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Compartmentation and Control of Arginine Metabolism in *Neurospora*

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The fate of [^{14}C]arginine derived from the medium or from biosynthesis has been examined in *Neurospora* growing in arginine-supplemented medium. In both cases the label enters the cytosol, where it is used efficiently for both protein synthesis and catabolism before mixing with the majority of the endogenous [^{12}C]arginine pool. Both metabolic processes appear to use the same cytosolic arginine pool. It is calculated that the nonorganellar cytoplasm contains approximately 20% of the intracellular arginine pool when the cells are growing in arginine-supplemented medium. The results suggest that compartmentation of arginine is a significant factor in controlling arginine metabolism in *Neurospora*. The significance of these results for studies of amino acid metabolism in other eukaryotic systems is discussed.

Amino acids are required precursors for protein biosynthesis, and yet they can serve as sources of nitrogen and/or carbon in many organisms. Catabolism is usually thought to be undesirable when cells are generating amino acids biosynthetically. Thus regulatory mechanisms have been evolved to control the metabolic distribution of amino acids between potentially competitive anabolic and catabolic pathways.

Most prokaryotic organisms solve this problem in a simple fashion. First, they fail to accumulate large pools of the amino acids. Second, catabolic enzymes are absent in cells growing in minimal medium but are induced when the pool expands in response to an exogenous supply of the amino acid. In many eukaryotic cells this simple mechanism does not appear to be sufficient to account for the control of amino acid utilization. Significant levels of catabolic enzymes and large pools of the amino acids are often observed during growth in unsupplemented medium. Enzyme levels and pool sizes increase only moderately (less than 10-fold) in the presence of an exogenous supply of the amino acid.

Arginine metabolism in *Neurospora* is an example of this phenomenon. During growth in minimal medium, a large arginine pool (8 mM in cell water) coexists with a significant level of its catabolic enzyme, arginase (EC 3.5.3.1). No catabolism is observed (6). During steady-state growth in arginine-supplemented medium, most of the metabolized arginine flows through the catabolic pathway (8). Under these conditions, the arginine pool is increased only 7.5-fold and arginase is induced only 3-fold (8).

We have recently demonstrated that most of the arginine pool of cells growing in minimal medium is sequestered within a membrane-bound vesicle (18). Pulse-labeling experiments have substantiated this observation and indicated that less than 2% of the total arginine pool is in the cytosol under these conditions (16). Both arginyl-transfer ribonucleic acid (tRNA) synthetase and arginase appear to be cytosolic enzymes (19). These compartmental features are summarized in Fig. 1. It has been suggested that this compartmentation plays a significant role in controlling arginine metabolism (18). In this hypothesis, the cytosolic arginine concentration plays a crucial role: during growth in minimal medium it is low, and arginine is channeled to protein because of the kinetic properties of the relevant enzymes (18); in arginine-supplemented medium it increases dramatically despite only a small change in the total arginine pool. This large increase results in induction of the catabolic enzymes (small) and rapid catabolism since the cytosolic arginine concentration now exceeds the kinetic requirements of arginase.

In this study I will further substantiate this hypothesis by showing that: (i) catabolism does not distinguish between exogenous or biosynthetically derived arginine; (ii) anabolic and catabolic products of arginine metabolism are derived from the same subcellular pool; and (iii) the cytosolic arginine concentration increases at least an order of magnitude more than the

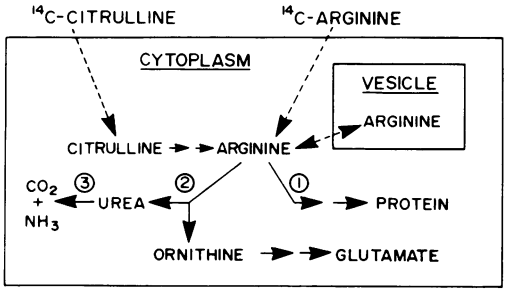

FIG. 1. *Simplified diagram of the biochemical steps and subcellular structures involved in arginine metabolism in Neurospora. The vesicle is a subcellular organelle distinct from mitochondria which may be analogous to the vacuole of higher plants. Metabolic steps: 1, arginyl-tRNA synthetase; 2, arginase (EC 3.5.3.1); 3, urease (urea amidohydrolase, EC 3.5.1.5).*

total arginine pool during growth in arginine-supplemented medium. The significance of these observations in regard to metabolic regulation in eukaryotic cells will be discussed.

