in cells (1), and it is likely that they bind to anionic cell constituents such as ribosomes, DNA, polyphosphates, and phospholipids (1-3). Second, while they are required for growth, spermidine and, in

higher organisms, spermine, are "dead-end" products: they are needed in few further biochemical reactions, and even catabolism of the polyamines may be restricted. Under these circumstances, it is odd that efficient feedback inhibition of ornithine decarboxylase, a key biosynthetic enzyme, has never evolved in any eucaryote. This is particularly unusual because high levels of polyamines may be toxic, even in an organism like *Neurospora crassa*, where little polyamine turnover takes place (1, 4, 5).

These peculiarities have led us to the present coordinated study of the management of polyamine pools of *N. crassa* *in vivo*, focusing on uptake, intracellular binding, and excretion during growth.

MATERIALS AND METHODS

Strains and growth. The *N. crassa* strains used were a wild-type strain, ORS-6a; a prototrophic, arginaseless (*aga*) strain, IC3 (6); a strain lacking both orni-

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³ Abbreviations used: ODC, ornithine decarboxylase; PUT, putrescine; SPD, spermidine.

thine decarboxylase and arginase (*spe-1*, *aga*), IC1894-53a (7); and strain IC2572-4a, carrying the *aga* mutation and another mutation, LV105, which blocks polyamine biosynthesis between putrescine and spermidine. The alleles of the *spe-1* and *aga* genes were LV10 and UM906, respectively, and are available from the Fungal Genetics Stock Center, University of Kansas Medical Center, Kansas City, Kansas. The LV105 mutation has normal levels of the ornithine and *S*-adenosylmethionine decarboxylases, and is probably blocked in the spermidine synthase reaction.⁴

The growth medium used was Vogel's medium N (8). Standing cultures were made in 10 ml medium in 50-ml Erlenmeyer flasks held at 32°C. Exponential cultures were heavily inoculated in medium in boiling flasks, with forced air for aeration and agitation, as described previously (9). The medium was supplemented as indicated under Results section. Dry weights were monitored by taking measured samples of culture, collecting the mycelia by filtration, acetone-drying, and weighing (9). Inoculations were made with conidia thoroughly washed in 0.25 M NaCl to remove external polyamines (5). Similarly, all harvests of mycelial samples, particularly those grown in the presence of polyamines, were washed in 0.25 M NaCl before extraction.

Polyamine uptake. Uptake of ¹⁴C-polyamines (putrescine and spermidine) was measured as described previously, but in the growth medium; that is, without transfer to the dilute medium used to characterize the saturable uptake systems (5).

Cell permeabilization. Retention of polyamines by permeabilized cells was measured by resuspending cells in a Na⁺ 3-[*N*-morpholino]propanesulfonic acid buffer (20 mM Na⁺, pH 7.2) with 0.2% glucose (5). The suspension was split in half, and *n*-butanol (7.5% by volume, final concentration) was added to one portion. After 5 min, cells were collected from both halves by centrifugation, and the medium and cell pellets were analyzed for putrescine and spermidine. The values for pellets were corrected for the amounts of extracellular polyamine in medium entrained by cell pellets.

Polyamine determinations. Cellular polyamines were extracted with 0.4 M HClO₄ which contained 2 mM EDTA. Extracts (usually 100 μl of a 1-ml extract) were dansylated and determined by high-performance liquid chromatography as previously described (10). Samples of supernatants from the permeabilization medium, after addition of HClO₄ and EDTA to 0.4 M and 2 mM, respectively, were analyzed similarly. Polyamines of the growth medium were isolated by application to AG50W cation-exchange

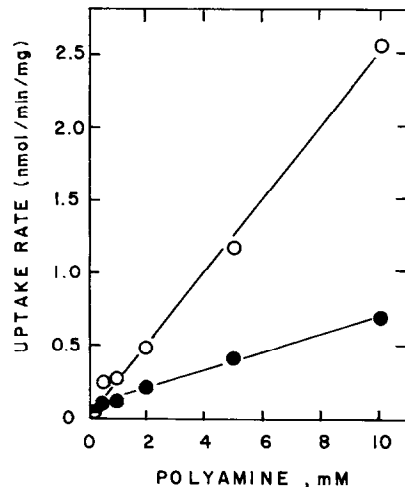


FIG. 1. Concentration dependence of polyamine uptake by the wild-type strain, ORS-6a, in Vogel's medium during growth. Each point was the average of two determinations after incubations of 5 min (putrescine, open circles) or 15 min (spermidine, closed circles).

columns, removing salts with 1.5 N HCl and eluting polyamines with 6 N HCl. These were evaporated, taken up in HClO₄-EDTA, and determined as above. 1,7-Diaminoheptane was used as an internal standard. Hydrolysis of medium and cell extracts in 6 N HCl yielded little or no more polyamines than unhydrolyzed samples, and no acetylpolyamines were detected in wild-type cells by direct analysis. The latter was done by thin-layer chromatography as described by Seiler and Knodgen (11).

