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Regulation of Polyamine Synthesis in Relation to Putrescine and Spermidine Pools in *Neurospora crassa*

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Polyamine pools were measured under various conditions of high and low concentrations of cytosolic ornithine with the wild-type and mutant strains of *Neurospora crassa*. In minimal medium, the wild-type strain has 1 to 2 nmol of putrescine and approximately 14 nmol of spermidine per mg (dry weight); no spermine is found in *N. crassa*. Exogenous ornithine was found to cause a rapid, but quickly damped, increase in the rate of polyamine synthesis. This effect was greater in a mutant (*ota*) unable to catabolize ornithine. No turnover of polyamines was detected during exponential growth. Exogenous spermidine was not taken up efficiently by *N. crassa*; thus, the compound could not be used directly in studies of regulation. However, by nutritional manipulation of a mutant strain, *aga*, lacking arginase, cultures were starved for ornithine and thus ultimately for putrescine and spermidine. During ornithine starvation, the remaining putrescine pool was not converted to spermidine. The pattern of polyamine synthesis after restoration of ornithine to the polyamine-deprived *aga* strain indicated that, in vivo, spermidine regulates polyamine synthesis at the ornithine decarboxylase reaction. The results suggest that the regulatory process is a form of negative control which becomes highly effective when spermidine exceeds its normal level. The possible relationship between the regulation of polyamine synthesis and the ratio of free to bound spermidine is discussed.

In *Neurospora crassa*, ornithine is an intermediate in both the synthesis and the catabolism of arginine, and it is the sole precursor of putrescine (1,4-diaminobutane) (10). Putrescine, formed by the cytosolic enzyme ornithine decarboxylase (EC 4.1.1.17), is used in turn in a second reaction by which the aminopropyl moiety of decarboxylated S-adenosylmethionine is added, thus forming spermidine. Putrescine and spermidine are the major polyamines of *N. crassa*, typically having a 1:10 ratio. No spermine is found in this organism (16).

Our interest in the polyamine pathway originated in part with previous tracer work, which suggested that the rate of putrescine synthesis from ornithine was almost wholly unaffected by very large changes (0.2 to 5 mM) in the cytosolic ornithine concentration, even in the short term (2, 3). This is an appropriate feature of a metabolic system which might encounter low (anabolic) or very high (arginine catabolic) concentrations of cytosolic ornithine. The mechanisms by which the stability of flux through ornithine decarboxylase could be maintained are (i) substrate saturation of ornithine decarboxylase even at a low concentration or substrate inhibition at a high concentration; (ii) polyamine turnover, which could maintain constant pools of

polyamines despite higher rates of synthesis; and (iii) a negative control of the flux by putrescine or spermidine.

The data reported here indicate that substrate saturation and polyamine turnover are unlikely possibilities. Instead, the data strongly suggest a fast and highly efficient control of the ornithine decarboxylase reaction in vivo by spermidine.

MATERIALS AND METHODS

Strains and media. Wild-type *Neurospora crassa* (74A) and the mutant strains *ota* (UM-728) and *aga* (UM-906) are from the collection of R. H. Davis. Vogel medium N (19) with 1.5% sucrose was used for growth. Supplements of arginine or ornithine were 1 mM, unless otherwise indicated.

Growth and sampling. Mycelia were grown exponentially from a conidial inoculum (10^6 conidia per ml) in 500- or 1,000-ml cultures with forced air at 25°C. Dry weights were determined by harvesting 10 to 40 ml of culture by filtration on Whatman No. 540 filter circles and then acetone-drying the mycelial pad. Mycelia were prepared for extraction by harvesting 25 to 50 ml of culture (0.2 to 0.8 mg/ml) by filtration and then washing the mycelia with distilled water. The washed mycelia were suspended in 1 or 2 ml of 0.4 M perchloric acid with 2 mM EDTA (disodium salt) at 4°C. The suspensions were centrifuged, and the supernatants were saved for pool measurements.

