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Cellular Distribution of Ornithine in *Neurospora*: Anabolic and Catabolic Steady States

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During growth on minimal medium, cells of *Neurospora* contain three pools of ornithine. Over 95% of the ornithine is in a metabolically inactive pool in vesicles, about 1% is in the cytosol, and about 3% is in the mitochondria. By using a ureaseless strain, we measured the rapid flux of ornithine across the membrane boundaries of these pools. High levels of ornithine and the catabolic enzyme ornithine aminotransferase coexist during growth on minimal medium but, due to the compartmentation of the ornithine, only 11% was catabolized. Most of the ornithine was used for the synthesis of arginine. Upon the addition of arginine to the medium, ornithine was produced catabolically via the enzyme arginase. The biosynthetic production of ornithine quickly ceased due to arginine inhibition of an early enzyme of ornithine synthesis. The biosynthesis of arginine itself, from ornithine and carbamyl phosphate, was halted after about three generations of growth on arginine via the repression of carbamyl phosphate synthetase A. The catabolism of arginine produced ornithine at a greater rate than it had been produced biosynthetically, but this ornithine was not stored; rather it was catabolized in turn to yield intermediates of the proline pathway. Thus, compartmentation, feedback inhibition, and genetic répression all play a role to minimize the simultaneous operation of anabolic and catabolic pathways for ornithine and arginine.

Neurospora crassa has the metabolic capacity to synthesize arginine and to catabolize arginine if it is available in the medium. Figure 1 shows how these pathways are organized in this fungus. Note that the amino acid ornithine is an intermediate in both pathways.

Most of the enzymes of the arginine biosynthetic pathway are located in the mitochondrion (3, 19). The enzymes of the catabolic pathway, arginase and ornithine aminotransferase (OATase), are in the cytosol (19). When *N. crassa* is grown on minimal medium, arginine and ornithine are present at high levels (20 to 30 nmol/mg [dry weight]), and for both of these amino acids, over 95% of the total amount in the cell is located in intracellular vesicles (8, 13, 17).

We wish to compare the regulation of the arginine pathway in *Neurospora* with that seen in the yeasts *Saccharomyces cerevisiae* and *Candida utilis*. In these yeasts, the catabolic enzymes arginase and OATase show large amplitudes of repression and induction (10- to 20-fold) (9, 10). It has also been demonstrated in *S. cerevisiae* that six of the enzymes of arginine biosynthesis are repressible by high levels of arginine (20).

Unlike the yeasts, in *N. crassa* only one enzyme, carbamyl phosphate synthetase A (arginine specific) (CPSase A), is significantly repressible or inducible. Other enzymes in the pathway show only small, two- to fourfold, changes in enzyme specific activity with arginine supplementation or arginine limitation. The enzymes are not repressed below levels seen during growth on minimal medium (3, 4). Despite the fact that almost all of these enzymes are essentially constitutive in *N. crassa*, there is considerable evidence to indicate that the biosynthetic pathway and the catabolic pathway do not operate simultaneously. During exponential growth on minimal medium no urea is produced, indicating that no catabolism of arginine occurs (6). OATase catabolizes only small amounts of ornithine (5, 7, 8). The anabolic pathway does not appear to operate when cultures are supplemented with arginine. The evidence for this is that strains lacking arginase, when grown on arginine, require polyamines for maximal growth (5). Supplementa-

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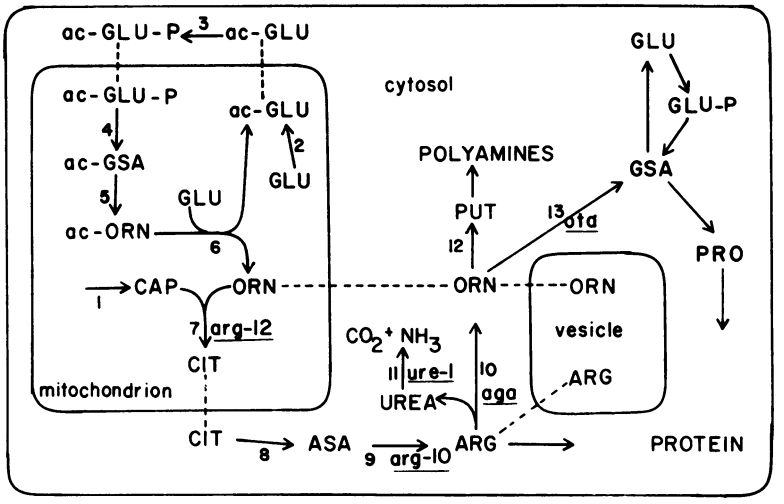

FIG. 1. *Diagram of the structural genes, biochemical steps, and cell compartments involved in arginine and ornithine metabolism in Neurospora. Enzymes: 1, CPSase A (EC 2.7.2.5, arginine specific); 2, N-acetylglutamate synthetase (EC 2.3.1.1); 3, N-acetyl-λ-glutamyl kinase; 4, N-acetylglutamic-λ-semialdehyde dehydrogenase; 5, acethylornithine δ-transaminase (EC 2.6.1.11); 6, N-acetylornithine-glutamate transacetylase; 7, OTCase (EC 2.1.3.3); 8, argininosuccinic acid synthetase (EC 6.3.4.5); 9, argininosuccinic acid lyase (EC 4.3.2.1); 10, arginase (EC 3.5.3.1); 11, urease; 12, ODCase (EC 4.1.1.17); 13, OATase (EC 2.6.1.13). Metabolites: GLU, L-glutamate; ac-GLU, N-acetyl-L-glutamate; ac-GLU-P, N-acetyl-λ-L-glutamyl phosphate; ac-GSA, N-acetyl-L-glutamic-λ-semialdehyde; ac-ORN, N-acetyl-L-ornithine; ORN, ornithine; CAP, carbamyl phosphate; CIT, citrulline; ASA, argininosuccinic acid; ARG, arginine; PUT, putrescine; GSA, glutamic-λ-semialdehyde; GLU-P, glutamyl phosphate; PRO, proline.*

tion with arginine can also repress CPSase A, halting flux through the last part of the anabolic pathway (4).

