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Energetics of Vacuolar Compartmentation of Arginine in *Neurospora crassa*

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The energy requirements for the uptake and retention of arginine by vacuoles of *Neurospora crassa* have been studied. Exponentially growing mycelial cultures were treated with inhibitors of respiration or glycolysis or an uncoupler of respiration. Catabolism of arginine was monitored as urea production in ureaseless strains. The rationale was that the rate and extent of such catabolism was indicative of the cytosolic arginine concentration. No catabolism was observed in cultures treated with an inhibitor or an uncoupler of respiration, but cultures treated with inhibitors of glycolysis rapidly degraded arginine. These differences could not be accounted for by alterations in the level or activity of arginase. Mycelia growing in arginine-supplemented medium and treated with an inhibitor or uncoupler of respiration degraded an amount of arginine equivalent to the cytosolic fraction of the arginine pool. The inhibitors and the uncoupler of respiration reduced the ATP pool and the energy charge. The inhibitors of glycolysis reduced the ATP pool but did not affect the energy charge. The results suggest that metabolic energy is required for the transport of arginine into the vacuoles but not for its retention. The latter is affected by inhibitors of glycolysis. The form of energy and the nature of the vacuolar transport mechanism(s) are discussed.

Metabolic compartmentation is an important phenomenon in cell physiology and regulation; it appears to be a universal phenomenon in lower as well as higher eucaryotic cells (6, 13, 14, 16). The mechanisms responsible for the translocation of various metabolites between different subcellular compartments (mitochondria, vacuoles, lysosomes, etc.) are only poorly understood. The intracellular translocations of the amino acid arginine in *Neurospora crassa* offer an excellent model system for the study of the phenomenon of compartmentation.

Synthesis of arginine is initiated within the mitochondria but culminates in the cytosol. The bulk of the intracellular arginine (>98%) is sequestered in the vacuoles during growth in minimal medium; approximately 2% of the total pool is in the cytosol (17, 19). These organizational features are diagrammed in Fig. 1. No catabolism of arginine takes place during growth in minimal medium despite a significant level of the cytosolic catabolic enzyme arginase (5).

Cytosolic arginine is approximately 20% of the total in mycelia growing in arginine-supplemented medium; catabolism of arginine (monitored as urea accumulation in ureaseless strains; see Fig. 1) is rapid (20). When such mycelia are transferred to minimal medium, catabolism of arginine quickly decreases and then ceases (21) Much of the cytosolic arginine is taken up into the vacuoles.

It is not clear how arginine is transported and retained in the vacuoles, but the movement of arginine against an apparent concentration gradient requires an accumulative transport mechanism. The existence of a nonspecific carrier capable of exchanging arginine across the vacuolar membrane has been reported for *N. crassa* (12) and yeast (3). Recent evidence suggests that arginine accumulation is driven by the proton motive force in vacuoles of *Saccharomyces cerevisiae* (15). Wiemken and co-workers have suggested a mechanism by which the vectorial accumulation of polyphosphate within the vacuoles of *S. cerevisiae* provides a polyanion which binds and thus retains previously accumulated vacuolar arginine (9). However, Davis and coworkers have shown that mycelia of *N. crassa* depleted of polyphosphate continue to sequester arginine within the vacuoles (4).

Several questions remain to be answered concerning vacuolar transport in *N. crassa*. First, is energy required for retention of arginine accumulated in the vacuoles? Second, what is the nature of the transporting mechanism? Third, is energy required for the operation of this mechanism? Fourth, if metabolic energy is required, what is the form of this energy? The present

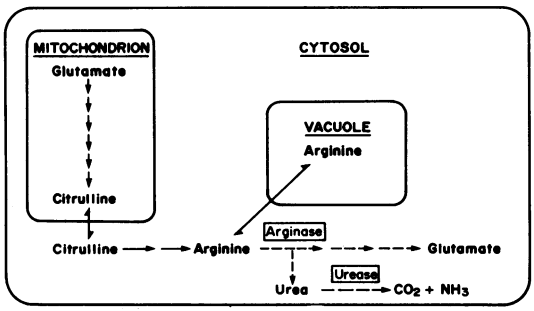

FIG. 1. Diagram of arginine metabolism and its organization in *N. crassa*.

report deals with the energy requirement for: (i) the retention of arginine previously accumulated in the vacuoles; and (ii) the uptake of arginine into the vacuoles. Intact mycelia were used to avoid the loss of essential cellular components which might be required for net accumulation. The effect of glycolytic and respiratory chain inhibitors (and an uncoupler) on the catabolism of arginine to urea (see Fig. 1) has been determined. The rationale was that treatments affecting sequestration of arginine in the vacuoles would result in an increased cytosolic arginine concentration sufficient to initiate or prolong catabolism (urea production). This method has been successfully used to study the mobilization of arginine (efflux from the vacuoles) upon nitrogen starvation (10).