Polyamine pools. Putrescine and spermidine pools were determined by a double-isotope dilution assay. The dilution of the specific activity of a known quantity of [^{14}C]putrescine or [^{14}C]spermidine by the sample solution was determined by derivatization with [^3H]dimethylamino-1-naphthalenesulfonyl chloride (DNS-Cl). The derivatized polyamines were separated by thin-layer chromatography, eluted in a scintillation vial, and counted. The ratio of ^{14}C to ^3H counts per minute in the derivatized mixture was compared to that of the undiluted, derivatized standard; the result was used to calculate the amount of nonradioactive polyamine in the sample solution. The technique is based on a recently described technique for quantitating amino acids (1).

In practice, a known amount of [^{14}C]polyamine was added to 0.1 ml of the perchloric acid extract (in a conical centrifuge tube) as the internal standard. A 1.0-nmol addition of [^{14}C]putrescine (20,000 dpm/nmol) and a 15.0-nmol addition of [^{14}C]spermidine (2,000 dpm/nmol) were used routinely. The mixture was made alkaline by the addition of 20 to 30 mg of Na_2CO_3 , and 0.1 ml of [^3H]DNS-Cl (2,500 dpm/nmol, 1.5 mg/ml of acetone) was added. The reaction mixture was thoroughly mixed, sealed with a rubber stopper, and incubated overnight in the dark at room temperature. The mixture was extracted with 0.5 ml of benzene. The benzene layer was removed, placed in a conical tube, and evaporated to dryness in a fume hood. The sample was redissolved in 50 μl of benzene, and 5 to 25 μl was spotted on a silica gel thin-layer chromatogram. The chromatogram was developed with ethyl acetate-cyclohexane (2:3, vol/vol), air dried, and sprayed with triethanolamine-isopropanol (1:4, vol/vol). The fluorescent spots were located with a low-energy UV lamp and marked with a pencil. The entire spot was cut from the chromatogram and placed in a scintillation vial with 5 ml of scintillation fluid (5 g of 2,5-diphenyloxazole per liter of toluene). After at least 4 h of elution, the chromatogram slice was removed from the vial. The samples were counted in a Beckman LS230 scintillation counter. The ratio of ^{14}C to ^3H was determined after subtracting the proportion of ^{14}C counts per minute in the ^3H channel (determined with nonradioactive DNS-Cl).

Polyamine pools determined by this method were reproducible, and they agreed with measurements previously made with an automated amino acid analyzer (15). Controls in which known amounts of nonradioactive polyamines were added to biological samples were within 10% of the expected values. The coefficient of variation for standards assayed over the ranges appropriate for biological samples was 9.8% for putrescine and 7.3% for spermidine. Further details of this technique will be published elsewhere (T. J. Paulus and R. H. Davis, submitted for publication); however, several points should be made here. First, spraying of the chromatogram with the triethanolamine reagent was found to be necessary for efficient elution of the derivatives into the toluene-based scintillation fluid. The spray also stabilized and enhanced the fluorescence. Second, the entire fluorescent spot had to be cut out. Although the fluorescent spots were visibly round and symmetrical, we detected an enrichment of the tritiated derivatives in the lower portion

of the spot. This is true of the DNS derivatives of commercially available polyamines as well as of those extracted from mycelia. Summing of the radioactivity in all portions of the spot yields the same results as cutting and eluting the entire spot. This interesting isotope effect warrants further investigation.

Fractionation of ornithine and polyamines. Polyamines were radioactively labeled during exponential growth of *N. crassa* with [^{14}C]ornithine (0.1 μM ; 266 mCi/nmol). Perchloric acid extracts were fractionated by ion-exchange chromatography as previously described (15). Samples of the ornithine and polyamine fractions were assayed for radioactivity. The scintillation fluid was 1 part Triton X-100 to 2 parts toluene (vol/vol); the latter contained 5 g of 2,5-diphenyloxazole per liter. Ornithine was quantitated colorimetrically by the method of Chinard (5).

