JOURNAL OF BACTERIOLOGY, Feb. 1977, p. 866-873

Vol. 129, No. 2
Printed in U.S.A.

Control of Arginine Utilization in *Neurospora*

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Received for publication 23 August 1976

The response of *Neurospora* to changes in the availability of exogenous arginine was investigated. Upon addition of arginine to the growth medium, catabolism is initiated within minutes. This occurs prior to expansion of the arginine pool or augmentation of catabolic enzyme levels. (Basal levels are approximately 25% of those found during growth in arginine-supplemented medium.) Catabolism of arginine is independent of protein synthesis, indicating that the catabolic enzymes are active but that arginine is not available for catabolism unless present in the medium. Upon exhaustion of the supply of exogenous arginine, catabolism ceases abruptly, despite an expanded arginine pool and induced levels of the catabolic enzymes. The arginine pool supports protein synthesis until the cells regain their normal capacity for endogenous arginine synthesis. These observations, combined with the known small level of induction of arginine catabolic enzymes, non-repressibility of most biosynthetic enzymes, and vesicular localization of the bulk of the arginine pool, suggest that compartmentation plays a significant role in controlling arginine metabolism in *Neurospora*.

Evidence consistent with the existence of multiple intracellular pools of a variety of amino acids in a large number of different cell types has appeared (12, 14-17, 20, 21, 25, 26). In most cases, the observation that amino acids from exogenous sources are preferentially incorporated into protein was made. In the absence of additional experimental evidence, the mechanism of such selective utilization has remained obscure.

Recent evidence indicates that a large fraction of the amino acid pool of fungi is sequestered within membrane-enclosed organelles; this includes the vesicle in *Neurospora crassa* (17, 20, 21) and the vacuole in *Saccharomyces cerevisiae* (25, 26). This compartmentation accounts for the preferential utilization of exogenous amino acids in *Neurospora* (17, 21) and yeast (7). In the absence of convincing direct evidence for similar compartmentation in mammalian cells, other models of subcellular organization cannot be eliminated (8).

Despite the overwhelming evidence for some level of metabolic compartmentation in many eukaryotic cells, no satisfactory explanation has been presented for the existence of such organizational complexity. In *Neurospora*, arginine is distributed in two subcellular pools: a small cytosolic pool and a large vesicular pool (20). Both anabolic (protein synthesis) and catabolic uses of arginine are carried out by cytosolic enzymes (22; Fig. 1).

During growth of *Neurospora* in minimal medium, no catabolism of arginine occurs despite a significant level of the catabolic enzyme arginase (EC 3.5.3.1). It has been suggested that the cytosolic arginine concentration is insufficient to satisfy the K_m of arginase (17). This is consistent with the observation that the cytosolic arginine concentration increases at least 75-fold when the organism is grown in arginine-supplemented medium (21), conditions that result in only a 3-fold induction of arginase and a 7.5-fold change in the total arginine pool (6). Endogenous arginine biosynthesis ceases (6), although carbamyl-phosphate synthetase A (arginine specific, EC 2.7.2.5), the only repressible enzyme of arginine biosynthesis, is repressed only fourfold (3). Arginine catabolism increases from essentially zero to a rate two to four times that of protein synthesis (21).

This study supports the compartmentation model (Fig. 1) by demonstrating that catabolism of arginine can occur in the absence of enzyme induction and prior to expansion of the total arginine pool. In addition, we also demonstrate that compartmentation of arginine allows *Neurospora* to respond within minutes to changes in the availability of arginine in the medium. Catabolism of arginine is initiated

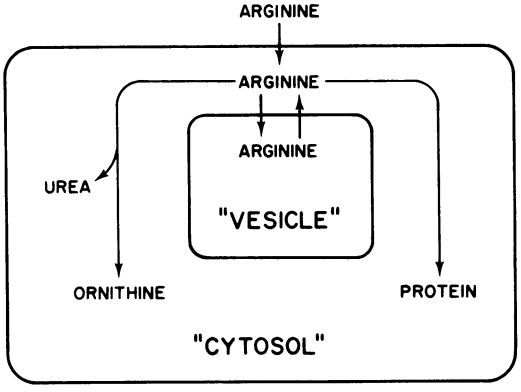

FIG. 1. *Schematic representation of the subcellular organization of the intracellular arginine pool and of the anabolic and catabolic enzymes of arginine metabolism. The vesicle(s) is a membrane-enclosed organelle that sequesters a number of amino acids in Neurospora.*

quickly upon arginine addition to the medium and ceases abruptly upon its exhaustion. The availability of exogenous arginine has no effect on the rate of protein synthesis.