MATERIALS AND METHODS

Strains, media, and chemicals. The ureaseless strain *ure-1* A (allele *ur9* of Kølmark [10]) and the double mutant *arg-5,ure-1* were obtained from Rowland H. Davis. Vogel minimal medium N (21) was used for growth. Arginine-supplemented medium had a final arginine concentration of 1.0 mM. L-Arginine, L-citrulline, and cycloheximide were purchased from Sigma Chemical Co. The isotopically labeled compounds L-[*guanido*-^{14}C]arginine (50 mCi/mmol) and L-[*carbamyl*-^{14}C]citrulline (62 mCi/mmol) were purchased from Amersham/Searle and purified by column chromatography before use. Ion-exchange resin AG 50W-X8 was obtained from Bio-Rad Laboratories.

Growth and sampling. All strains were grown at 25 C from a conidial inoculum (approximately 5×10^6 conidia/ml). Cultures were grown in 1-liter flat-bottomed boiling flasks with forced aeration (7). Dry weights were determined by collection of appropriate-sized samples on Whatman no. 54 filter paper, drawing 5 to 10 ml of acetone through the pad, followed by air drying for 1 to 2 min. Dried cells were scraped from the filter paper and weighed.

Cells were separated from the medium by filtration using Gelman GA-6 cellulose-acetate membrane filters (0.45-μm pore size). The filtrate was analyzed for urea and medium arginine. Control experiments indicated that more than 98% of the accumulated urea was present in the medium. The cells were washed three times with ice-cold distilled water. No loss of intracellular arginine occurred in this procedure. Cells were then scraped from the filter into 3 ml of 5% trichloroacetic acid. After centrifugation and re-extraction, the combined supernatants were extracted three times with ethyl ether to remove the trichloroacetic acid, evaporated, and used for column chromatography. The pellet was suspended in 4 ml of 3 N HCl, autoclaved for 1 h, and analyzed for arginine by the method of Van Pilsum et al. (20).

In the experiments involving radioactive amino acids, small (5 ml), fast harvests were made by using wide-bore 10-ml pipettes. A 5-ml sample was pipetted directly into 0.25 ml of 100% trichloroacetic acid. After a minimum of 1 h, the samples were centrifuged and the supernatants were prepared for chromatography as described above. The precipitate (protein) was collected on Whatman GF/C glass-fiber filter paper, washed five times with ice-cold 5% trichloroacetic acid, then washed three times with ethanol-ether (1:1), and finally washed with ether. The samples were air-dried overnight and then counted in toluene scintillation fluid.

A second 5-ml sample was also taken. This sample was filtered immediately, using the membrane filters. The collected cells were washed with ice-cold water, extracted, and analyzed for radioactivity and amount of soluble arginine. A portion of the filtrate was analyzed for radioactivity and amount of medium arginine.

Fractionation and chromatography. Urea and medium arginine were separated on AG 50W-X8 (H^+ form) as previously described (16). Intracellular arginine was first purified on AG 50W-X8 (Na^+ form) followed by desalting on the same columns in the H^+ form (16). Urea and arginine were determined after evaporation of HCl. Urea was estimated by the method of Koritz and Cohen (11), with chloride added to intensify and standardize color values (3). Arginine was estimated colorimetrically by the method of Van Pilsum et al. (20). Radioactivity of dry samples was estimated, using toluene scintillation fluid (4 g of 2,5-diphenyloxazole per liter of toluene) and aqueous samples in toluene-Triton X-100 scintillation fluid (16). Counting was done in a Beckman CPM-100 liquid scintillation counter.