Chemicals. Putrescine, spermidine, arginine, and ornithine were purchased as hydrochlorides from Sigma Chemical Co. All organic solvents were of reagent-grade quality. Triethanolamine was purchased from Aldrich Chemical Co. Triton X-100 was purchased from Research Products International. Polygram Sil G thin-layer chromatograms (20 by 20 cm, 0.25 mm thick) were purchased from Brinkmann Instruments Inc. Radioactive isotopes, [$1,4\text{-}^{14}\text{C}$]putrescine dihydrochloride (90.2 mCi/mmol), 5-[$\text{tetramethylene-1,4-}^{14}\text{C}$]spermidine trihydrochloride (98.7 mCi/mmol), 5-[$\text{methyl-}^3\text{H}$]DNS-Cl (26.08 Ci/mmol), and L-[$\text{U-}^{14}\text{C}$]ornithine, were purchased from New England Nuclear Corp.

RESULTS

Effect of ornithine and arginine on polyamine synthesis. Wild-type *N. crassa* was grown in minimal medium and in medium supplemented with arginine or ornithine. Previous work has shown that the cytosolic ornithine concentration ranges from approximately 0.2 mM during growth in minimal medium to 5 to 10 mM in arginine-supplemented medium (2, 3), where ornithine is derived from the cytosolic arginase reaction. The effect on the long-term steady-state pools of the polyamines putrescine and spermidine is shown in Table 1. Arginine had very little effect on polyamine pools, thus confirming calculations made from previous tracer experiments (2, 3). Ornithine, however, had a small but significant effect. The effect of ornithine can be rigorously tested with a mutant strain of *N. crassa* (*ota*) defective in the enzyme ornithine aminotransferase. This strain is unable to catabolize ornithine and accumulates large amounts of ornithine in the cell when arginine or ornithine is added to the medium (11). In addition, ornithine causes a partial inhibition of growth in the *ota* strain (9). The results for the *ota* strain are also shown in Table 1. Ornithine, when added to the medium, caused a 50 to 70% increase of total polyamines in the *ota* strain, in contrast to the minor effect of arginine.

Polyamine pools were determined during the period immediately after the addition of arginine or ornithine to cultures growing in minimal medium. A rapid increase in the rate of polyamine synthesis would be indicative of changes in enzyme activity rather than of synthesis of more enzyme. The results (Fig. 1) show that the additions caused changes in 0.5 to 1 h and that values approaching steady-state values (Table 1) were achieved within a generation. The effects are slightly greater in the *ota* strain (Fig. 1B); however, the time course of the increase in wild type and *ota* was the same. Putrescine pools were generally larger after the addition of ornithine, but the time course was not reproducible. Arginine had little or no effect on polyamine pool sizes in the short term. The manner in which polyamine accumulations are related to growth rates (Table 1) is not known.

Conservation of radioactively labeled polyamines during exponential growth.

TABLE 1. Steady-state polyamine pools in wild-type and *ota* strains^a

Strain	Addition to minimal medium ^b	Generation time (h)	Polyamine pool (nmol/mg, dry wt) ^c	
			Putrescine	Spermidine
74A (wild-type)	None	3.0	1.4 ± 0.2	13.7 ± 0.7
	Ornithine	2.9	2.0 ± 0.5	17.3 ± 1.3
	Arginine	3.0	1.6 ± 0.2	15.5 ± 0.8
<i>ota</i>	None	3.3	1.5 ± 0.3	16.0 ± 0.9
	Ornithine	5.0	3.5 ± 0.5	26.8 ± 1.2
	Arginine	3.5	3.4 ± 1.0	16.6 ± 0.5

^a The media indicated were inoculated with conidia, and at least four samples were taken during exponential growth.

^b Additions were at a concentration of 1 mM.

^c With standard deviation ($n \geq 4$).

Polyamine turnover under conditions of excess ornithine was investigated in a pulse-chase experiment. A wild-type culture growing in minimal medium was given a trace of L-[U-¹⁴C]ornithine at a high specific radioactivity, and after 10 min it was given a large excess of unlabeled ornithine. The amount of radioactivity in putrescine and spermidine during the chase period was measured. Figure 2 shows that after a brief period of continued entry of radioactivity into polyamines no further label entered, and no loss of ¹⁴C-labeled polyamines occurred for over a generation.