MATERIALS AND METHODS

Strains, media, and chemicals. The ureaseless strain *ure-1* A and the double mutant *arg-5 ure-1* A were used. The *arg-5* mutation prevents the synthesis of ornithine, and such strains are arginine auxotrophs. Strains were used after purification by a single conidial isolation. The medium used was Vogel minimal medium N (19) supplemented with 1.5% sucrose. When the medium was supplemented with arginine, the concentration was indicated in the legends to the appropriate figures. Cycloheximide and L-arginine were purchased from Sigma Chemical Co. L-[*guanido*-^{14}C]-arginine (59 mCi/mmol) was purchased from the Amersham/Searle Corp. and purified by column chromatography prior to use. Analytical grade AG 50W-X8 was purchased from Bio-Rad Laboratories. Triton X-100 was obtained from Research Products International. Scintillation fluid contained 2 parts of toluene (vol/vol) containing 0.4% (wt/vol) 2,5-diphenyloxazole (PPO) per 1 part of Triton X-100.

Growth and sampling. Exponentially growing cultures were obtained from a conidial inoculum (10^6 conidia/ml) and grown at 25°C in flat-bottomed, boiling flasks with forced aeration (4). When cultures were treated with cycloheximide, they were exposed to the drug for 10 min prior to addition of arginine. Labeling experiments were initiated when the cultures had reached a dry weight of 0.30 to 0.60 mg/ml. Dry weights were determined by collection of mycelia by filtration followed by acetone drying (4). Sampling and preparation of radioactive samples for ion-exchange chromatography were performed as previously described (17, 21).

Fractionation and chromatography. Purification of urea and arginine from the medium and from the acid-soluble pool, solubilization of protein arginine, and determination of radioactivity were performed as previously described (17, 21). Urea was estimated by the method of Koritz and Cohen (9), with chloride added to intensify and standardize color values (1). Arginine was estimated colorimetrically by the method of Van Pilsum et al. (18).

Enzyme assays. Arginase was assayed by one of two methods. For enzyme induction experiments, arginase was assayed using permeabilized cells. Cell density was monitored using a no. 54 filter in a Klett-Summerson colorimeter. Cells were collected in chilled tubes containing sufficient cycloheximide to give a final concentration of 10 μg/ml, centrifuged, and washed once with 0.02 M potassium phosphate buffer, pH 7.4, containing 10 mM ethylenediaminetetraacetic acid (buffer A). The washed cells were collected by centrifugation and frozen overnight at −20°C. Thawed cells were suspended in 2.5 ml of buffer A. A 0.125-ml amount of toluene-ethanol (1:4) was added to each sample. The samples were mixed thoroughly for 2 min using a Thermolyne Maxi-Mix. The permeabilized cells were washed once with buffer B (buffer A without ethylenediaminetetraacetic acid) and suspended in an appropriate volume of buffer B. The permeabilized cell suspension was used directly for determination of enzyme activity. Control experiments confirmed the validity of this technique (R. L. Weiss, manuscript in preparation). In other cases, enzyme assays were performed using cell extracts. Arginase was assayed by the method of Davis and Mora (5). Assays were done in duplicate at 37°C, and the correction was made for non-enzymatic activity. Unless otherwise indicated, one unit of activity is defined as the formation of 1 μmol of ornithine in 1 min. Protein was determined by the method of Lowry et al. (10).