In this study, we investigated the metabolic behavior of ornithine in cells growing on minimal and arginine-supplemented medium. The strain *ure-1*, which is unable to degrade urea arising from arginine catabolism, was used extensively. By monitoring the metabolism of labeled ornithine, we directly measured the flux through anabolic and catabolic pathways under the different growth conditions. As in wild-type *N. crassa*, we found that ornithine can exist within three pools in the cell, and we have measured the flux of ornithine between these pools. Our data demonstrate that compartmentation plays a key role in regulating the synthesis and catabolism of arginine and ornithine.

MATERIALS AND METHODS

Strains and chemicals. The following strains were used; *ure-1*, *arg-12*, *ota*, and the double mutants *aga ota*, *arg-10 aga*, and *arg-12 ota* (all from the laboratory collection of R.H.D.). Vogels medium N (15) with 1.5% sucrose was used for growth. Supplements of arginine were sometimes used, as indicated in Results. Triton X-100 was purchased from Research Products International. The scintillation fluid used was 1 part Triton X-100 to 2 parts toluene (vol/vol); the latter contained 0.5% (wt/vol) 2,5-diphenyloxazole. Uniformly labeled [^{14}C]ornithine (261 mCi/mmol) was obtained from New England Nuclear Corp., [*guanidino*-^{14}C]arginine (25 mCi/mmol) was obtained from Calatomic, and uniformly labeled [^{14}C]arginine (216 mCi/mmol) was obtained from Calbiochem. All isotopes were purified before use by column chromatography (8).

In one experiment, a mixture of guanidino-labeled arginine and uniformly labeled arginine was used. In this case, the specific activity of the guanidino moiety and ornithine moiety of the mixture was determined by cleaving the arginine with bovine liver arginase (7). The products of the reaction, urea, ornithine, and unreacted arginine, were separated by column chromatography, and the radioactivity of each was determined.

Growth and sampling. Mycelia were grown exponentially from a conidial inoculum (10^6 conidia per ml) in 800- or 200-ml cultures of minimal medium with forced air at 25°C. The auxotrophic strains were supplemented with 200 mg of arginine per liter. Except for the experiment shown in Table 2, the cultures were used when they had attained a dry weight of 0.3 to 0.5 mg/ml. Dry weights were determined by harvesting and acetone-drying the mycelia from a 30- to 40-ml sample of culture. In the experiments using [^{14}C]ornithine, the sampling was done as previously described (8). In the experiments using [^{14}C]arginine, 40-ml samples were pipetted and quickly filtered on Gelman Ga-6 cellulose acetate membranes (0.45-μm pore size) and washed

with 4 ml of distilled water. The filtrate was collected for determinations of arginine and urea. The washed mycelia were suspended in 3 ml of 5% trichloroacetic acid (0.05 g/ml) at 4°C.

Fractionation. Extracts of the samples were prepared and fractionated by ion-exchange chromatography as previously described (8). Fractions containing urea were separated on 15-cm-long columns with Dowex AG50W resin in the H^+ form, according to the method of Watts et al. (16). The pH of the samples was brought to about 1 with 4 N HCl, and they were applied to the columns. The columns were then washed with 10 ml of 0.1 N HCl and eluted successively with 5 and 6 ml of 4 M NaCl in 0.1 N HCl. All urea emerges in the 6-ml fraction. Recovery of urea, about 85%, was not as good as that achieved for other compounds, and all values were adjusted accordingly.

As a test of the fractionation procedure for the neutral and acidic amino acids (8), a mutant lacking ornithine transcarbamylase (OTCase) (*arg-12*) and a mutant lacking OATase (*ota*) were each grown on [^{14}C]ornithine. The data, shown in Table 1 in Results, demonstrate that the method efficiently separated the OATase products from the OTCase products.

Paper chromatography of the fractions containing citrulline and argininosuccinate was performed. These fractions can be evaporated and used directly because they elute in 6.0 N HCl and are free of salts. One-dimensional ascending paper chromatography was used with two different solvent systems: phenol-water (100:20, vol/vol, with NH_3 atmosphere) or ethanol-NH_3 (77:23, vol/vol). Unfortunately, neither of these systems will completely separate citrulline from one of the two anhydride forms of argininosuccinate.

The level of polyamines was kindly determined by David Morris for the wild-type strain, 74A, in samples harvested at several stages of growth and on minimal and arginine-supplemented media. The concentration of polyamines per milligram (dry weight) remained constant, and the value found, the sum of putrescine (0.6 nmol per mg), spermidine (21 nmol per mg) and spermine (0.2 nmol per mg), was used throughout in calculations (8).

Calculations. The details of the calculations used have been described (8). Briefly, the specific radioactivity of a "new product" (e.g., "new polyamines") made during a small interval of time was calculated from a knowledge of the steady-state concentration of the product per milligram (dry weight) and the exponential rate of growth. Thus, the amount of polyamines synthesized in a given interval, ΔX, can be found by the formula $\Delta X = X_0(e^{kt} - 1)$, where t is the time of the interval in minutes and k, for a doubling time of 171 min, is 0.004 min^{-1}. The specific radioactivity is then determined by dividing the change in the counts per minute determined by the change in nanomoles (ΔX) in a given interval. For these determinations, the best-fit curves of counts per minute versus time were used.