Calculations. The specific radioactivities of medium and pool arginine were determined by direct measurement. The specific radioactivities of new protein arginine and of newly synthesized urea were determined as follows (16): (i) best-fit lines were derived from the individually determined values for the incorporation of radioactivity into protein or urea; (ii) these lines were used to calculate the amount of radioactivity incorporated into protein or urea for each 5-min interval; (iii) the absolute amount of new protein arginine or new urea was calculated by the equation $\Delta X = X_0 (e^{kt} - 1)$, where ΔX is the amount of new protein arginine or new urea, X_0 is the amount at the beginning of the interval, k is the growth constant, and t is the time interval in minutes (X_0 and k were determined by monitoring the accumulation of protein arginine and urea during growth); and (iv) the specific radioactivities were calculated as the change in total radioactivity during the time interval divided by ΔX (16).

RESULTS

Urea accumulation as a measure of arginine catabolism. The simplest method of deter-

mining the flux through a metabolic reaction is to measure the accumulation of a stable reaction product. The anabolic and catabolic products of arginine metabolism are shown in Fig. 1. The anabolic product, protein arginine, is a reasonably stable end product that accumulates during growth. The catabolic products, urea and ornithine, are further metabolized, and their components are ultimately reincorporated into a wide variety of cellular constituents or liberated as CO_2.

Individual mutants are available that lack the enzyme necessary for the degradation of urea or ornithine, respectively. Because ornithine is required for polyamine synthesis (8), the urease-negative mutant *ure-1* was examined as a tool for measuring flux through the catabolic pathway. Three criteria were evaluated to determine whether urea accumulation in *ure-1* was a specific measure of arginine catabolism. First, no other sources of urea must exist during steady-state exponential growth. This has been shown to be the case (6). Second, no alternative means of urea degradation must exist. This has been shown to be the case (6). Third, accumulation of urea must occur exponentially and parallel the accumulation of other stable cellular constituents during steady-state growth in arginine-supplemented medium.

The accumulation of urea, protein arginine, and dry weight during exponential growth of *ure-1* in arginine-supplemented medium is shown in Fig. 2. During exponential growth, *ure-1* accumulated both urea and protein arginine. These accumulations paralleled the increase in dry mass of cells throughout the period of exponential growth. During such growth, all the metabolized arginine from the medium could be accounted for in the accumulated protein arginine and urea. This observation is consistent with the observation that feedback inhibition prevents endogenous arginine synthesis during growth in arginine-supplemented medium (8). In a similar experiment, the exogenous arginine was isotopically labeled in its guanido-carbon. The specific radioactivities of the accumulated urea and protein arginine were found to be identical with that of the exogenous arginine. These results indicate that urea accumulation is an accurate and specific measure of arginine catabolism and suggest that increased accumulation does not affect the rate of such catabolism.

Source of arginine used for protein synthesis and catabolism. A culture of *ure-1* was grown in arginine-supplemented medium. The accumulation of urea and protein arginine was followed (see Fig. 2). At the middle of exponential growth, [*guanido*-^{14}C]arginine was added to the medium. At short intervals thereafter, samples were removed and the radioactivity in arginine (soluble and protein) and urea was determined (Fig. 3). The soluble arginine pool was rapidly labeled, but the rate of labeling declined at later times as the pool tended toward equilibrium with the medium. The rate of labeling of protein arginine and urea increased rapidly in the first 20 min, but appeared to become almost linear by 30 min. The increasing rate of incorporation was consistent with the increasing label in the arginine pool. The long lag before the achievement of a steady-state rate of incorporation suggests that [^{14}C]arginine is substantially diluted before incorporation into both urea and protein. The steady-state ratio of accumulation of urea and protein determined over a 6-h period averaged 2.9 ± 0.1 (standard error of the mean [SEM]). This ratio varied from 2.6 to 4.2 between independent experiments. The corresponding ratio of radioactivity was 2.8 ± 0.1 (SEM). The results indicate that both anabolism and catabolism are derived from the same intracellular pool of arginine when arginine is from the medium.

