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Mobilization of Sequestered Metabolites into Degradative Reactions by Nutritional Stress in *Neurospora*

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The pools of arginine and ornithine rapidly disappear during nitrogen starvation of *Neurospora crassa*. Much of this disappearance can be accounted for by degradation catalyzed by preexisting catabolic enzymes. Purine degradation is also initiated by nitrogen metabolic stress. Mobilization of these compounds into degradative reactions does not appear to be a general response to nutritional stress since neither carbon starvation nor inhibition of protein synthesis elicits this response. It is suggested that nitrogen starvation may specifically alter the distribution of arginine and ornithine between vesicles and cytosol. This would be sufficient to initiate and maintain their degradation. These results suggest that compartmentation of amino acids provides a metabolic reserve to be utilized during periods of specific nutritional stress.

The amino acid pools of a variety of eucaryotic microorganisms do not appear to be distributed randomly throughout the cell water. In *Neurospora*, the bulk of the basic amino acids are localized within a subcellular structure which has been called the vesicle (16). In yeast, similar compartmentation of amino acids occurs in the vacuole (8, 18). Compartmentation within a subcellular organelle allows the cell to accumulate large pools of amino acids without concomitant induction of their catabolic enzymes or significant degradation by preexisting enzymes. The latter is particularly important since the basal (uninduced) level of catabolic enzymes is often very high in eucaryotes. Arginine metabolism in *Neurospora* exhibits these phenomena. Arginine is degraded via ornithine and urea to glutamate, carbon dioxide, and ammonia. Arginine is an excellent nitrogen source but is an extremely poor carbon source. The relevant enzymes and organizational features are shown in Fig. 1. The cytosolic degradative enzymes are present in significant amounts in cells growing in unsupplemented medium (5-7). They are induced less than fivefold in the presence of exogenous arginine (5). The concentrations of arginine and ornithine in cells growing in unsupplemented medium would greatly exceed the K_m's of their respective catabolic enzymes if they were distributed uniformly throughout the cell water (16). Little catabolism of either amino acid occurs under these conditions because the bulk of the pool is sequestered within the vesicles (5, 16). The vesicles have been shown to sequester all of the basic amino acids in addition to arginine and ornithine (16).

The precise mechanism and function of such subcellular compartmentation have not been determined in either metabolic or structural terms. It seems unlikely that the vesicles function exclusively to prevent catabolism of accumulated basic amino acids. This could be achieved more easily (as in procaryotic organisms) by maintaining low intracellular pools of these amino acids and low, or negligible, basal levels of their respective catabolic enzymes.

Catabolism of arginine and ornithine can be initiated in the absence of enzyme induction (17). This results from increases in the cytosolic concentrations of these amino acids much smaller than that potentially available from the vesicles (17). This suggests that the vesicles might serve as a reservoir of nutrients to be mobilized in times of metabolic stress.

These reserves might be mobilized as alternative sources of carbon or nitrogen, or both. All that would be required for the degradation of amino acids (especially arginine and ornithine) by preexisting catabolic enzymes would be a change in amino acid distribution between the cytosol and vesicles. The suggestion by Sumrada and Cooper that nutrient deprivation or inhibition of protein synthesis alters the permeability of the yeast vacuole (13) is consistent with this idea.

We have examined the metabolic fate of the intracellular pools of arginine and ornithine under conditions of nitrogen and carbon starvation. The approach has been to examine the effect of nitrogen and carbon starvation on the mobilization of vesicular arginine and ornithine into degradative (nitrogen- and carbon-yielding) reac-