RESULTS

Independent measurement of the three routes of ornithine metabolism. By following the fate of radioactive amino acids added to the culture medium, it is possible to show that the enzymatic steps shown in Fig. 1 are the only ones involved in arginine metabolism under the growth conditions employed here. The data in Table 1 show the distribution of radioactivity derived from [^{14}C]ornithine in various mutant strains. In *arg-12* strains lacking OTCase,

TABLE 1. *Distribution of ornithine and arginine in mutant strains*[a]

Genotype	Enzyme deficiency	Isotope	Products (%)		Ornithine and polyamines	Total cpm
			OATase	OTCase		
ure-1	Urease	[^{14}C]ornithine[b]	27	38	35	143,460
ota	OATase	[^{14}C]ornithine[b]	1	52	46	75,180
arg-12	OTCase	[^{14}C]ornithine[c]	53	<1	47	232,500
ota arg-12	OATase and OTCase	[^{14}C]ornithine[c]	<1	0	99	122,000
ota aga	OATase and arginase	[^{14}C]ornithine[b]	<1	44	56	60,930
ota aga	OATase and arginase	[^{14}C]arginine[d]	<1	99	<1	42,770
aga arg-10	Arginase and argininosuccinate lyase	[^{14}C]arginine[d]	<1	99[e]	<1	16,790

[a] All values are given for 20 min after the addition of isotope.

[b] A 3-min pulse of high-specific-activity ornithine was given, followed by the addition of a large amount of unlabeled arginine.

[c] Arginine auxotrophs were washed free of arginine-supplemented medium, and then grown in labeled ornithine.

[d] Enough arginine was added to sustain catabolism for at least 30 min.

[e] All radioactivity was in arginine, none was in citrulline or argininosuccinic acid.

there is no measurable radioactivity found in citrulline, argininosuccinate, or arginine. Thus, OTCase is the only route from ornithine to arginine.

The *ota* mutants, lacking OATase, are unable to catabolize radioactive ornithine, which is added to the medium. In Table 1, the *ota* mutant, given [^{14}C]ornithine, yielded less than 2% of the total radioactivity in products other than polyamines or intermediates of the arginine pathway. Similar results are obtained with the double mutant lacking arginase and OATase, *aga ota*. Less than 2% of the total radioactivity is found in OATase products.

A double mutant lacking both OTCase and OATase should be unable to metabolize [^{14}C]ornithine, except for polyamine synthesis. Table 1 shows that this is indeed the result. The data demonstrate that OTCase, OATase, and ornithine decarboxylase (ODCase) are the only routes of ornithine utilization under the growth conditions used here.

A double mutant lacking arginase and argininosuccinate lyase, the last enzyme of the arginine biosynthetic pathway, does not metabolize added [^{14}C]arginine except for protein synthesis. Thus, arginase appears to represent the only degradative route for arginine. The data from this strain do not allow us to completely rule out other pathways because of possible repression during long-term growth on arginine. The presence of other pathways is made unlikely by the data obtained from the double mutant lacking arginase and OATase. This strain was grown in minimal medium and then supplemented with labeled arginine. Radioactivity could be found in only two fractions, arginine and the fraction containing citrulline and argininosuccinate. No degradative products could be found. This last piece of data indicates that the arginine pathway is at least partially reversible. Because OATase is missing in this strain, any ornithine that might be produced could be trapped, but no radioactive ornithine was found. Thus, the biosynthetic pathway is not reversible beyond citrulline.

In fact, it is doubtful that even much citrulline is formed via a back reaction. The reaction that converts citrulline to argininosuccinate is essentially irreversible in other organisms (12). Citrulline and argininosuccinate are difficult to separate, because the argininosuccinate forms two anhydrides during the fractionation procedure. Paper chromatography of a labeled citrulline-argininosuccinate fraction showed at least 88% of the radioactivity in two peaks identifiable as argininosuccinate. The remaining 12% was in a peak in which citrulline and an argininosuccinate anhydride overlap.

Three intracellular ornithine pools in cultures growing on minimal medium. To see how ornithine is distributed in cells that are actively making ornithine and arginine, an experiment similar to one done previously in this laboratory with wild-type *N. crassa* was performed (8). A small pulse of highly labeled [^{14}C]ornithine (0.1 μM, 2.5 $\times$ 10^8 cpm/μmol) was added to a culture of *ure-1* in the log phase of growth on minimal medium. The uptake and subsequent distribution of the labeled ornithine are shown in Fig. 2.

The pattern of ornithine distribution in *ure-1* is the same as that shown for wild-type 74A (8). Specifically, the following results should be noted: (i) the labeled ornithine is rapidly taken up by the cells. Eighty-five percent of the isotope enters during the first 3 min. (ii) During this 3-min period, half of the labeled ornithine is metabolized. (iii) In contrast, the rate of metabolism of [^{14}C]ornithine falls abruptly when uptake ceases. For each 3-min period after the completion of uptake, only 1% of the [^{14}C]ornithine is converted to other products.

These data indicate that ornithine entering the cell flows through a metabolically active compartment, presumably the cytosol, where ODCase and OATase are located (19). From there, it enters the mitochondrion to be used in the OTCase reaction; it also enters a metabolically inactive pool in the vesicle. After all the [^{14}C]ornithine has entered the cell, incorporation of radioactivity into all of these products continues, but at a greatly reduced rate, as labeled ornithine from the vesicle exchanges with the ornithine pool in the cytosol (8, 17).

The relative sizes of the cytosolic and total ornithine pools have been determined to be essentially the same as in the wild type (8). Briefly, the specific radioactivity of the "new" polyamines made during the first 20 s of the experiment was 20,650 cpm/nmol. The average radioactivity of the total ornithine in the cell during this period was only 297 cpm/nmol. Thus, the polyamines were made from a cytosolic pool that was 70-fold more radioactive. This comparison demonstrates that isotope was diluted by, at most, one-seventieth, or 1.4%, of the total intracellular ornithine.