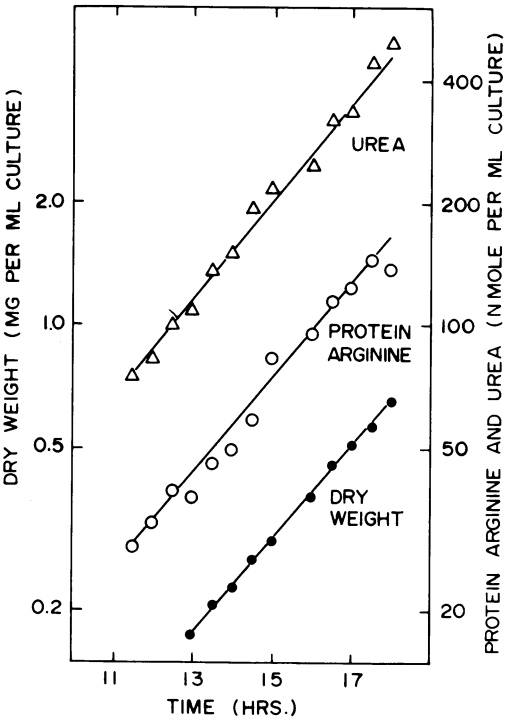

FIG. 2. *Accumulation of cellular dry mass, protein arginine, and urea during exponential growth of Neurospora in minimal medium supplemented with 1.0 mM arginine.*

A similar experiment was performed using

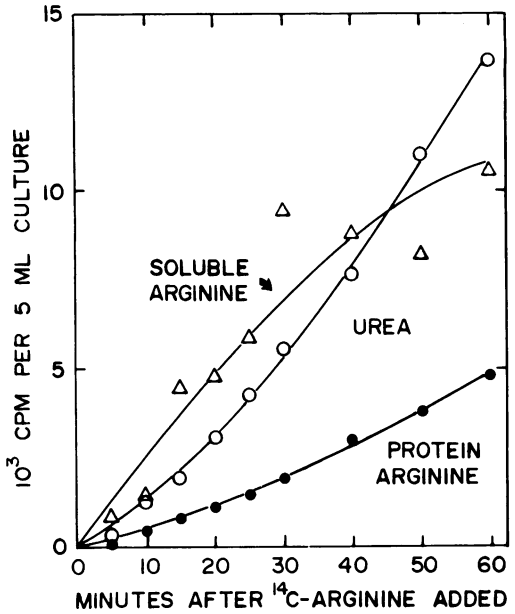

FIG. 3. *Uptake of [guanido-^{14}C]arginine from the medium and its appearance in soluble arginine, protein arginine, and urea. Five microcuries of [guanido-^{14}C]arginine was added to a 200-ml culture of Neurospora equivalent to that shown in Fig. 2 at 15.5 h.*

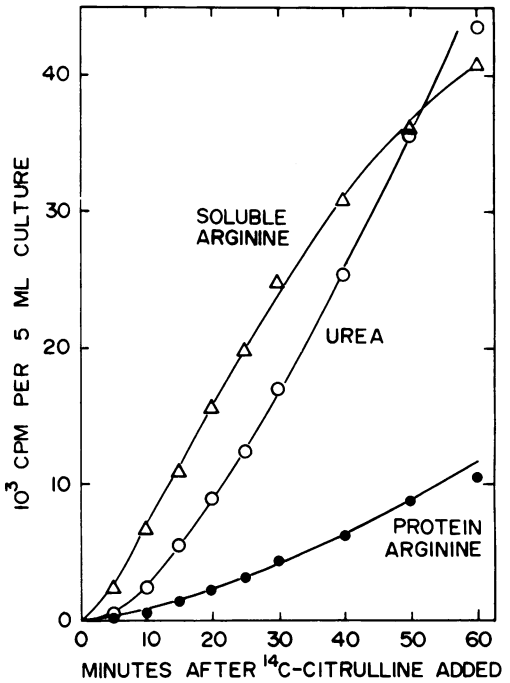

FIG. 4. *Uptake of [carbamyl-^{14}C]citrulline from the medium and its appearance in soluble arginine, protein arginine, and urea. Five microcuries of [carbamyl-^{14}C]citrulline was added to a 200-ml culture of Neurospora equivalent to that shown in Fig. 2 at 15.5 h.*