RESULTS

Uptake of Polyamines from the Growth Medium

Uptake of putrescine and spermidine by wild-type (strain ORS-6a) or *aga* (strain IC3) cells from Vogel's growth medium is slow because the process lacks a saturable component seen in a dilute buffer (5). This can be attributed to the cations of the medium, particularly Ca²⁺, which are inhibitory to the saturable system. The remaining uptake activity is largely nonsaturable, suggesting a diffusional mechanism (Fig. 1). This system transports putrescine and spermidine at 0.25 and 0.15 nmol/min/mg dry weight, respectively, at 1 mM. (In dilute

⁴ J. Pitkin and R. H. Davis, unpublished experiments.

TABLE I
POLYAMINE CONTENT OF CELLS AND MEDIUM DURING GROWTH OF STANDING CULTURES
OF THE WILD-TYPE STRAIN ORS-6a

Time hr	Dry weight (mg/ml)	Polyamine content of					
		Cells		Cells		Medium	
		PUT	SPD	PUT	SPD	PUT	SPD
		(nmol/mg) ^a		(nmol/ml culture)		(nmol/ml culture)	
0	0					0.1	0.4
24	1.7	0.5	15.9	0.9	27.0	0.7	1.6
48	3.0	0.4	13.5	1.3	40.7	0.7	7.4
72	3.0	0.4	15.2	1.1	45.6	1.3	10.7

^a 1 nmol/mg dry wt = 0.4 mM (apparent) in cell water.

buffer, the saturable system contributes rates of about 1.8 and 3.0 nmol/min/mg at 1 mM putrescine and spermidine, respectively (5.) The initial uptake rate in Vogel's medium is short-lived (5 to 30 min), suggesting equilibration across the cell membrane or a negative control of further uptake.

Excretion of Polyamines during Growth

If the mechanism of uptake from the growth medium is diffusional, one might expect efflux of polyamines from the cell during growth. This was tested in two situations: either during standing, long-term growth or during logarithmic growth of young cultures (Table I). Standing cultures (10 ml) were inoculated with conidia of the wild-type strain, and the media and cells were analysed at 24, 48, and 72 hr growth at 32°C. The cultures were swirled twice each day to prevent aerial growth and conidiation. As growth proceeded, putrescine and spermidine, particularly the latter, were found in the medium (Table I). The greatest increase was seen as growth stopped. Although rather little putrescine was in the cells at any time, a higher proportion of it was lost to the medium, in keeping with its lower valence. The maintenance of the cellular spermidine pool per milligram dry weight argues against cell breakage or cell death being responsible

for "excretion." Instead, it suggests that spermidine is retained by cells by a mechanism that can be saturated, that synthesis continues after that point, and that the excess is lost to the medium. Cultures that were not swirled showed the same pattern of appearance of polyamines in the medium and in the cells, indicating that the differentiation involved in conidiation had little effect.

Excretion of polyamines during logarithmic growth of germinating conidia was tested in three cultures (Table II). In the case of wild type mycelia, virtually no polyamines were excreted. The LV105, *aga* strain (IC2572-4a), which accumulates putrescine, lost over 10% of its putrescine to the medium (Table II). Spermidine was not seen in the medium, in part because little was present in the cell. Finally, the arginase-less *aga* strain (IC3), grown on arginine and later given ornithine, was tested. During growth on arginine, this strain cannot make ornithine because its biosynthesis is feedback inhibited and the arginase reaction, an alternate source of ornithine, is missing (6). Under these conditions, ornithine decarboxylase reaches about 70-fold its normal activity in the cells. (Growth continues, albeit slowly, because a small amount of the putrescine analog, cadaverine, appears by the decarboxylation of lysine (12).) The arginine-grown cells were given ornithine, leading to a