Measurement of ornithine flux across the mitochondrial membrane. We have assumed that the strain *ure-1* has the same level of intracellular polyamines as the wild-type strain (22 nmol/mg [dry weight]). Using this value, the flux through ODCase is calculated to be 0.09 nmol/min · mg for the experiment shown in Fig. 2. To maintain the arginine pool and provide arginine for protein the flux through OTCase must be 0.86 nmol/min · mg. It has pre-

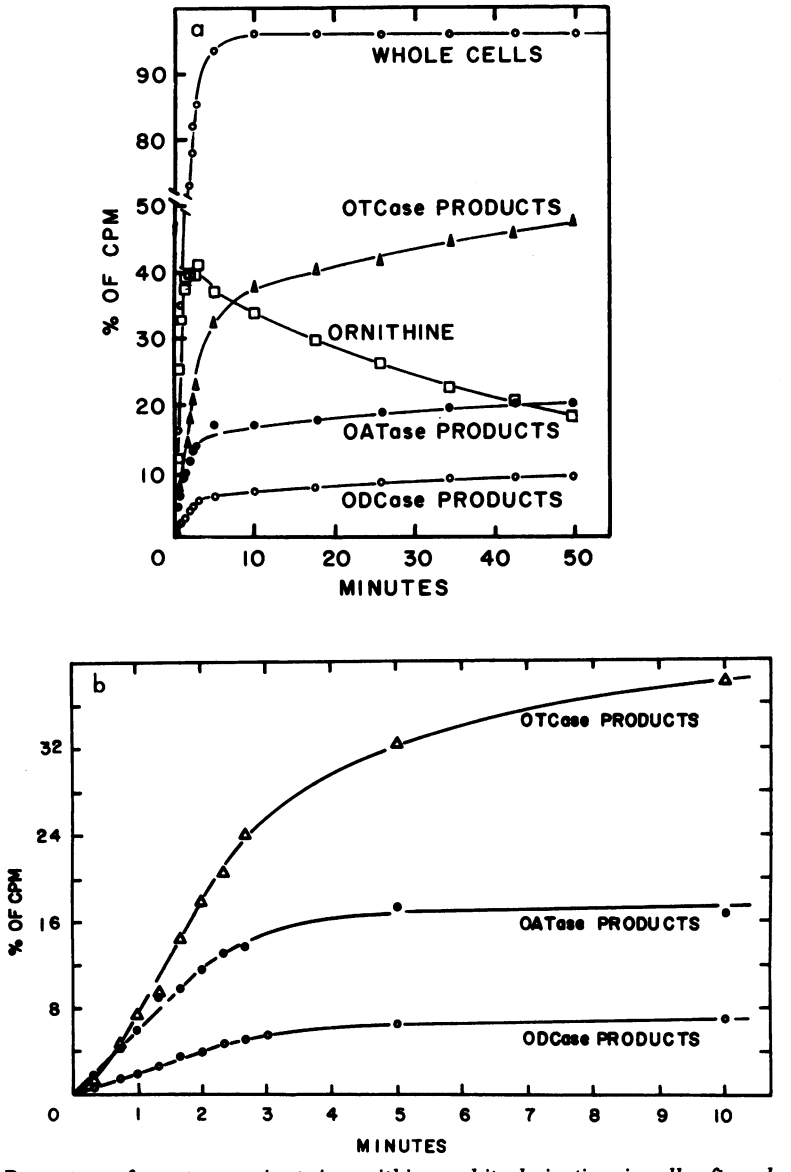

FIG. 2. *(a) Percentage of counts per minute in ornithine and its derivatives in cells after administration of [^{14}C]ornithine (0.1 μM in the medium, 500,000 cpm per 20-ml sample) at 0 min. ODCase products include putrescine, spermidine, and spermine, and the values shown are corrected for the loss of $^{14}CO_2$ in the ODCase reaction. Products of OTCase and OATase include not only the immediate products of these enzymes but also the sum of all subsequent metabolic derivatives. (b) Expansion of the data for the first 10 min of the experiment shown in (a).*

viously been shown (8) that even though OTCase and the final enzymes of ornithine synthesis are located in the mitochondrion, most of the ornithine produced leaves, flows through the cytosol, and then reenters the mitochondrion before being used for citrulline synthesis. In Fig. 2a, it can be seen that most of the radioactive ornithine in the cell is ultimately converted to arginine. One objective of the experiment shown in Fig. 2 was to determine the net flux of ornithine from the cytosol into the OTCase reaction. This rate will provide an important basis of comparison with later experiments in which cytosolic ornithine arises from the catabolism of arginine.

When the first few minutes of the experiment are examined closely, as shown in more detail in Fig. 2b, a lag of 40 s is seen before a maxi-

mal, linear, rate of incorporation of radioactivity into OTCase products is attained. Incorporation of radioactivity into polyamines occurs with a lag of only 5 s. The difference between the curves for OTCase products and polyamines is evidence that these two products have different precursor pools. The [^{14}C]ornithine enters the cytosol and is used for polyamine synthesis before its entry into the mitochondrion, where it is used by OTCase. The linear portion of the curve for OTCase products intercepts the abscissa at 20 s, and this 20-s "wash out" time (8) corresponds to the use of 0.83 nmol of ornithine per mg (dry weight). A pool of ornithine of this size within the mitochondrion represents 3% of the total ornithine in the cell.

The presence of this small mitochondrial pool must be kept in mind when attempting to calculate the net flux of ornithine from the cytosol through the OTCase reaction. The specific radioactivity of new polyamines is a measure of the specific radioactivity of the ornithine in the cytosol. Because of dilution of labeled ornithine by the mitochondrial pool, the specific radioactivity of new OTCase products is lower than that of new polyamines (Fig. 3). For the period from 40 s to 150 s, however, the incorporation of isotope is essentially linear for OTCase and ODCase products (Fig. 2b), and the specific radioactivity of the products is constant (Fig. 3). This shows that the rate of isotope use by OTCase is equal to the net rate of isotope entry into the mitochondrion.