The role of ornithine as the sole precursor for polyamines in *N. crassa* was established by the

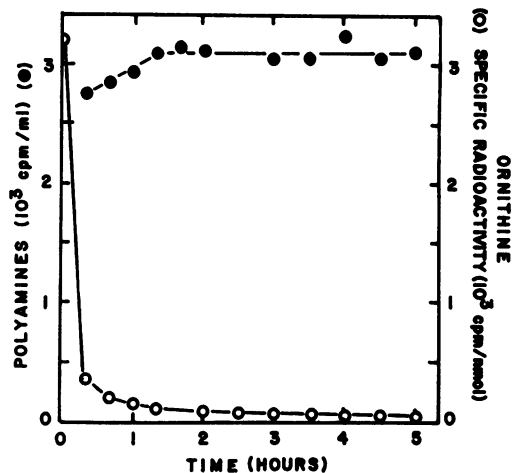


FIG. 2. Conservation of radioactively labeled polyamines. A wild-type culture was given L-[U-¹⁴C]ornithine (0.1 μ M) at a high specific radioactivity (266 mCi/mmol) for 10 min before a chase of 2 mM unlabeled L-ornithine (time zero).

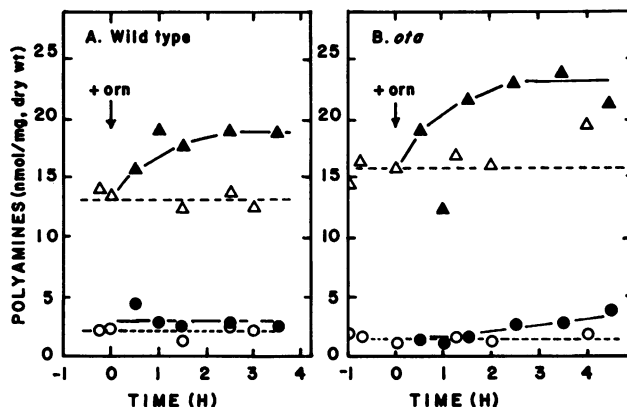


FIG. 1. Polyamine pools in the wild-type strain and the *ota* mutant after the addition at time zero of ornithine (1 mM) to cultures growing exponentially in minimal medium. Putrescine (○) and spermidine (Δ) in culture growing on minimal medium (control); putrescine (●) and spermidine (▲) after the addition of ornithine.

properties of a mutant strain, *aga*, lacking arginase activity (10). The *aga* mutant grows well in minimal medium but requires putrescine or spermidine when arginine is added to the culture. The requirement is imposed by ornithine deprivation: the synthesis of ornithine is feedback inhibited by arginine (13), and the *aga* mutation blocks ornithine formation by the arginase reaction (10). We wished to use the *aga* strain to test polyamine turnover under conditions of ornithine deprivation.

A culture of *aga* cells was grown in minimal medium. Polyamine pools were normal. Arginine (1 mM) was then added, and the polyamine pools were monitored thereafter (Fig. 3). The intracellular ornithine level dropped 10-fold in the first 0.5 h (data not shown). Although there was no immediate effect on growth (Fig. 3, bottom), polyamine accumulation ceased immediately after the addition of arginine. The polyamine pools, expressed as nanomoles per milliliter of culture, were stable for several hours after