[*carbamyl*-^{14}C]citrulline as a source of endogenously synthesized radioactive arginine (Fig. 1). This experiment is possible since the presence of arginine in the medium does not prevent transport of citrulline (17) nor repress the enzymes that convert citrulline to arginine (1). The results of such an experiment are shown in Fig. 4. After a short lag, radioactivity appeared in arginine and accumulated rapidly for almost the entire 60-min labeling period. Radioactivity appeared in protein and urea after a similar lag. The rate of their accumulation became relatively constant only after 20 to 30 min. This result is consistent with a significant dilution of the newly synthesized radioactive arginine by an unlabeled arginine pool. The ratio of incorporation of radioactivity into urea and protein was constant, averaged 4.0 ± 0.1 (SEM), and was not significantly different from the steady-state ratio of 3.9 ± 0.1 (SEM). No distinction was observed therefore between anabolic and catabolic utilization of newly synthesized arginine. Similar conclusions have been reached by Castaneda et al. (2).

The results described above are consistent with the observation that both arginase and arginyl-tRNA synthetase are soluble enzymes (19). Both protein and urea are therefore derived from the cytosolic arginine pool.

Relation of cytosolic to total intracellular arginine pool. The data in Fig. 3 and 4 measure the amount of radioactive arginine incorporated into protein and urea during relatively short time intervals. These data and the steady-state parameters (as Fig. 2) for these experiments have been used to calculate the average specific radioactivity of the cytosolic arginine pool during the intervals (Materials and Methods; 16). These figures are plotted at the midpoint of the intervals and compared with the specific radioactivity of the total intracellular arginine pool (Fig. 5 and 6).

In the experiment in which the cells were labeled with [^{14}C]arginine (Fig. 5), the specific radioactivity of the total arginine pool increased linearly for approximately 20 min and more slowly thereafter, and had not reached equilibrium after 60 min. In the citrulline experiment (Fig. 6), a brief lag preceded the linear increase in specific radioactivity of the total arginine pool. This reflected the time necessary for the conversion of citrulline to arginine. In both experiments, the cytosolic arginine pool (source of protein arginine and urea) was more rapidly labeled than the total arginine pool.

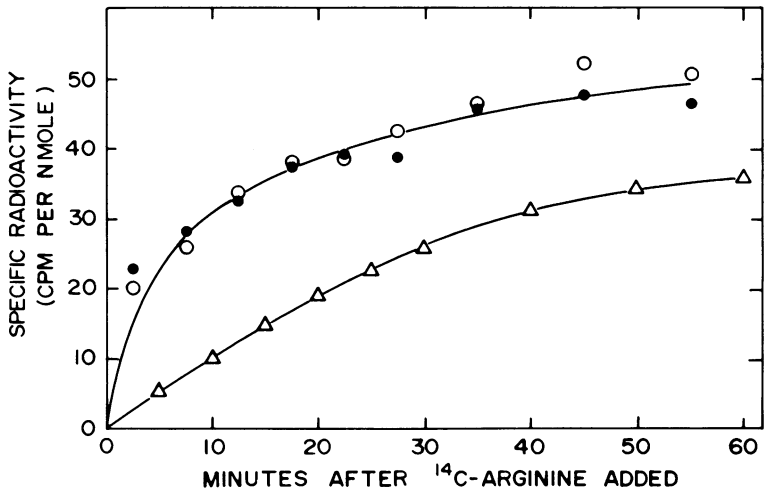

FIG. 5. *Specific radioactivity of soluble arginine and the arginine being used for protein synthesis and catabolism (urea) after [^{14}C]arginine addition (see Fig. 3). New protein arginine and urea are plotted at the midpoints of the respective intervals. Soluble arginine is plotted at the time at which samples were taken for analysis. Symbols: ●, New protein arginine; ○, new urea; △, soluble arginine.*