During this period, the flux of ornithine from the cytosol into the mitochondrion can be calculated. To satisfy the ornithine-consuming reactions in these cells, ornithine is made, within the mitochondrion, at the rate of 1.17 nmol/min·mg. As seen in Fig. 3, however, the ornithine used by the OTCase reaction in the mitochondrion is 53% as radioactive as the ornithine in the cytosol. Therefore, the flux of ornithine into the mitochondrion must be 1.32 nmol/min·mg [1.32/(1.32 + 1.17) = 53%]. The flux of ornithine out of the mitochondrion must be even higher than the flux in, because there are ornithine-consuming processes in the cytosol. As shown in Fig. 5, the flux out of the mitochondrion must be 1.63 nmol/min·mg. Thus, ornithine is exchanged across the mitochondrial membrane at a higher rate than it is synthesized.

The flux of ornithine from the cytosol through the OTCase must be 0.53 (0.86) = 0.46 nmol/min·mg. There is another way of calculating this rate that may be more intuitively obvious. For example, during the interval from 60 to 80 s in Fig. 2 and 3, the specific radioactivity of new polyamines (and thus also of cytosolic ornithine) was 20,600 cpm/nmol. For this same interval, 17,700 cpm appeared in OTCase products, representing 0.86 nmol of cytosolic ornithine (this number is only coincidentally the same as the OTCase flux.) This portion of culture had a dry weight of 5.6 mg, and the flux can thus be calculated: 0.86 nmol/(5.6 mg × 0.33 min) = 0.46 nmol/min·mg.

The results show that there are two phases of the experiment in which a steady state is achieved with regard to the entry of isotope into the mitochondrion and into polyamines. Figure 4 represents an approximation of the ratio of the slope of the curve in Fig. 2 for OTCase

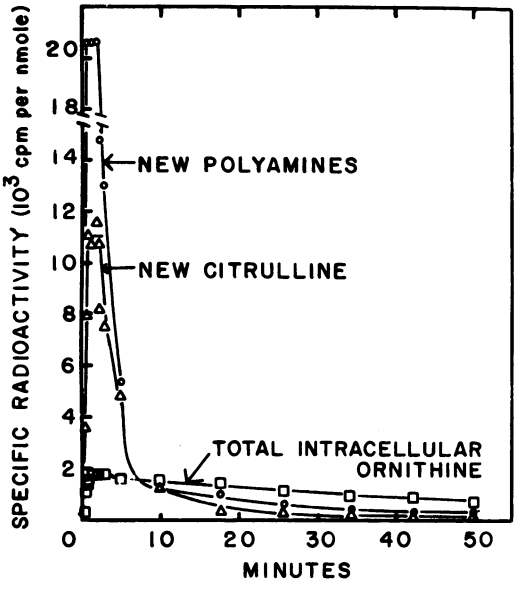

FIG. 3. *Specific radioactivity of the products of ornithine metabolism. The data are from the experiment shown in Fig. 2.*

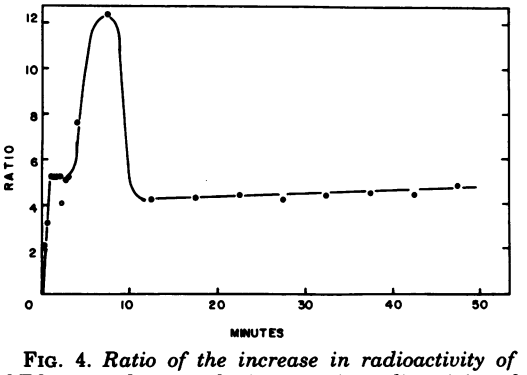

FIG. 4. *Ratio of the increase in radioactivity of OTCase products to the increase in radioactivity of ODCase products. The ratios were calculated for 20-s intervals from 0 to 3 min, a 2-min interval from 3 to 5 min, and 5-min intervals thereafter.*

products to that for ODCase products. It was obtained by plotting the ratios of the increment of counts per minute in the two curves for many small intervals of time. The use of isotope by OTCase is approximately 5.3-fold that of ODCase during both linear phases of the experiment. This demonstrates that whether the isotope ultimately comes from the medium or the vesicle, it must pass through a single cytosolic pool used by both ODCase and the mitochondrion. The deviations from the 5.3 value occur when the label is first added to the medium and when it is exhausted from the medium: both are periods of reequilibration between mitochondrial and cytosolic pools.

Measurement of ornithine flux across the vesicle membrane. For the experiment shown in Fig. 2, the cell had a total ornithine pool of 21 nmol/mg. To maintain this level of ornithine during exponential growth, there must be a net flux of ornithine into the vesicle of 0.09 nmol/min·mg. However, several features of the data show that there must be an exchange of ornithine between vesicle and cytosol occurring at a much higher rate. One compelling piece of evidence is the extent to which isotope entering the cell is diluted by unlabeled ornithine in the cytosol. The specific radioactivity of new polyamines quickly reaches and maintains its maximum value during the uptake of isotope (Fig. 3). This value for the new polyamines (20,600 cpm/nmol) tells us that cytosolic ornithine is only one-twelfth as radioactive as the ornithine being taken up from the medium (250,000 cpm/nmol). The amount of unlabeled, newly synthesized ornithine emerging from the mitochondrion is insufficient to account for this 12-fold dilution of isotope. Using the flux numbers calculated above (Fig. 5), 66% of the ornithine in the mitochondrion flows into the cytosol [1.63/(1.63 + 0.86)], equivalent to 0.77 nmol/min·mg (0.66 × 1.17). From 60 to 80 s in the experiment (Fig. 2) 62,500 cpm of exogenous ornithine enters the cell, corresponding to a flux of 0.13 nmol/min·mg. Thus, exogenous ornithine enters the cell at $^1/_6$ the rate at which unlabeled ornithine is emerging from the mitochondrion, yet the specific radioactivity of new polyamines shows cytosolic ornithine to be diluted 12-fold.