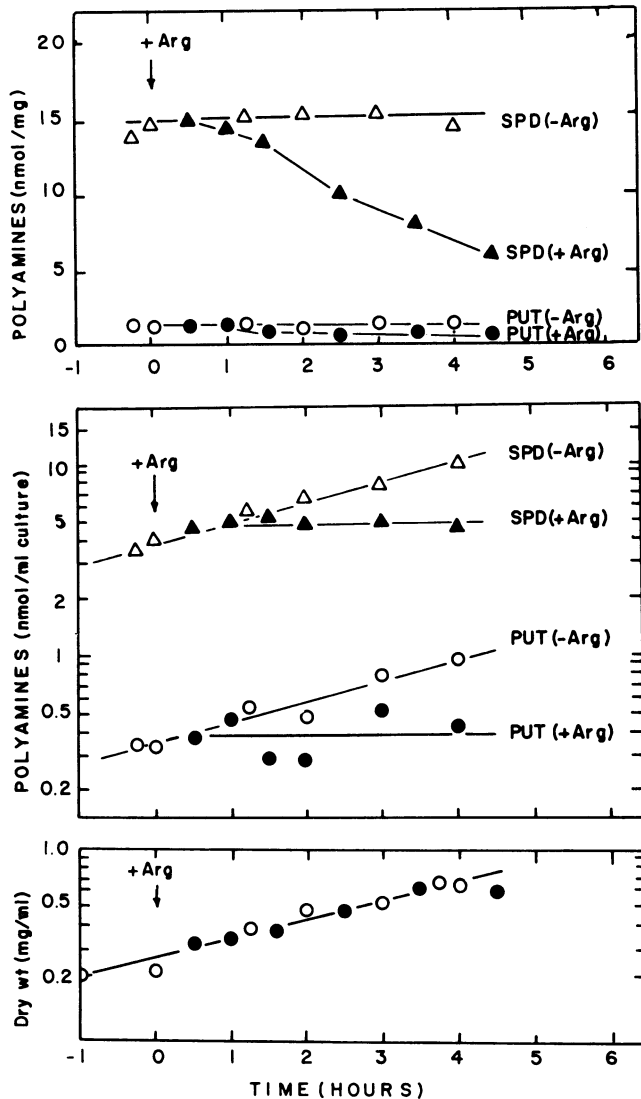


FIG. 3. Effect of arginine addition on the polyamine pools of the *aga* strain. The *aga* strain was grown in minimal medium, and arginine was added to a final concentration of 1 mM at time zero. (Bottom) Dry weight increase with time; (middle) behavior of polyamine pools with time; (top) behavior of polyamine pools, expressed on a dry weight basis. (Open symbols) No arginine added; (closed symbols) arginine added. SPD, Spermidine; PUT, putrescine.

synthesis stopped (Fig. 3, middle) and were accordingly diluted by further growth (Fig. 3, top). This result and the similar data acquired in conditions of excess ornithine indicate that polyamine turnover does not occur during exponential growth.

It is of interest that after the cessation of total polyamine synthesis, the putrescine pool was not significantly converted to spermidine. This may be an important feature of the polyamine biosynthetic pathway and will be discussed further below.

Polyamine synthesis during relief of ornithine deprivation. We wished to study the pattern of polyamine synthesis when ornithine was added to an *aga* culture grown with arginine. Growth of an *aga* mutant in arginine-supplemented medium was slow (doubling time, 4 to 6 h), and polyamine pools were approximately 1/10 normal. When ornithine was added to an arginine-grown *aga* culture, a burst of polyamine synthesis began within the first 15 min (Fig. 4). The rapid synthesis of polyamines was probably due to high levels of the biosynthetic enzymes (17). The accumulation of polyamines (per milliliter of culture) virtually ceased after 2 h. The putrescine pool increased most rapidly, continuing for nearly 2 h, and then declined. The synthesis of spermidine continued after 2 h, but only at the expense of the accumulated putrescine; the total accumulation of polyamines per milliliter of culture remained constant.

When the same data are normalized to a dry weight basis (Fig. 5), the burst of polyamine synthesis after the ornithine addition can be compared to data in Table 1. Several important features of the data become apparent. (i) The polyamine levels of the control culture were, as noted, very low. (ii) Total polyamines accumulated after the ornithine addition to three- to fourfold the normal level (Table 1) before synthesis stopped at 2 h. (iii) Putrescine accumulated to 30 times its normal level before its synthesis ceased at 2 h. (iv) At 2 h, the spermidine pool was approximately equal to the normal level of 15 to 20 nmol/mg (dry weight). At this point, putrescine synthesis stopped. Spermidine continued to be formed from the large putrescine pool, accumulating to twofold its normal level by the end of the experiment. The data indicate that spermidine controls putrescine synthesis from ornithine, but not spermidine synthesis from putrescine. Putrescine, moreover, does not appear to control its own synthesis.

Effect of exogenous spermidine on polyamine pools. To test the effect of excess spermidine on putrescine levels, wild-type mycelia were grown in the presence of 1 mM spermidine.