RESULTS

Initiation of arginine catabolism. The relation between the size of the intracellular arginine pool and the initiation of arginine catabolism was investigated by supplementing an exponential culture of strain *ure-1* with 0.2 mM [*guanido*-^{14}C]arginine. At various times thereafter, samples were removed and analyzed for intracellular arginine and for radioactivity in protein and urea. The results are shown in Fig. 2. The arginine pool expands relatively slowly, and it reaches the value characteristic of steady-state growth in arginine-supplemented medium after a period of only 25 to 30 min. Radioactivity appears in both protein and urea at the first time point (5 min), and the rate of accumulation increases steadily throughout most of the experimental period. This is consistent with an increasing specific radioactivity of the precursor arginine pool or continued cell growth during the labeling period or both. The most significant and surprising observa-

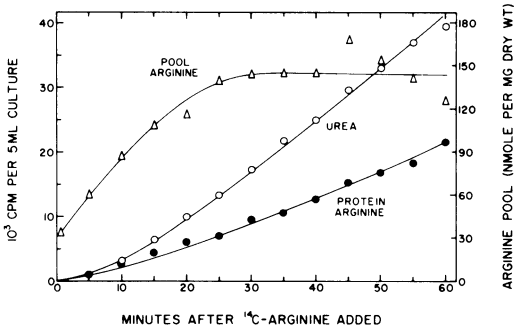

FIG. 2. *Uptake of [guanido-^{14}C]arginine (0.2 mM, 0.125 mCi/mmol) from the medium and its incorporation into protein and degradation to form urea in strain ure-1. Radioactive arginine was added at time zero when the cells had reached 0.6 mg/ml of dry weight.*

tion is the rapidity with which the organism responds to exogenous arginine. Since both protein synthesis and catabolism are derived from the same cytosolic arginine pool (11, 22), it is clear that the rate of catabolism increases from zero and quickly exceeds the rate of protein synthesis.

The results indicate that urea accumulation begins before complete arginine pool expansion. Two possible explanations might account for the rapidity with which arginine catabolism is initiated. First, the catabolic enzyme, arginase, might be induced or activated rapidly upon addition of arginine to the growth medium. Second, the arginine concentration available to arginase might rise dramatically and trigger arginine catabolism. The latter possibility appears more likely, since a high basal level of arginase exists in cells grown in minimal medium, but most of the intracellular arginine pool is sequestered within the vesicles (20) and is inaccessible to arginase (22).

To further substantiate this hypothesis, the level of arginase was examined at various intervals after the addition of 2 mM arginine to a culture growing in minimal medium (Fig. 3). The higher arginine concentration was used to maximize uptake and enzyme induction. The arginine pool rises quickly but reaches its maximum level after 2 to 3 h. In comparison to the results in Fig. 2, the larger pool expansion was due to a 10-fold increase in the amount of arginine supplementation. Note the high basal level of arginase activity and the long lag prior to an increase in the level of this enzyme (40 to 60 min). It is obvious that arginine catabolism is initiated long prior to the induction of arginase.

Because of changing arginine-specific radioactivities during the experimental period, it is not possible to tell from Fig. 2 how the rate of catabolism is affected by the expansion of the arginine pool during the experimental period. It was previously shown that protein synthesis and catabolism are derived from the same cytosolic arginine pool (11, 21). The rate of protein synthesis is unaffected by the presence of exogenous arginine. Thus, a plot of the radioactivity appearing in urea versus that in protein arginine will indicate the relative rates of the two processes independent of cell growth and of any changes in precursor pool specific radioactivity. Such a plot, derived from the data in Fig. 2, is shown in Fig. 4. After a short lag, accumulation of radioactivity in the two products of arginine metabolism increases coordinately with a slope of approximately two. Since the first point represents an interval of 5 min after the addition of arginine to the medium, it is apparent that after an approximately 4-min lag, the rate of catabolism is constant with respect to protein synthesis during the remainder of the experimental period (60 min). This occurs despite continually increasing arginine pool during this time (Fig. 2).

To examine the possible activation of the preexisting arginase, the effect of pretreatment of an exponential culture of strain *ure-1* with the protein synthesis inhibitor, cycloheximide, on arginase activity in vivo was examined. Activity was measured by the appearance of ra-