The additional dilution can easily be accounted for if unlabeled ornithine emerges from the huge pool in the vesicle at the rate of 0.66 nmol/min·mg [0.13/(0.77 + 0.13 + 0.66) = $^1/_{12}$]. The rate of flux from the cytosol back into the vesicle would then have to be 0.75 nmol/min·mg (0.09 + 0.66) to account for the net growth of the vesicular pool. These rates are summarized in Fig. 5.

Although the values for the flux across the vesicular membrane have been chosen "to fit" the isotope dilution data, their validity can be demonstrated. Several predictions about the behavior of labeled ornithine in this experiment can be made based on the scheme shown in Fig. 5. The first prediction is that even though the net consumption of ornithine by the vesicle is small, a large proportion of exogenously added isotope will end up in this pool. (Because labeled ornithine entering the vesicle is diluted by a huge excess of unlabeled ornithine, the isotope is trapped there to a great extent.) For example, the rate of flux into the vesicle is 1.6-fold the flux from the cytosol through the OTCase reaction (0.75/0.46). The data in Fig. 2b show that, at the 2-min point, near the middle of the uptake phase, there is 195,000 cpm in intracellular ornithine and 90,000 in OTCase products, a ratio of 2.2:1. A second example is that the rate of flux into the vesicle is 8.4-fold the rate of the ODCase reaction (0.75/0.09). At the 2-min point, 19,700 cpm is found in ODCase products, yielding a ratio for vesicle versus ODCase of 9.9:1.

A second possible prediction from the rates in Fig. 5 concerns the relative specific radioactivities of the various products after all the labeled ornithine has entered the cell. For points beyond 10 min in the experiment in Fig. 2, the vesicle is the only source of [^{14}C]ornithine. Radioactive ornithine should emerge from the vesicle and mix with unlabeled ornithine coming from the mitochondrion, yielding cytosolic ornithine that is 46% as radioactive as vesicular ornithine [0.66/(0.66 + 0.77)]. The data for the

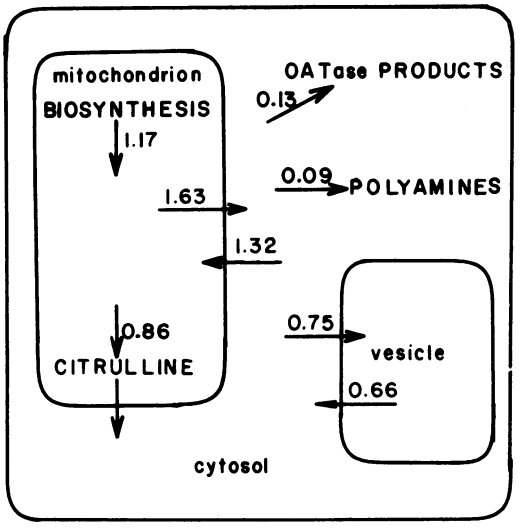

FIG. 5. *Flux of ornithine, during growth on minimal medium, into its metabolic products and across organellar membranes.*

last five points in Fig. 3 show the specific radioactivity of polyamines, which are made from cytosolic ornithine, to be 46% of the specific radioactivity of intracellular ornithine. The flux of radioactive ornithine from the vesicle into the mitochondrion will be 46% of 1.32 nmol/min·mg. OTCase products should thus be 24% as radioactive as vesicular ornithine [(0.46 × 1.32)/(1.32 + 1.17)]. For the last five points in Fig. 3, the specific radioactivity of OTCase products is 22% that of intracellular ornithine. Thus, the agreement between the prediction and the data is excellent.

A third type of prediction based on the rates in Fig. 5 is the rate at which the labeled ornithine will be lost from the vesicular pool. The calculation is as follows. Besides flux into the vesicle, there are three reactions that remove ornithine from the cytosol, at a rate of 0.68 nmol/min·mg (0.13 + 0.09 + 0.46). Forty-six percent of cytosolic ornithine is labeled ornithine from the vesicle. Therefore, labeled ornithine will be lost at a rate of 0.31 nmol/min·mg (0.46 × 0.68). Because these cells have an ornithine pool of 21 nmol/mg, this rate of loss corresponds to 1.5% per min. The data for the last five points in Fig. 3 show that labeled ornithine is disappearing at a rate of 1.4% per min.

Catabolism of ornithine by cells growing on minimal medium. As can be seen in Fig. 2a and 2b, isotope incorporation into OATase products exhibits a pattern somewhat different than that seen for polyamines and OTCase products. For the first 2 min of the experiment, as [^{14}C]ornithine enters the cell, incorporation of isotope into OATase products is 300% higher than incorporation into polyamines. As uptake ceases, the rate of incorporation into OATase products falls abruptly, even more sharply than for the other two pathways. In the period from 10 to 50 min, the OATase products are incorporating label at a rate only 50% higher than that seen for polyamines.

This pattern of rapid utilization during uptake, followed by a relatively slow use once the labeled ornithine is in the cell, has been observed for wild type (8). Cell fractionation experiments in this laboratory have shown OATase to be a "soluble" enzyme, presumably in the cytosol (19). The rapid incorporation of ornithine as it enters the cell may indicate that this enzyme has preferential access to ornithine coming through the cell membrane. Confirmation of this hypothesis will require further experiments.

Since it is not clear what pool of ornithine OATase may be using during the uptake phase of the experiment, a calculation of the flux cannot be obtained for this interval. If, for the period from 10 to 50 min, we assume that OATase uses the same pool as that used for polyamine synthesis, then the flux through OATase is 1.5 times that through ODCase. The value obtained, 0.13 nmol/min·mg, represents a rate of ornithine catabolism that is only 11% of the rate of ornithine production. (Biosynthesis produces 1.17 nmol/min·mg.) Thus, on minimal medium, the biochemical apparatus of the cell directs 89% of its endogenous ornithine into anabolic reactions or storage.