TABLE II

EXCRETION OF POLYAMINES DURING GROWTH OF YOUNG, EXPONENTIAL CULTURES: WILD TYPE (ORS-6a) ON MINIMAL MEDIUM (Min); LV105, *aga* (IC2572-4a) ON MINIMAL MEDIUM; AND *aga* (IC3), GROWN UNTIL $T = 0$ ON ARGININE (Arg), AFTER ADDITION OF 5 mM ORNITHINE (ORN)^a

Strain, Medium	Time (h)	Dry weight (mg/ml)	Polyamine content of					
			Cells		Cells		Medium	
			PUT	SPD	PUT	SPD	PUT	SPD
			(nmol/mg) ^b		(nmol/ml culture)		(nmol/ml culture)	
ORS-6a, Min	0	0.43	0.7	16.4	0.3	7.0	0.4	0.2
	1	0.55	0.7	16.6	0.4	9.2	0.3	0.3
	2	0.69	0.6	15.5	0.4	10.7	0.3	0.3
	3	0.86	1.1	18.6	1.0	16.0	0.3	0.2
IC2572-4a, Min	0	0.3	3.3	5.0	10	0.6	0.8	0.5
	2.5	0.6	53	1.3	32	0.8	5.1	0.2
	5.2	1.0	58	1.2	58	1.2	8.5	0.2
IC3, Arg; ORN added at $T = 0$	0	0.56	1.2	1.3	0.7	0.8	0.2	0.3
	1	0.68	19.7	4.4	13.3	3.0	1.5	0.4
	2	0.86	31.3	10.7	26.9	9.2	4.7	0.7
	3	1.08	27.8	12.2	30.0	13.2	9.2	1.7
	4	1.25	19.8	11.9	24.7	14.9	8.5	0.6
	5	1.50	26.0	13.6	39.0	20.4	9.4	0.8

^a Growth of strain IC3 on arginine leads to a state of ornithine (and thus polyamine) starvation, and severe derepression of ornithine decarboxylase.

^b 1 nmol/mg = 0.4 mM (apparent) in cell water.

large burst of putrescine synthesis (4). About one-third of the putrescine made appears in the medium. None of the spermidine is excreted, but in fact the spermidine pool never much exceeds that found in wild-type cells.

Extracts of standing and logarithmic cultures have only traces of acetylpolyamines or other polyamine conjugates, and very little of the polyamines are found conjugated to macromolecules (data not shown). Little polyamine catabolism has been detected in cultures of this type, and polyamines do not serve as a nitrogen source (5). Only in strains that take up excessive amounts of putrescine does acetylputrescine appear, and it is not a large fraction of the putrescine absorbed.⁵

⁵ R. H. Davis and J. L. Ristow, unpublished observations.

Therefore, polyamine excretion is the only known means of disposing of a large excess of polyamines.

Relation of Internal and External Spermidine

Steady-state cultures of the wild-type and the *spe-1* mutant strains were tested for the effect of spermidine supplementation upon the internal spermidine pool (Fig. 2). Because little spermidine is synthesized, spermidine is almost a nonmetabolizable intermediate, as noted above. In varying the spermidine concentration in the medium, the tests of the two strains differed at the lowest concentration: wild type was grown in minimal medium, while the *spe-1* mutant was grown necessarily with 0.5 mM spermidine. The slope of the internal vs external spermidine concentra-

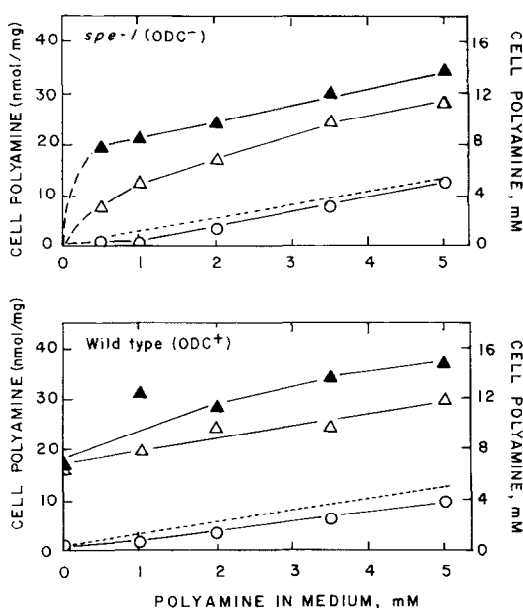


FIG. 2. Effect of varying the external concentration of putrescine or spermidine on the internal polyamine pools of the *spe-1*, *aga* (IC1894-53a, top), and *aga* (IC3, bottom) strains during growth. Circles, putrescine; triangles, spermidine. Open symbols, abscissa represents putrescine-supplemented cultures; closed symbols, abscissa represents spermidine-supplemented cultures. The dotted line represents equal concentrations in the medium and in cell water, assuming 2.5 ml cell water per gram cells, dry weight (right ordinate).