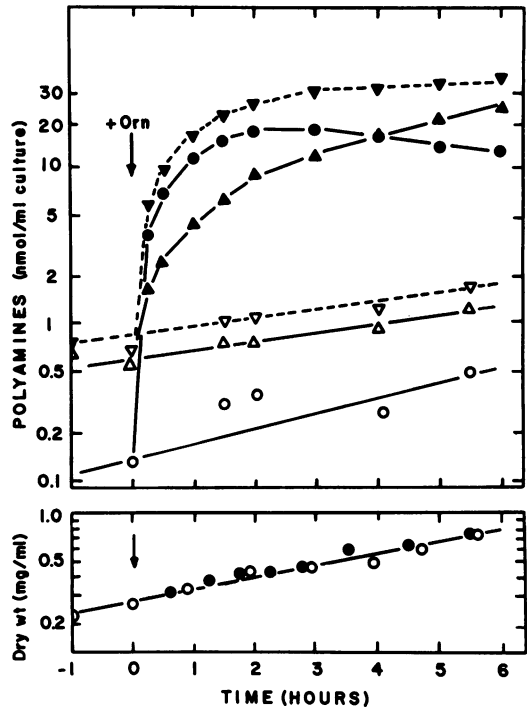


FIG. 4. Effect of ornithine addition on the *aga* strain after growth in minimal medium supplemented with 1 mM arginine. At time zero, L-ornithine was added to a 5 mM final concentration. (Bottom) Increase of dry weight with time in control (○) and ornithine-treated (●) cultures; (top) polyamine pools of control (open symbols) and ornithine-treated (closed symbols) cultures. Symbols: ○ and ●, putrescine; △ and ▲, spermidine; ▽ and ▾, total polyamines (sum of putrescine and spermidine).

No differences were seen in either the putrescine or the spermidine pools of the spermidine-supplemented and control cultures. The inability to expand the endogenous pool of spermidine suggests poor uptake of spermidine from the medium. Radioactive tracer data (not shown) confirmed this.

DISCUSSION

Our results indicate that spermidine exerts a form of negative control over polyamine synthesis in *N. crassa*. The control, moreover, affects the ornithine decarboxylase reaction and is responsible for maintaining stable rates of polyamine synthesis over a wide range of cytosolic ornithine concentrations. The significance of this work is that the regulatory response has been defined *in vivo* in wild-type *N. crassa* and well-characterized mutant strains.

We have shown that polyamine turnover does not occur during exponential growth. We have

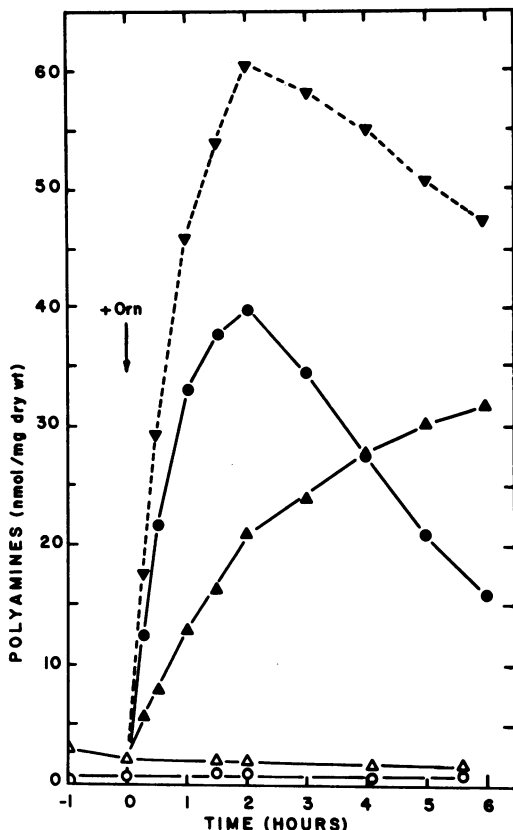


FIG. 5. Effect of ornithine on polyamine pools of the arginine-grown arginaseless (*aga*) strain. Same data as in Fig. 4 (top) expressed on a dry weight basis. Symbols: open, control; closed, ornithine addition; \circ and \bullet , putrescine; Δ and \blacktriangle , spermidine, \blacktriangledown , total polyamines in ornithine-treated culture.