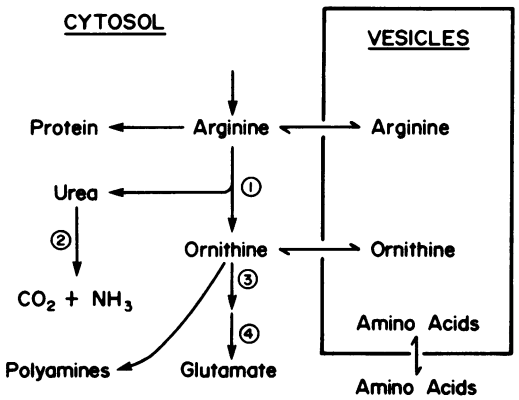

FIG. 1. *Diagram of the biochemical steps and subcellular structures involved in the degradation of arginine and ornithine in Neurospora. The vesicle is a subcellular organelle distinct from mitochondria which may be related to the vacuole of yeast and higher plants. Enzymes: 1, arginase (EC 3.5.3.1); 2, urease (EC 3.5.1.5); 3, ornithine transaminase (EC 2.6.1.13); 4, pyrroline-5-carboxylate dehydrogenase (EC 1.5.1.12).*

tions (Fig. 1). The results are consistent with the hypothesis that the vesicles serve as a specific nitrogen reserve to be mobilized in times of nitrogen metabolic stress.

MATERIALS AND METHODS

Strains, media, and chemicals. The mutant strains used in these experiments are listed in Table 1. Multiply mutant strains were constructed by standard genetic techniques (4). Strains were purified by single conidial isolation before use. The medium used was Vogel's minimal medium N supplemented with 1.5% sucrose (15). Nitrogen-free medium was the same medium without NH_4NO_3. Carbon-free medium lacked sucrose. L-[*guanido*-^{14}C]arginine (28 mCi/mmol), L-[*U*-^{14}C]ornithine (139 mCi/mmol), and Aquasol were obtained from New England Nuclear Corp. Analytical-grade AG 50W-X8 cation-exchange resin was purchased from Bio-Rad Laboratories. Triton X-100 was obtained from Research Products International. All other chemicals were reagent grade or equivalent. Dry radioactive samples were counted in toluene scintillation fluid containing 0.4% (wt/vol) 2,5-diphenyloxazole (PPO). Aqueous samples were counted in Aquasol or in toluene–Triton X-100 (2:1) scintillation fluid.

Growth and sampling. Exponential cultures were obtained as previously described (1). In experiments where radioactive isotope was used, 1 μCi of labeled amino acid was added to the culture 5 min before transfer to fresh medium. Uptake was essentially complete within this time (2). For transfer to new medium, the cells were collected by filtration on Whatman no. 540 filter paper, washed with 100 ml of prewarmed new medium, and suspended in 150 ml of the prewarmed medium. They were incubated at 30°C with vigorous agitation. At appropriate intervals, cells were collected by filtration on Whatman no. 540 filter paper. Amino acid pools were solubilized by boiling-water extraction as previously described (1). The filtrate (medium) was analyzed for urea. The extracted cells were either resuspended in 0.5 N NaOH overnight to solubilize protein or hydrolyzed as described below. Liberated CO_2 was collected by passing the escaping culture air through a solution of ethanolamine.

Fractionation and chromatography. The boiling-water extracts containing the soluble arginine, ornithine, and polyamines were evaporated to dryness and were separated on columns of AG 50W-X8 (Na^+ form) as previously described (1, 9). Arginine was determined by the method of Van Pilsum et al. (14). Ornithine was determined by the method of Chinard (2). Urea was purified on columns of AG 50W-X8 (H^+ form) as previously described (17). Urea was determined by the method of Koritz and Cohen (10), with chloride added to intensify and standardize the color values (3). Protein solubilized as described above was determined by the method of Lowry et al. (11). In some cases, the protein was hydrolyzed overnight at 100°C in 6 N HCl. It was then filtered through 3.0-μm membrane filters (Millipore Corp.). Protein arginine was determined as described above for boiling-water extracts.

Measurement of radioactivity. Fractions containing urea or arginine were assayed for radioactivity by counting portions in toluene–Triton X-100 scintillation fluid. The arginine samples were first neutralized by addition of acid. Ethanolamine samples and total cells were counted in Aquasol. Labeled standards showed that no appreciable quenching occurred under these conditions.

Enzyme assays. Arginine and ornithine transaminase were assayed in toluenized cells by the method of Davis and Mora (6) as previously described (1).