Arginine inhibition of the biosynthetic production of ornithine. The data presented thus far show how ornithine, produced in the mitochondion during growth on minimal medium, is distributed throughout the cell. The addition of arginine to a culture that had been growing on minimal medium inhibits this mitochondrial production of ornithine, as shown in Fig. 6. When labeled arginine (0.22 mM) is added to the medium, further increases in the internal arginine pool or the protein-arginine of the cell come almost entirely from uptake. In fact, the amount of arginine derived from unlabeled ornithine within the cell is difficult to detect

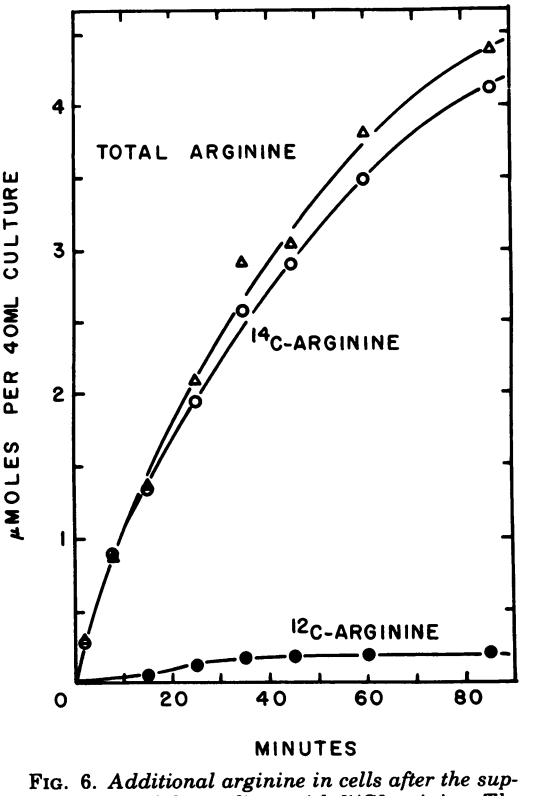

FIG. 6. *Additional arginine in cells after the supplementation of the medium with [^{14}C]arginine. The points for [^{12}C]arginine represent the difference between the other two curves.*

against the large background of radioactive material that quickly accumulates. However, all such experiments have been consistent in that no additional unlabeled arginine is found after 35 min of [^{14}C]arginine uptake, and the total amount of unlabeled arginine made during this time is about 10 nmol per mg (dry weight). It is entirely possible that this 10 nmol represents the conversion of the "on-line" intermediates between the feedback-inhibitable reaction (probably acetylglutamate kinase [2]) and arginine. This is an amount of arginine that could support growth for only 7% of a generation.

The data in Fig. 6 do not address the possibility that ornithine synthesis may continue, with unlabeled ornithine cryptically leaving the mitochondrion for catabolic fates. An experiment with the *ota aga* mutant permitted a test of the possibility. This mutant can neither catabolize ornithine nor produce ornithine from the catabolism of arginine. If ornithine synthesis continued after the addition of arginine to the medium, and if it flowed out of the mitochondrion at the rate seen on minimal medium, it would be expected that ornithine should accumulate in this strain. The results showed that it did not accumulate. The ornithine pool fell, after a lag of about 5 min, and there was no detectable addition of unlabeled ornithine. This argues that feedback inhibition of ornithine synthesis is very effective.

Arginine eventually eliminates the cycling of ornithine from catabolic to anabolic use. Although feedback inhibition effectively removes the biosynthetic source of ornithine, citrulline synthesis could possibly continue by using the ornithine arising from catabolism of arginine. For citrulline (and arginine) synthesis to be completely stopped, it may be that the other substrate for the OTCase reaction, carbamyl phosphate, must become limiting. The enzyme that makes carbamyl phosphate, CPSase A, is not sensitive to feedback inhibition (14, 21), but it is repressed after long periods of growth on arginine (3).

Table 2 illustrates the results of an experiment designed to see how long the use of ornithine for arginine synthesis continues. The amount of labeled ornithine produced via catabolism of labeled arginine can be estimated by measuring the amount of urea produced. Equimolar amounts of urea and ornithine are made in this reaction. The failure to recover the expected amount of labeled ornithine as ornithine or in the products of the OATase and ODCase reactions is attributed to use in the OTCase reaction. Thus, the flux through OTCase after various periods of growth on arginine can be measured. The growth curve for this experiment, shown in Fig. 7, demonstrates that all samples were taken during a period of exponential increase, with a generation time of 2.8 h.

For the 30-min period beginning 1 h after the addition of arginine to the culture, about 25% of the labeled ornithine produced in the cell is being recycled back to arginine synthesis. This

TABLE 2. *Distribution of ^{14}C from arginase products*[a]

Time (h)	Dry wt (mg/ml)	Urea (=ornithine produced) (cpm)	OATase products, ornithine, and polyamines		% Assumed to be cycled (OTCase products)
			cpm	%	
0	0.095				
1.0	0.123	7,575	5,706	75	25
3.9	0.250	9,825	7,218	74	26
6.7	0.505	16,954	15,042	89	11
8.4	0.735	7,995	8,551	106	0

[a] At zero time, the culture was divided into four flasks, each containing unlabeled arginine. At the times indicated, [^{14}C]arginine was added to one of the flasks and the culture was harvested 30 min later. The distribution of radioactivity to urea and products of arginine catabolism is shown. This arginine had equal radioactivity in the urea and ornithine moieties. The amount of radioactive arginine added, less than 0.1 μmol, was insufficient to significantly change the arginine concentration.