also shown that, although quickly damped, the ornithine decarboxylase reaction responds rapidly to sudden increases in cytosolic ornithine. The effect was not large in the wild-type strain, but it was enhanced in the mutant strain unable to catabolize ornithine. This suggests that ornithine decarboxylase is not substrate saturated at the low ornithine concentrations characteristic of mycelia grown in minimal medium. This hypothesis is consistent with a previous estimate of the K_m of ornithine decarboxylase for ornithine (12). The enzyme was assayed in crude, desalted extracts; the saturation curve was Michaelian, with a K_m of approximately 0.5 mM for ornithine (12). The cytosolic ornithine concentration of mycelia grown in minimal medium has been estimated from tracer experiments to be approximately 0.2 mM (2, 3). We cannot eliminate substrate inhibition by excess ornithine (6) as an accessory mechanism for limiting the response to ornithine.

Arginine had no effect on the rate of polyamine synthesis. The reason for the difference between the effects of arginine and ornithine is not clear, because the addition of both compounds leads to an increase in cytosolic ornithine pools (2, 3). It is possible that ornithine is more effective than arginine in this regard. However, there may be other effects of arginine on polyamine metabolism. Sikora and McDougall (17) have reported that arginine causes a fourfold induction of ornithine decarboxylase in wild-type *N. crassa* and a 100-fold induction of ornithine decarboxylase in the *aga* mutant strain. The induction of ornithine decarboxylase in the *aga* mutant strain grown in arginine-supplemented medium is readily explained by starvation for ornithine, and subsequently for polyamines, as we have shown. The induction of ornithine decarboxylase in wild-type *N. crassa* is not easily understood; further work will be necessary to confirm this phenomenon.

The data presented in this study are consistent with several models for ornithine decarboxylase control, including simple feedback inhibition, protein modification, enzyme inactivation, and inhibitory protein (antizyme) activity (4, 18). However, two features of the control process can be deduced from the pool measurements in Fig. 5. The control process must be rapid and effective over a narrow range (less than twofold) of spermidine concentrations in the cell. (The normal pool of spermidine is 6 mM, if referred to cell water. No polyamine synthesis occurs at double this level, and a twofold increase occurs at one-half this level [Fig. 4].) How is fine control exerted by relatively small changes in a large pool? It is possible that polyamine pools are not freely diffusible but exist in bound and unbound states. Polyamines are known to bind readily to many cell constituents *in vitro* (7). Spermidine has been reported to maintain the conformational integrity of tRNA (8). Polyamines also bind and impart stability to membranes (14, 18). Small changes in the total spermidine pool near the point of saturation of intracellular binding sites may lead to proportionately large changes in a free pool. The large changes in the free pool of spermidine could then exert control over the ornithine decarboxylase reaction.

The data reported in this investigation do not distinguish between free and bound pools of polyamines. However, several of our observations suggest the presence of such pools. First, when arginine was added to the *aga* mutant growing in minimal medium (Fig. 3), polyamine synthesis stopped, but the remaining putrescine was not converted to spermidine. This would not be expected of a freely diffusible putrescine

pool, but instead it indicates that a significant portion of the normal putrescine pool is inaccessible for spermidine synthesis. In addition, during a similar pulse-chase experiment designed to measure polyamine turnover (Fig. 2), the specific activity of putrescine decreased by only 50% in one generation (data not shown). This observation would also not be expected of a small, freely metabolizable putrescine pool.

Further work will test several features of the system described here. First, the binding capacity of spermidine-deprived and spermidine-sufficient cell extracts for added spermidine will be tested. Beyond testing the binding hypothesis, such experiments will allow us to probe the possible differences in the abilities of arginine and ornithine to displace spermidine to the free state, as suggested by their effect on pool size. Second, ornithine decarboxylase will be studied in vitro to determine the mechanism by which spermidine exerts negative control.

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