RESULTS

Effect of nutrient deprivation on the intracellular pools of arginine and ornithine. To determine the effect of nitrogen or carbon starvation on the intracellular pools of arginine

TABLE 1. *N. crassa strains*

Genotype	Allele	Source
ure-1[a]	*9* (FGSC 1230)[b]	R. H. Davis
aga	UM906 (FGSC 1699)	R. H. Davis
alc-1	WRR1 (FGSC 2699)	G. A. Marzluf
aln-1	U3 (FGSC 2701)	G. A. Marzluf
aga ure-1		This work
alc-1 ure-1		This work
aln-1 ure-1		This work
aga alc-1 ure-1		This work

[a] Neither the *ure-1* mutation nor the accumulation of urea by such a strain affects the growth or metabolic behavior of the organism (12, 17).

[b] FGSC, Fungal Genetics Stock Center, Arcata, Calif.

and ornithine, cells germinated in minimal medium were transferred to minimal, nitrogen-free, or carbon-free medium. Samples were collected at various intervals thereafter and analyzed as described in Materials and Methods. Under conditions of carbon or nitrogen starvation, net growth (protein synthesis) ceased within minutes. The effect of these transfers on the pools of ornithine and arginine is shown in Fig. 2. In nitrogen-free medium, the ornithine pool began to decline immediately and was less than 15% of its normal pool size by 100 min (Fig. 2B). The arginine pool remained relatively constant for 15 to 30 min and then declined to low levels by 100 min (Fig. 2A). When cells were transferred to carbon-free medium, the ornithine pool was essentially constant for 100 min (Fig. 2B). The arginine pool doubled within 80 min of the initiation of carbon starvation (Fig. 2A). On transfer to fresh minimal medium, the arginine pool increased slowly (relative to carbon starvation) throughout the experimental period (Fig. 2A), whereas the ornithine pool remained unchanged (Fig. 2B).

The disappearance of arginine and ornithine during nitrogen starvation suggested that they might be mobilized into the appropriate degradative reactions (Fig. 1). Alternatively, ornithine might be converted to arginine.

Metabolic fate of arginine and ornithine upon nutrient deprivation. The metabolic fate of arginine was examined by measuring the accumulation of urea in the culture medium with a mutant strain lacking urease (*ure-1*). Upon nitrogen starvation, urea accumulated rapidly after an initial lag of about 20 min (Fig. 3). The lag corresponds to that observed for the decline in the arginine pool (Fig. 2A), but considerably more urea was produced than could be accounted for by the disappearance of arginine. Urea was also produced to a limited extent under conditions of carbon starvation after a much longer lag period (Fig. 3).

To confirm that the appearance of urea is, at least partially, the result of arginine degradation,

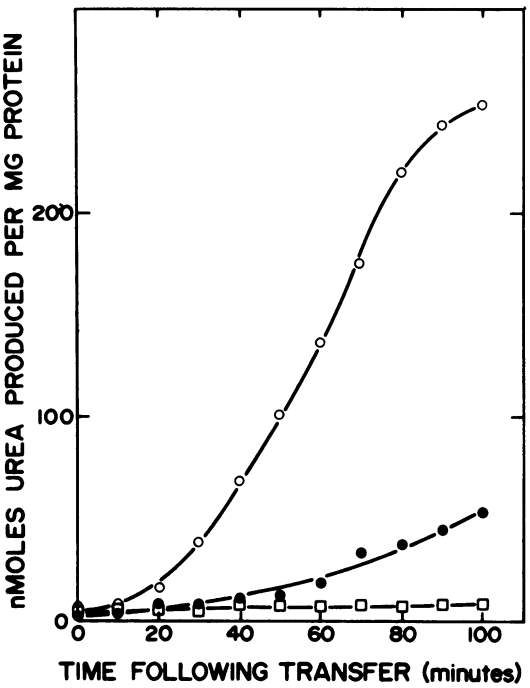

FIG. 3. *Accumulation of urea in the medium after transfer of ure-1 cells from minimal medium to fresh minimal* (□), *nitrogen-free* (○), *or carbon-free* (●) *medium.*