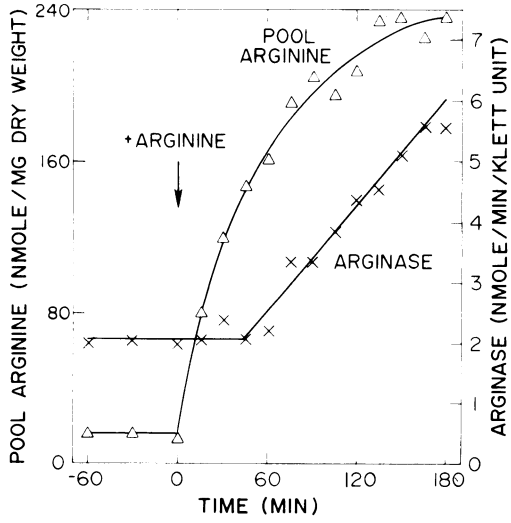

FIG. 3. *Induction of arginase. Cells were grown for 8 h in minimal medium. At time zero, arginine was added to a final concentration of 2.0 mM. At various intervals, samples were withdrawn and analyzed for intracellular arginine and arginase activity.*

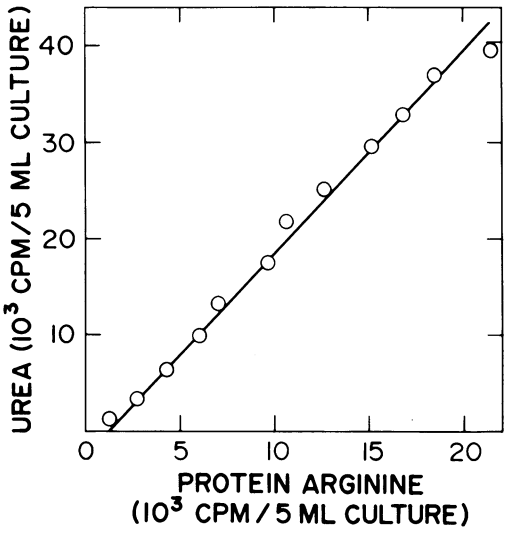

FIG. 4. *Differential rate of urea accumulation. The incorporation of [guanido-^{14}C]arginine into protein and urea (see Fig. 2).*

dioactivity in urea when cells were exposed to moderate levels of [*guanido*-^{14}C]arginine. The results are shown in Fig. 5. Cycloheximide inhibits protein synthesis by more than 98%. Under these conditions, no increase is observed in the activity of arginine catabolic enzymes in extracts (R. L. Weiss and G. P. Anterasian, unpublished observations). Despite the inhibition of protein synthesis and the lack of arginase induction, radioactivity appears in urea after a 5-min lag. Radioactivity in urea increases linearly, despite the continued expansion of the total acid-soluble arginine pool (Fig. 5). The next question addressed was how the organism responds to exhaustion of arginine from supplemented medium.

Cessation of catabolism. The double mutant *arg-5 ure-1* was grown in limiting arginine-supplemented medium. At 30-min intervals, samples were withdrawn and analyzed for medium, intracellular and protein arginine concentrations, dry weight, and accumulated urea. The results are shown in Fig. 6. As long as arginine remains in the medium, urea accumulates exponentially and parallel to protein arginine. Both these accumulations parallel that of cellular dry weight (21). The intracellular arginine pool remains constant at the value characteristic of cells growing in arginine-supplemented medium (approximately 150 nmol/mg of dry weight; 6), and the specific activity of arginine remains constant at the induced level. Upon exhaustion of arginine from the medium, catabolism ceases abruptly and the arginine pool decreases rapidly thereafter. Protein arginine accumulation, however, continues at the same rate until exhaustion of intracellular arginine. At this point, net protein synthesis ceases, since the *arg-5* mutation prevents the endogenous production of arginine. Cessation of catabolism upon depletion of arginine from the growth medium occurs despite an induced level of arginase. (Although the specific activity of arginase declines after depletion of arginine from the medium, it is still more than twice the basal [uninduced] level at the time protein synthesis ceases [R. L. Weiss and G. P. Anterasian, unpublished observations].) Urea

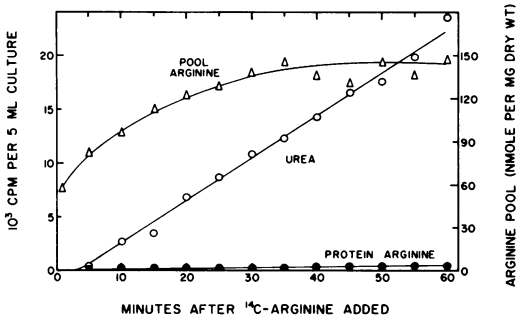

FIG. 5. *Uptake of [guanido-^{14}C]arginine (0.1 mM, 0.14 mCi/mmol) and incorporation into urea in the presence of cycloheximide. An exponential culture of Neurospora was pretreated for 10 min with 10 μg of cycloheximide per ml when it had reached 0.3 mg/ml of dry weight.*