tion was consistent with equilibration of spermidine across the membrane. However, the y -intercept of this function in the *spe-1* mutant, in which all spermidine comes from the medium, is about 18 nmol/mg dry weight. This reflects very efficient uptake of spermidine from the medium at low concentrations. It is very likely that this efficient uptake at low external levels of spermidine is driven by intracellular binding to anionic cell constituents. At higher concentrations of extracellular spermidine, these sites would be saturated, and free equilibration of diffusible, intracellular polyamines with those in the medium takes place.

It is noteworthy that the y -intercept of the *spe-1* spermidine curve, when spermidine is varied, is equal to the amount of this metabolite found in wild-type cells

grown in minimal medium (Fig. 2). This suggests that the same intracellular binding capacity largely determines the spermidine pool size of wild-type cells, and that there is little diffusible spermidine, in keeping with previous tracer work (1).

Relation of External Putrescine and Internal Polyamines

When external putrescine is varied in wild-type cultures, the cellular putrescine level roughly follows what is expected of equilibration across the cell membrane, with the curve passing close to the origin (Fig. 2). However, because the wild-type strain continues to make putrescine and spermidine even in the presence of external putrescine, the contribution of uptake to the internal pool is not clear. In the *spe-1* mutant, the synthesis of spermidine is clearly limited by putrescine uptake at low putrescine concentrations, and spermidine achieves its normal pool size only when the medium has over 2 mM putrescine (Fig. 2). Only at this concentration of external putrescine, in fact, does equilibration across the cell membrane keep pace with demand. Intracellular binding of putrescine (a divalent amine) cannot be seen in this experiment, probably because the trivalent spermidine competes successfully for binding sites.

There is a significant discrepancy between the polyamine pools of wild type and the *spe-1* mutant. Whereas wild type can maintain a normal pool of spermidine (18 nmol/mg, dry weight) in minimal medium with 0.8 nmol of putrescine, the *spe-1* mutant is unable to do so until the internal putrescine pool reaches 3 to 4 nmol/mg dry weight (Fig. 2). Because the putrescine in wild type is drawn from the ODC reaction, and that of the *spe-1* mutant is transported from the medium, ornithine decarboxylase appears to deliver putrescine to the spermidine synthase reaction more efficiently than the transport system does. Whether this reflects an organization of enzymes in wild type or uptake and sequestration at unfavorable locations in the mutant cannot be decided.

TABLE III
RETENTION OF POLYAMINES BY WILD-TYPE CELLS PERMEABILIZED WITH *n*-BUTANOL^a

Growth medium	Butanol	Putrescine (nmol)		Spermidine (nmol)	
		Supernatant	Cells (%) ^b	Supernatant	Cells (%)
Minimal	—	0.2	4.4 (96)	0.05	46.7 (99)
	+	1.2	2.1 (60)	2.1	46.6 (96)
+5 mM PUT	—	1.4	12.2 (90)	0	63.3 (100)
	+	5.0	5.6 (53)	2.8	57.1 (95)
+5 mM SPD	—	0.9	1.1 (55)	1.4	84.0 (98)
	+	1.7	0.5 (23)	4.7	75.2 (94)

^a Cells were grown and processed as described under Materials and Methods. Suspensions of approximately 2.8 mg cells (dry weight) were used.

^b Numbers in parentheses refer to percentage of total polyamine (cells + supernatant) found in the cells.

Polyamine Retention by Permeabilized Cells

Cells of wild type, grown in minimal medium or in medium supplemented with 5 mM putrescine or 5 mM spermidine, were tested for their ability to retain their polyamine pools after permeabilization with *n*-butanol (Table III). The technique is known to remove all soluble arginine, 99% of which is in the vacuoles (13), from cells (5). The permeabilization medium has rather low ionic strength, and the degree of binding of polyamines by permeabilized cells does not necessarily reflect the state of polyamines *in vivo*. Nevertheless, all permeabilized cells appeared to retain over 90% their spermidine, and over half their putrescine. This was true even of cells preloaded during growth with putrescine or spermidine. Only in the case of spermidine-loaded cells was putrescine retained poorly, but the values are too low to be really meaningful. The experiment demonstrates that there is significant anionic material in cells capable of binding polyamines, the strength of binding being related to the charge of the polyamine, as predicted from the observations of living cultures. It is significant that the retention of total polyamines in this experiment was much better than that of isotopically labeled polyamines introduced into cells just before permeabilization in previous experiments (5).