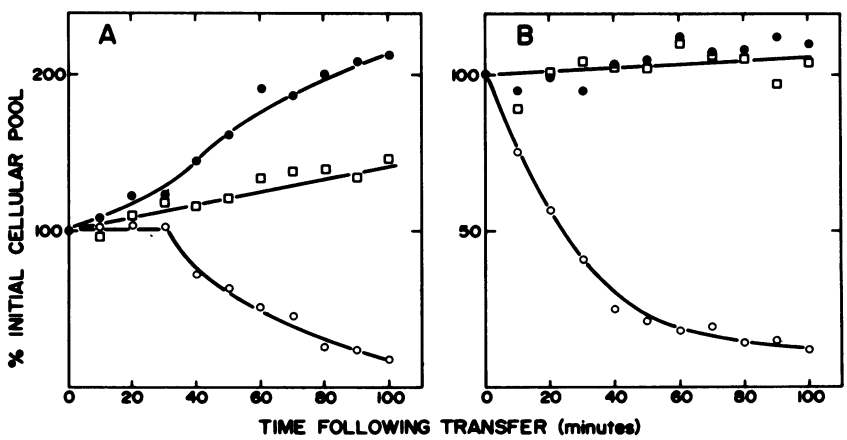

FIG. 2. *Arginine and ornithine pools after transfer of ure-1 cells to fresh minimal* (□), *nitrogen-free* (○), *or carbon-free* (●) *medium. (A) Arginine pool; (B) ornithine pool.*

the arginine pool was labeled with [*guanido*-^{14}C]arginine before transfer to starvation conditions. The results are shown in Fig. 4. In the case of nitrogen starvation, a loss of radioactivity from the arginine pool occurred with concomitant appearance of radioactive urea in the medium (Fig. 4A). Loss of radioactivity from the arginine pool occurred immediately. A short delay was observed before the appearance of radioactive urea in the medium. Most of the radioactivity lost from the arginine pool, and not appearing in urea, was found in protein arginine. In contrast to nitrogen starvation, carbon starvation resulted in the production of only a small amount of radioactive urea (Fig. 4B).

Ornithine is a precursor of both arginine and polyamines. Both are required for the normal growth of *Neurospora* (5). Ornithine may also be degraded through a series of reactions leading ultimately to glutamate (Fig. 1). It exhibits compartmental behavior similar to arginine (9). The fate of ornithine under conditions of nitrogen or carbon starvation was determined. The ornithine pool was labeled with [*U*-^{14}C]ornithine before transfer to fresh medium. The amount of radioactivity in the ornithine pool was measured immediately after transfer. Evolved CO_2 was collected for 2 h after the transfer. Radioactivity in biosynthetic and degradative products was determined. The results are shown in Table 2. Less than 4% of the radioactive ornithine appeared in CO_2 during incubation in minimal medium. The corresponding values for carbon and nitrogen starvation were 15.5 and 32.2%, respectively. Nitrogen starvation resulted in a considerable increase in degradation. The small amount of degradation in minimal medium has been previously described (1a).

Effect of nutrient deprivation on the level of arginase and ornithine transaminase. The degradation of arginine and ornithine in nitrogen-free medium might result from the induction or derepression of arginase and ornithine transaminase (Fig. 1). Sumrada and Cooper have reported the internal induction of allophanate hydrolase in *Saccharomyces cerevisiae* (13) under similar conditions. Such enzyme induction or derepression does not appear to be necessary in *Neurospora* since significant basal levels of both enzymes are present in cells growing in minimal medium. The specific activities of the enzymes were determined after the transfer of cells to minimal, carbon-free, or nitrogen-free medium. The results are shown in Table 3. Arginase activity did not increase after the transfer to starvation conditions. Ornithine transaminase activity doubled after 2 h in nitrogen-free medium.

Other sources of urea during starvation. The amount of urea produced during nitrogen starvation exceeded the loss of arginine (Fig. 2 and 3). Newly synthesized arginine did not ap-

TABLE 2. *Metabolic fate of ornithine*[a]

Medium	Initial radioactivity (%)		
	Biosynthetic products[b]	Degradation products[c]	CO_2[d]
Minimal	79.6	20.4	3.3
Carbon-free	57.7	42.3	15.5
Nitrogen-free	35.0	65.0	32.2

[a] Cells were labeled with [*U*-^{14}C]ornithine before transfer to the indicated medium for 2 h.