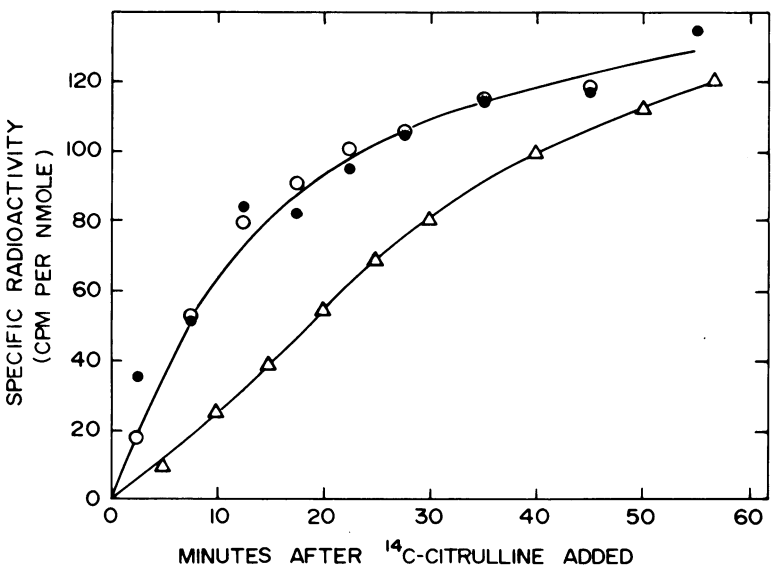

FIG. 6. *Specific radioactivity of soluble arginine and the arginine being used for protein synthesis and catabolism (urea) after [^{14}C]citrulline addition (see Fig. 4). New protein arginine and urea are plotted at the midpoints of the respective intervals. Soluble arginine is plotted at the time at which samples were taken for analysis. Symbols: ●, New protein arginine; ○, new urea; △, soluble arginine.*

This occurred whether the radioactivity was derived from exogenous arginine or from endogenous biosynthesis.

These results are consistent with the previous observations that at least two locationally distinct arginine pools exist in *Neurospora*—a metabolically active cytosolic pool and a metabolically inactive vesicular pool (16, 18). The size of the cytosolic arginine pool during growth in arginine-supplemented medium could be calculated from the data in Fig. 5 and 6. The specific radioactivity of the total arginine pool was expressed as [counts per minute (total)/arginine (total)]. The specific radioactivity of the cytosolic arginine pool was expressed as [counts per minute (cytosolic)/arginine (cytosolic)]. Since the radioactivity must pass through the cytosolic pool before entry into the

vesicle (16), the counts per minute (cytosolic) will equal the counts per minute (total) at early times. When this assumption is true, then the specific radioactivity of the total arginine pool divided by the specific radioactivity of the cytosolic arginine pool will yield the fraction of arginine that is cytosolic. As radioactivity equilibrates with the vesicular pool, counts per minute (cytosolic) will be less than counts per minute (total). As the time of exposure to radioactivity increases, the apparent value for the fraction of arginine that is cytosolic will also increase. The data in Fig. 5 and 6 have been used to calculate this fraction for each 5-min time between 5 and 30 min. Extrapolation of the calculated values to zero time yields values for the fraction of arginine in the cytosol of 0.20 (citrulline labeling) and 0.18 (arginine labeling). Thus I conclude that approximately 20% of the arginine pool is cytosolic in *Neurospora* growing in arginine-supplemented medium.

DISCUSSION

The results indicate that the same arginine pool is used for both protein synthesis and catabolism. This pool represents only a fraction of the total arginine pool and is preferentially labeled by both exogenous and newly synthesized radioactive arginine. The following observations indicate that this metabolically active pool is cytosolic: (i) arginine synthesis from citrulline is performed by soluble enzymes (19); (ii) both arginase and arginyl-tRNA synthetase are soluble enzymes (19); (iii) a large proportion of the arginine pool is not in the cytosol but is sequestered in a membrane-enclosed organelle, the vesicle (18); and (iv) arginine taken up from the medium enters the cytosol.