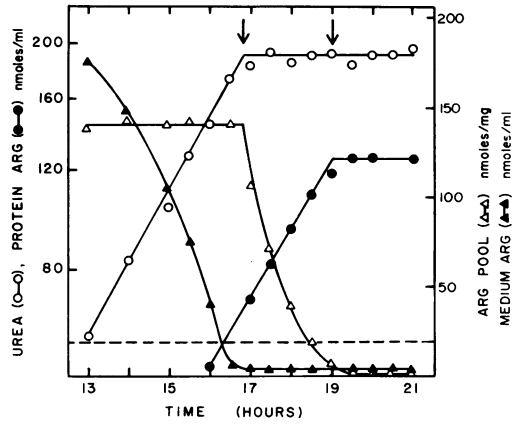

FIG. 6. *Response of strain arg-5, ure-1 to exhaustion of arginine from the medium. The mutant was grown on a limiting (0.3 mM) concentration of arginine. At various intervals, samples were withdrawn and analyzed for medium, acid-soluble, and protein arginine concentrations and for accumulated urea. The dashed line indicates the size of the acid-soluble arginine pool of wild-type cells grown in minimal medium.*

accumulation recommences within 5 min if additional arginine is added to the medium at any time after the cessation of catabolism. This occurs even in the presence of cycloheximide (see below).

To assess more accurately the transition to arginine-depleted medium, a similar experiment was performed using strain *arg-5 ure-1* with the addition of a small amount of [*guanido*-^{14}C]arginine to the medium approximately 90 min before the expected depletion. Samples were removed at 10-min intervals and analyzed for radioactivity in urea, protein, and arginine remaining in the medium. The results are shown in Fig. 7. Protein synthesis continues throughout the experiment. The rate of urea synthesis begins to decrease at about 90 min and is zero at 120 min. The decrease in rate occurs concomitantly with the decreasing rate of arginine uptake from the medium. By 120 min, more than 95% of the added radioactivity has been taken up by the cells, the medium arginine concentration has fallen to less than 10 μM, further uptake proceeds only slowly, and arginine is used exclusively for protein synthesis. The data further substantiate the observed abrupt cessation of catabolism in response to a substantial reduction in arginine uptake from the medium.

Utilization of the endogenous arginine pool. The results shown in Fig. 6 and 7 indicate that the expanded endogenous arginine pool formed during growth in arginine-supplemented medium is readily available for protein synthesis upon exhaustion of arginine from the growth medium. The effect of the large arginine pool on growth of the prototrophic strain *ure-1* was examined during the transition from growth in arginine-supplemented medium to growth in arginine-free medium (Fig. 8). Upon exhaustion of arginine from the medium (approximately 9 h), the soluble arginine pool declines rapidly, reaching a minimum at 13.5 h. The decrease in the pool is slower than that observed in the arginine auxotroph (Fig. 6). After reaching a minimum, the soluble arginine pool begins to level off at a value similar to that observed in cells growing in minimal medium. During the entire transition period, growth continues at the normal rate (Fig. 8). At the end of the period, endogenous arginine biosynthesis can support maximal growth.

Catabolism and arginase activity. A major premise used in interpreting the experimental results is that activities measured in cell extracts or permeabilized cells are related to the potential activity in intact cells. The experiments shown in Fig. 2, 3, and 5 imply that activity measured in extracts is expressible in