[b] Sum of radioactivity in ornithine, arginine (including protein), and polyamines.

[c] Initial radioactivity in ornithine minus radioactivity in biosynthetic products.

[d] Radioactive CO_2 was measured as described in the text.

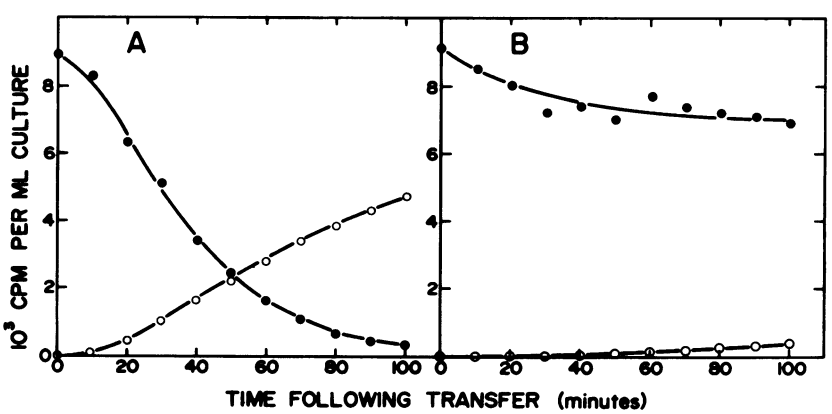

FIG. 4. *Radioactivity in arginine* (●) *and urea* (○). *Cells (ure-1) were labeled with [guanido-*^{14}*C]arginine 5 min before transfer to fresh medium. (A) Nitrogen-free; (B) carbon-free.*

TABLE 3. *Specific activities of arginase and ornithine transaminase*[a]

Time after transfer (h)	Sp act (U/mg of protein)	
	Ornithine transaminase	Arginase
0	0.010	0.94
2	0.021	0.91

[a] Cells were transferred to nitrogen-free medium at zero time. Enzyme activities were determined immediately before transfer and after 2 h at 30°C in the new medium. Enzyme activities were measured as described in the text.

pear to be sufficient to account for the discrepancy. Therefore, attempts were made to identify additional sources of urea. The degradation of purines can yield urea (Fig. 5). The accumulation of urea in various mutants was examined. All strains carried the *ure-1* mutation. The *aga* mutation rendered cells incapable of degrading arginine to urea (Fig. 5). Similarly, the *alc* and *aln* mutations prevented purine degradation to urea (Fig. 5).

After germination in minimal medium, the mutant strains were transferred to starvation conditions. The accumulation of urea in the medium was measured. The results are shown in Table 4. Very little urea was produced in the mutant strain incapable of arginine or purine degradation. During carbon starvation, most of the urea appeared to be produced by the degradation of arginine. In nitrogen-free medium the urea appeared to arise almost equally from arginine and purines. The non-additivity of the urea production between the double mutant strains and *ure-1* probably arises from partial relief of starvation conditions by arginine or purine degradation in the latter.

DISCUSSION

The results of these experiments indicate that arginine and ornithine are degraded rapidly during nitrogen starvation (Fig. 2 through 4; Table 4). Degradation occurs to a more limited extent during carbon starvation (Fig. 4; Table 4). The initiation or acceleration of degradation does not appear to be due to internal induction or derepression of the catabolic enzymes. Both nitrogen and carbon starvation lead to a reduction of growth and protein synthesis, but nitrogen starvation results in more rapid and extensive degradation. Thus, mobilization does not appear to be a general response to adversity. This is consistent with the observation that inhibition of protein synthesis with cycloheximide does not result in significant degradation of arginine (12).

Purines appear to respond in a manner similar to arginine and ornithine. Their degradation is greatly stimulated by nitrogen starvation. They are not degraded during growth in minimal medium and only to a small extent upon carbon starvation (Table 4). These results suggest that purines might be compartmentalized within the vesicles. This possibility is under investigation.