This compartmentation of arginine appears to play a decisive role in controlling arginine catabolism in *Neurospora*. During growth in minimal medium, the cytosolic arginine pool is very small—less than 2% of the total pool (16). Assuming that 80% of the cell water is cytosolic, the cytosolic arginine concentration would be 0.2 mM. As demonstrated here, during steady-state growth in arginine-supplemented medium approximately 20% of the arginine pool appears to be cytosolic. Using the same assumption as above, one obtains a cytosolic arginine concentration of 15 mM, a 75-fold increase. This increase is an order of magnitude larger than the 7.5-fold increase in the total arginine pool or the 3-fold induction of arginase observed for cells grown in arginine-supplemented medium. These results suggest that the metabolic fate of arginine is more sensitive to changes in cytosolic arginine concentrations than to changes in the total arginine pool or in the level of the catabolic enzymes. A similar conclusion has been reached from experiments involving ornithine metabolism in *Neurospora* (J. N. Karlin et al., J. Biol. Chem., in press).

Compartmentation would also appear to solve one major metabolic problem anticipated to occur in cells containing large pools of a catabolic substrate. By separating the majority of the pool from a catabolic enzyme in the cytosol, catabolism is prevented during growth in minimal medium (16, 18, 19). This is unnecessary in most prokaryotic cells that do not have large amino acid pools during growth in minimal medium and whose basal level of catabolic enzymes is essentially zero.

The large arginine pool found in cells growing in unsupplemented medium would also appear to present additional regulatory and metabolic problems. Cellular efficiency requires that cells be able to respond quickly to changes in the availability of exogenous arginine. Because of the large arginine pool, significant changes in the intracellular arginine concentration require long time periods; e.g., 30 min of exposure to exogenous arginine is required for the 7.5-fold pool expansion. If the cells were to respond to the total arginine pool, it is obvious that regulatory (feedback inhibition, repression, and induction) and metabolic (catabolism) responses would be very sluggish. Wasteful continued biosynthesis of arginine might continue for a considerable time despite the presence of an ample supply of exogenous arginine. Similarly, the cell's ability to efficiently utilize the exogenous arginine as an additional source of carbon and/or nitrogen would be delayed significantly while the arginine pool expanded.

Compartmentation of the arginine pool appears to provide a means of overcoming these potential inefficiencies. The small cytosolic arginine pool could expand and contract quickly in response to changes in the availability of exogenous arginine. Catabolism, feedback inhibition, induction, and repression would be initiated or cease long before large changes in the total arginine pool occurred. In this regard it is interesting to note that the only early arginine biosynthetic enzyme that is soluble is acetylglutamate kinase (EC 2.7.2.8), the feedback-inhibitable enzyme (4); all others are mitochondrial (5, 19). Additional experimental verification of these expectations will be the subject of future publications.

Similar compartmental features are likely to occur in most eukaryotic cells. An apparent preferential utilization of exogenous amino acids has been observed in plants (13), animal cells (12), and other eukaryotic microorganisms

(15). In some cases, radioactive exogenous amino acids do not appear to be diluted by the entire intracellular pool before incorporation into protein. These observations are consistent with those described here for *Neurospora*. It has been hypothesized that this selective utilization of amino acids occurs because amino acid transport and aminoacyl-tRNA synthetases are somehow coupled at the plasma membrane (9). In the case of *Neurospora*, both exogenous and newly synthesized arginine are preferentially incorporated into protein and preferentially catabolized. If the coupling hypothesis were correct, this would require an association between arginine transport, arginine biosynthesis from citrulline, arginyl-tRNA synthetase, and arginase. In view of the evidence for a vesicular pool of arginine (18), the compartmentation model appears to be correct. However, in the absence of convincing biochemical evidence for the existence of similar compartmentation of amino acids in mammalian cells, it is impossible to distinguish between the hypothetical models for the selective utilization of amino acids. Kinetic experiments of radioactive amino acid utilization are unlikely to resolve this issue.

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