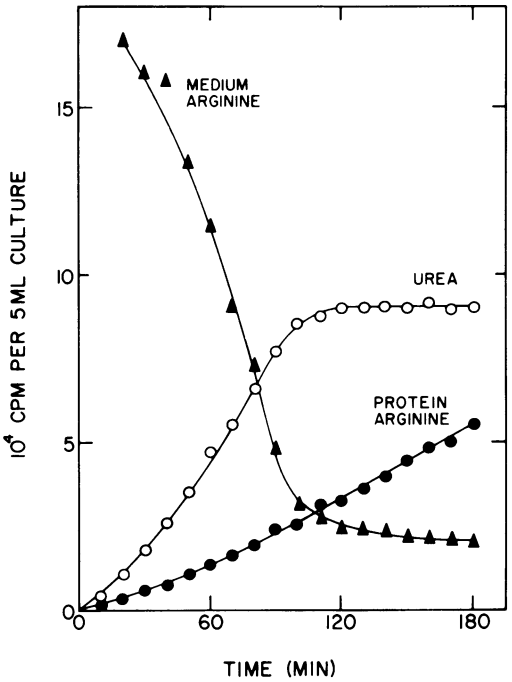

FIG. 7. *Utilization of [guanido-^{14}C]arginine by strain arg-5, ure-1. The basic experimental details are the same as in Fig. 6. Approximately 90 min prior to exhaustion of medium arginine (time zero), 5 μCi of [guanido-^{14}C]arginine was added to 200 ml of the culture. At 10-min intervals, samples were withdrawn and analyzed for radioactivity in urea, medium, and protein arginine.*

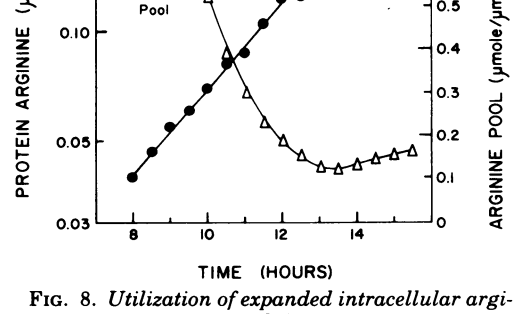

FIG. 8. *Utilization of expanded intracellular arginine pool by strain ure-1. Cells were grown in minimal medium supplemented with 0.2 mM arginine. At various intervals, samples were withdrawn and analyzed for medium, acid-soluble, and protein arginine concentrations. The medium arginine was exhausted between 8.5 and 9 h.*

intact cells. In fact, experiments similar to that shown in Fig. 5 can provide a quantitative measure of cellular arginase activity. We have measured the relative activities of uninduced and induced levels of arginase in both cell extracts and intact cells. The uninduced culture was strain *ure-1* grown in minimal medium. The induced culture was strain *arg-5 ure-1* grown in arginine-supplemented medium for 16 h and then washed and transferred to minimal medium for 1.5 h (cells equivalent to those at 18.5 h in Fig. 6). The two cultures were diluted to equal concentrations and treated with cycloheximide (final concentration of 10 μg/ml) for 10 min. A portion of each culture was then extracted and assayed for arginase activity. A second portion was exposed to arginine (5 mM) for 60 min, and the culture was then assayed for accumulated urea. The activities of both induced and uninduced cultures were compared. The uninduced culture accumulated urea at the rate of 0.925 nmol/min per mg of protein, whereas the induced culture rate was 1.57 nmol/min per mg of protein. The ratio is 1.70. The specific activities of the arginase measured in cell extracts were 1.56 and 2.62 units/mg of protein, respectively. The ratio of specific activities is 1.68. The results indicate that activities measured using cell extracts are proportional to the potential activity existing in intact cells. Similar conclusions have been obtained for permeabilized cells (R. L. Weiss, manuscript in preparation).

DISCUSSION

Arginine catabolism, as measured by the appearance of urea in *ure-1*, commences very soon after the addition of arginine to minimal medium (Fig. 2). Catabolism does not require enzyme induction, since it occurs prior to arginase induction (Fig. 3) and in the presence of cycloheximide (Fig. 5). The rate of arginine catabolism becomes constant within 4 min after arginine addition (Fig. 4), whereas the total arginine pool has only increased to 60 nmol/mg of dry weight, or approximately 40% of the pool found in arginine-grown cultures. These results suggest that: (i) neither arginase induction nor total arginine pool expansion is necessary for arginine catabolism; (ii) the concentration of the arginine pool accessible to arginase either saturates the enzyme or remains constant during total pool expansion.