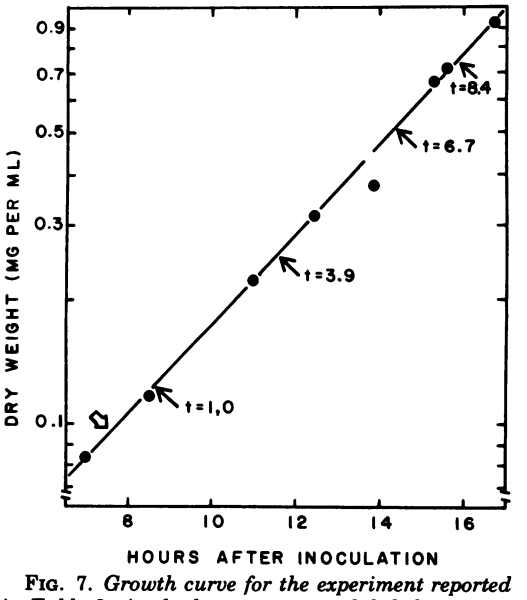

FIG. 7. *Growth curve for the experiment reported in Table 2. At the large arrow, unlabeled arginine was added to the cultures. The other arrows correspond to the addition of [^{14}C]arginine to each of the cultures, with the hours of growth in arginine noted.*

confirms the previous finding that CPSase A is not inhibited by arginine. These data also agree with previous experiments (14, 21) in that complete repression of the enzyme occurs rather slowly, because cycling continues for several hours and at least two generations. By the third generation, however, all the labeled ornithine can be recovered in products of reactions other than those leading to arginine synthesis.

Utilization of ornithine by OATase during growth on arginine. After three generations of growth on arginine-supplemented medium, urea and ornithine are being produced by the arginase reaction at the rate of 1.3 nmol/min · mg. Thus, 37% of the arginine being taken up by the cell is being catabolized. As shown in Table 3, the flux of ornithine through ODCase is 0.11 nmol/min · mg, not appreciably different from the rate seen on minimal medium. The rate through OATase increases eightfold, to a level of 1.07 nmol/min · mg. This reaction is the major fate of ornithine within the cell during periods of arginine catabolism. In these cultures, in which arginine did not serve as the limiting nitrogen source, ornithine was used mostly for proline synthesis.

DISCUSSION

Genetic control, feedback inhibition, and compartmentation all play a role in regulating the arginine pathway in *N. crassa*. By using labeled amino acids, we have demonstrated that the arginine biosynthetic pathway and the arginine catabolic pathway do not usually operate simultaneously, even though almost all the enzymes for both pathways are always present in the cell.

During growth on minimal medium, the catabolic pathway barely functions. In the strain *ure-1*, which lacks the ability to degrade urea, no urea accumulates, demonstrating that no arginine is degraded. The strain *ure-1* does catabolize a small portion of the ornithine made in the cell, a phenomenom also found in the wild type (8). However, the flux through the catabolic enzyme, OATase, amounts to only 11% of the total ornithine used in the cell. Even though large quantities of ornithine and arginine are present in these cells, catabolism is avoided by sequestration of more than 95% of these amino acids in vesicles.

TABLE 3. *Flux through reactions of the arginine pathway*

Enzyme	Flux (nmol/min · mg) in medium:	
	Minimal	Arginine-supplemented[a]
OTCase	0.86	ND[b]
ODCase	0.09	0.11
OATase	0.13	1.07
Arginase	ND	1.30

[a] Nine hours after arginine was added.
[b] ND, Not detectable.

With the strain *ure-1*, we have confirmed and extended our findings on the compartmentation of ornithine and have demonstrated high rates of flux across the membrane boundaries of the ornithine pools. Flow of ornithine across the mitochondrial membrane provides substrate for polyamine synthesis. Uptake by the vesicles keeps the cytosolic concentration at a level that avoids significant catabolism while providing for the storage of ornithine for later use.

Upon arginine supplementation of the medium, the catabolic pathway begins to operate. After three generations of growth on arginine, nearly 40% of the arginine taken up by the cell is being catabolized. Flux through the OATase reaction increases eightfold, and most of the ornithine in the cell is directed into proline synthesis.

The availability of arginine in the medium makes the continued synthesis of arginine unnecessary. Ornithine is still required for polyamine synthesis, but the catabolism of arginine provides a source of ornithine. Thus, it is not surprising to see that very effective feedback inhibition of ornithine synthesis occurs in vivo. However, the second segment of the anabolic pathway, begining with the production of carbamyl phosphate, could use the ornithine that arises from catabolism and thus produce arginine, even in the absence of ornithine biosynthesis. CPSase A is not sensitive to feedback inhibition (14, 21). It can be repressed by high levels of arginine, however, and the experiment in Table 2 shows that arginine synthesis completely stops after several generations of growth on arginine-supplemented medium. This agrees with an earlier report by Thwaites (14). Thwaites showed that the strain *arg-12*^{s} *pyr-3a*, in which the mitochondrial CPSase A provides the carbamyl phosphate required for uridine synthesis, would show 10-fold growth (dry weight) after the addition of arginine. These data demonstrate that considerable time is required before the repression of CPSase A stops the production of carbamyl phosphate. The data in Table 2 indicate that the cycling of ornithine from catabolic to biosynthetic reactions can occur in this interval. The extent to which this occurs is investigated more fully in the following paper.

Thus, feedback inhibition of acetylglutamate kinase and repression of CPSase A seems well designed to fit the organization of this pathway in the cell. The localization of a feedback-in-

hibitable step in the cytosol permits this step to be effectively regulated by the level of arginine in the cytosol where most of this amino acid is used. The other enzyme to be regulated, CPSase A, is in the mitochondrion where the level of arginine might not be representative of what is present in the cytosol. Controlling this enzyme by repression permits the cytosol to regulate the level of enzyme in the mitochondrion, a distinct compartment.

These experiments directly demonstrate that there is little simultaneous operation of arginine anabolic and catabolic pathways. The essentially constitutive character of these enzymes may be the means that *Neurospora* uses to prepare itself for changes in its nutritional environment. Lack of genetic control over the pathways, as compared with yeast, is more than compensated for by the compartmentation of the enzymes and substrates within the cell. Regulating the access of enzyme to substrate can control a pathway as effectively as regulating enzyme amount or enzyme function.

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