DISCUSSION

We have shown that in the growth medium, the saturable polyamine uptake systems seen in dilute buffer are virtually inactive, and that entry of polyamines is largely diffusional. The nonconcentrative nature of this system was shown by analysis of steady-state cultures in which the external polyamine concentration was varied. Over most of the concentration range tested, the slope of internal vs external concentration had the relationship expected of equilibration across the cell membrane. Moreover, excess polyamines are excreted into the medium, although not necessarily at a rate that maintains a normal pool size.

Of greatest interest was the finding that a considerable, apparently concentrative uptake of spermidine into *spe-1* cells took place when the medium had a low concentration (<0.5 mM) of spermidine. Above this concentration, the internal and external concentrations varied as expected of equilibration. We interpret the apparent concentrative uptake at low concentration as evidence for spermidine sequestration by anionic binding sites within the cell, such as nucleic acids (especially of ribosomes (2)), vacuolar polyphosphate (1), and phospholipids (3). This is consistent with our previous tracer work showing that at least 70–85% of the spermidine of *N. crassa* cells is sequestered from biosyn-

thetic reactions producing and consuming it (1). It also fits with the finding that butanol-permeabilized, wild-type cells release hardly any endogenous spermidine.

Quantitatively, the amount of spermidine trapped by *spe-1* cells at low external concentration approximates that found in wild-type cells grown in minimal medium. This implies that in exponential cultures of *N. crassa*, the spermidine synthetic rate saturates spermidine binding sites, without much excess. The small excess, in fact, is the pool used as an intermediate between putrescine and spermidine synthesis, and as the metabolic signal for regulating ornithine decarboxylase synthesis and turnover (10, 14). Because the diffusible pool is so small, it is highly responsive to the rate of spermidine synthesis, and is well suited to the role of a regulatory signal.

It was not possible to decide whether putrescine might bind to intracellular sites *in vivo*. Under all conditions in which internal and external concentrations were compared, the anionic binding sites inferred from the spermidine experiments were either occupied with the stronger cation, spermidine, or, if not, little putrescine was available owing to its rapid conversion to spermidine. The behavior of putrescine was that expected of equilibration with cell water, as though no binding sites remained. Only in the logarithmic cultures of the LV105, *aga* strain, having little spermidine, was there a large amount of intracellular putrescine which might have been bound internally (Table II). Moreover, some putrescine is bound by permeabilized cells, even those that contain normal amounts of spermidine.

The control of polyamine pool size involves a number of factors. In terms of polyamine synthesis, the control of the synthesis and the turnover of ornithine decarboxylase are the most important (14). It is noteworthy that these mechanisms are the sole known mechanisms of controlling this enzyme in *N. crassa*, and that feedback inhibition does not prevail. The latter mechanism has not been found in any organism, in fact, and it may be dispensable in view of the adequacy of the other mecha-

nisms. In addition, intracellular binding of the potential effectors (e.g., spermidine and spermine) may make them erratic allosteric signals.

We may speculate that during periods of polyamine insufficiency, the needs of cells for polyamines can be met for a time by drawing on the sequestered fraction. This would allow time for derepression or enzyme stabilization to restore a normal rate of synthesis. When polyamines are in excess, *N. crassa* appears to dispose of excess polyamines by excretion. This is seen most clearly in spermidine excretion by normal stationary cultures, but putrescine can also be excreted when it is made in great excess, as noted above. In the absence of a substantial polyamine catabolic route, excretion of polyamines prevents their accumulation to toxic levels.

The mechanism of control of the pools in *N. crassa* is very similar to that for the putrescine pool of *Escherichia coli* proposed recently by Kashiwagi and Igarashi (15). They found that excess putrescine was readily excreted, especially in strains that overproduced it. Spermidine was not excreted by *E. coli*, unlike *N. crassa*, in which it is lost to the medium when growth stops. The difference is probably due to the difference in the efficiency of feedback control of *S*-adenosylmethionine decarboxylase in *E. coli*, which readily prevents excess spermidine biosynthesis, even when the gene for the enzyme is present in multiple copies. It is very likely, however, that polyamine excretion is part of the way in which many organisms and cell types manage the size of their polyamine pools (16), and that this comes into play when intracellular binding sites for the higher polyamines are saturated.

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