When arginine is depleted from the growth medium, catabolism ceases abruptly (Fig. 6). Such cessation does not appear to be due to inactivation of arginase, since readdition of arginine in the presence of cycloheximide results in a rapid restoration of arginine catabolism. The rate of catabolism begins to decrease as the medium arginine nears exhaustion and the rate of uptake declines (Fig. 7). When the rate of arginine uptake becomes low enough, the concentration of arginine available to arginase appears to drop below the threshold required for catabolism. This occurs despite the presence of an induced level of arginase and an expanded arginine pool. This suggests that the rate of catabolism is determined, in part, by the rate at which arginine enters the cell. The continued incorporation of arginine into protein (Fig. 6) indicates that the arginine concentration is sufficient to maintain a normal rate of protein synthesis. Only when the arginine pool is almost completely exhausted does the arginine concentration decrease sufficiently to affect the rate of protein synthesis.

These results are consistent with compartmentation of the bulk of the arginine pool (Fig. 1; 17, 20, 21). The concentration of arginine in the cytosol will depend on the rate of its entry into the cytosol (from the medium or vesicular pool or both) relative to its consumption (protein synthesis and withdrawal into the vesicular pool). When arginine from the medium is entering the cell, the consumption processes appear to be overwhelmed and the cytosolic arginine concentration increases. This increase is now sufficient to satisfy the K_m (21, 22) of arginase (5 mM). In the absence of uptake from the medium, the cytosolic concentration of arginine decreases below the threshold required for catabolism but still is sufficient to satisfy the K_m (13) for arginyl-transfer ribonucleic acid synthetase (20 μM).

In *Neurospora*, arginine biosynthesis is controlled by feedback inhibition of acetylglutamate kinase (EC 2.7.2.8; 2) and repression of carbamyl-phosphate synthetase (3). All biosynthetic enzymes except carbamyl-phosphate synthetase A are nonrepressible (3). Both regulatory responses react to the cytosolic arginine concentration (R. L. Weiss, unpublished observations). Although feedback inhibition can be released almost instantaneously, derepression of carbamyl-phosphate synthetase will require a significant period of time. The data in Fig. 6 indicate that the vesicular compartmentation of arginine provides an arginine pool capable of supporting almost an entire generation of normal growth (2.5 h) before the cells would require biosynthetically derived arginine to support continued normal growth. The experiment shown in Fig. 8 indicates that vesicular compartmentation provides a means of smoothing the transition between growth in arginine-supplemented and minimal medium. Normal growth continues throughout the transition, as

the cells come to rely on biosynthesis for their arginine.

The results presented here suggest that compartmentation is the primary factor controlling arginine catabolism. During growth in minimal medium, the cytosolic arginine concentration remains low and no catabolism occurs (17). When the organism is exposed to arginine, the small cytosolic pool expands quickly and the preexisting arginase begins to metabolize arginine. The increase in the cytosolic arginine concentration results in the cessation of endogenous arginine biosynthesis (R. L. Weiss, manuscript in preparation) probably by feedback inhibition of the cytosolic enzyme acetylglutamate kinase (2, 3) and repression of carbamylphosphate synthetase A (3). When the exogenous arginine disappears, the cytosolic arginine concentration drops abruptly, catabolism ceases, and it appears that feedback inhibition is relieved and derepression commences. The vesicular arginine pool supports growth as the cell's ability to synthesize arginine is restored.

Similar mechanisms would appear to be operative in yeast, since similar compartmentation has been observed (25, 26) and certain regulatory responses described are also paralleled in this organism (23, 24). However, the large magnitude of induction of the catabolic enzymes and the observed repression of biosynthetic enzymes suggests that, in this organism, the control of enzyme levels is a more significant regulatory feature than in *Neurospora*. The existence of similar compartmental features in many mammalian cells (12) suggests that these features may be a common occurrence in many eukaryotic cells. Thus, compartmentation of amino acids in eukaryotic cells would appear to provide a means of accumulating significant reservoir pools of amino acids without affecting a cell's ability to respond quickly to environmental alterations. The physiological role of these reservoir pools is under investigation.

ACKNOWLEDGMENTS

This investigation was supported in part by National Science Foundation research grants GB-43240 (R.L.W.) and GB-13398 (R.H.D.) and by a grant (3072) from the Committee on Research, Academic Senate, University of California at Los Angeles.

We thank George P. Anterasian and Jane S. Taber for technical assistance and Joann R. Basabe for criticism of the manuscript.

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