The delay in the decline of the arginine pool upon transfer to nitrogen-free medium suggests several possibilities. First, pools of nitrogen metabolites may be sufficient to maintain adequate nitrogen status for the observed delay period (10 to 30 min). This seems unlikely since metabolic changes characteristic of nitrogen starvation are initiated within minutes after the transfer to nitrogen-free medium (e.g., Fig. 2B). Second, the level of the arginine pool might be maintained during this period by continued biosynthesis from ornithine. This would replenish any arginine lost through degradation. The appearance of radioactivity in urea (from [*guanido*-^{14}C]arginine) during this period (Fig. 4) is consistent with this hypothesis. However, even this process exhibits a lag period (Fig. 4). Thus, it is possible that some cellular process, such as protein syn-

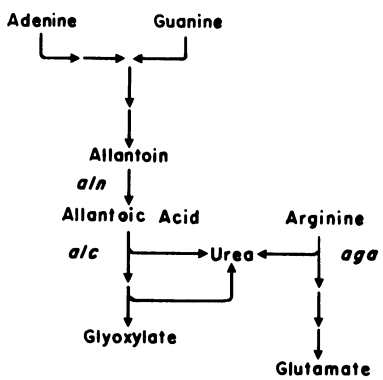

FIG. 5. *Diagram of arginine and purine degradative pathways of Neurospora and location of mutational blocks.*

TABLE 4. *Urea production in mutant strains*[a]

Strain[b]	nmol of urea/mg of protein per 2 h		
	Minimal	Nitrogen-free	Carbon-free
ure-1	7	253	105
aga ure-1	7	159	53
alc ure-1	12	128	101
aln ure-1	ND[c]	136	89
aga alc ure-1	ND	14	25

[a] Cells growing in minimal medium were transferred to the indicated medium for 2 h. Urea accumulated in the medium was determined as described in the text.

[b] Mutations are those indicated in Fig. 5.

[c] ND, Not determined.

thesis or depletion of an inhibitor of arginase, must occur before rapid mobilization of arginine into the degradative pathway. The degradation of ornithine is also accelerated by nitrogen starvation, although some ornithine degradation is known to occur in cells growing in minimal medium (1a).

Results of the carbon starvation experiments are consistent with the observation that neither arginine nor ornithine serves as an efficient carbon source for *Neurospora*. Arginine degradation occurs to a limited extent. Similarly, ornithine degradation is slightly enhanced by carbon starvation. Purine degradation exhibits behavior similar to that of arginine and ornithine. Degradation is likely to be a secondary consequence of the accumulation of these compounds in the absence of macromolecular syntheses rather than a specific response to nutrient deprivation.

The results of these experiments suggest some interesting regulatory features. Nitrogen starvation initiates or accelerates the degradation of arginine, ornithine, and purines. This alteration in metabolism is consistent with the abilities of these compounds to serve as sole nitrogen sources (7). In contrast, carbon starvation fails to elicit this response and the compounds are poor carbon sources. These observations are consistent with the internal induction of allophanate hydrolase upon nitrogen starvation of *S. cerevisiae* (3).

At least two possible mechanisms might account for the mobilization of arginine and ornithine. First, nitrogen starvation might alter the distribution of arginine and ornithine between vesicles and cytosol, thus rendering them more accessible to the preexisting degradative enzymes. Second, the activities of the preexisting degradative enzymes might be altered dramatically in vivo by nitrogen starvation, but this alteration might not be observed in in vitro assays. Experiments are in progress to distinguish between these possibilities.

It is an unusual feature of fungi such as *Neurospora* that these organisms maintain intracellular pools of many metabolites far in excess of their immediate metabolic needs. In some cases, these metabolites appear to be isolated physically from the functioning synthetic and degradative pathways of the cell. This requires the expenditure of considerable metabolic energy. Therefore, it seems unlikely that such compartmentalization would function solely to prevent wasteful degradation. The behavior of the intracellular pools of arginine and ornithine during metabolic stress suggests that these pools may operate as nitrogen reserves. These reserves are made available (mobilized) to the preexisting metabolic machinery of the cell under conditions of specific metabolic stress. We are continuing these studies in an attempt to understand the mechanisms by which this mobilization occurs.

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