MATERIALS AND METHODS

Strains, media, and chemicals. Two mutant strains of *N. crassa* were used: LA44 (*ure-1*, FGSC 1230) and LA401 (*arg-10 aln ure-1*). The latter was constructed by mating LA89 (*aln ure-1* [10]) and LA13 (*arg-10*, FGSC 122). Both LA44 and LA13 were originally obtained from the Fungal Genetics Stock Center, Arcata, Calif. Crosses and genetic analyses were made as described by David and de Serres (7). Strains carrying the *ure-1* mutation lack the enzyme urease and are unable to degrade urea. Such strains accumulate one molecule of urea for every molecule of arginine degraded by arginase (Fig. 1). Strains carrying the *aln* mutation lack allantoinase and are unable to degrade purines. The latter have been shown to be the only other significant source of urea in *N. crassa* starved for nitrogen (10). Strains carrying the *arg-10* mutation lack argininosuccinate lyase and are unable to synthesize arginine or to produce argininosuccinate from arginine. Growth of *N. crassa* was carried out in Vogel's minimal medium N (18) supplemented as indicated in the appropriate tables or figures. All chemicals were obtained from common sources and were reagent grade or equivalent. Amino acids and metabolic inhibitors were obtained from Sigma Chemical Co. L-[*guanido*-^{14}C]arginine (55 mCi/mmol) was obtained from International Chemical and Nuclear. Analytical-grade AG50W-X8 cation-exchange resin was obtained from Bio-Rad Laboratories. Partisil-10 SAX strong anion exchanger was purchased from Whatman. AS Pellionex SAX pellicular strong anion exchanger was a gift from D. E. Atkinson. Aqueous radioactive samples were counted in toluene-Triton X-100 (2:1) scintillation fluid containing 0.4% (wt/vol) 2,5-diphenyloxazole.

Growth and treatment with inhibitors or an uncoupler. Conidia were inoculated into appropriate liquid medium (10^6 conidia per ml) and germinated overnight at 16°C (2). Experiments were initiated after 1 h of growth at 30°C, at which time the germinated conidia were in steady-state exponential growth. Retention of vacuolar arginine was studied with the mutant strain LA44. An exponentially growing culture was pulsed with [*guanido*-^{14}C]arginine (2 μCi/100 ml) for 10 min. Uptake of radioactive arginine and its equilibration with the vacuolar pool were complete in this period. The bulk of the radioactive arginine was in the vacuoles. The mycelia were then harvested, washed with minimal medium, and resuspended in fresh minimal medium containing 20 mM glucose as the sole carbon source. This low concentration of glucose was used to allow competition with 2-deoxyglucose and resulting inhibition of glycolysis. This low glucose concentration did not have any effect on the growth of the germinated conidia. This culture was then divided into several flasks containing various inhibitors or an uncoupler, as specified. Samples were withdrawn at various times and analyzed for radioactivity in urea and for the size of the arginine pool.

Transport of arginine into the vacuoles was studied with the triple mutant strain LA401. Germinated conidia of this strain growing in medium supplemented with 1 mg of arginine per ml were transferred to fresh medium supplemented with 0.1 mg of arginine per ml. This lower arginine concentration did not affect the size of the arginine pool or its distribution between the cytosol and vacuoles (20% cytosolic; 20). The fresh culture was pulsed with [*guanido*-^{14}C]arginine (2 μCi/100 ml) for 30 min. After 30 min, the mycelia were harvested, washed with minimal medium, and resuspended in fresh minimal medium (without arginine). The germinated conidial culture was divided and treated with various inhibitors or an uncoupler, as specified. A culture with no inhibitor and one with cycloheximide were included as controls. Sampling started immediately after transfer to minimal medium. Samples were analyzed for radioactivity in urea and protein arginine and for the size of the arginine pool.

Sampling. Three-milliliter samples were withdrawn from the liquid cultures at the indicated times. The samples were boiled for 10 min to extract urea and the arginine pool. Precipitated protein was collected by centrifugation for 2 to 3 min in a clinical centrifuge. Radioactivity in protein arginine was determined by counting a portion of this protein precipitate, when appropriate. Urea and arginine were purified by cation-exchange chromatography and analyzed as previously described (2, 8).

Amino acid analysis. Samples for amino acid analysis (25 ml) were collected by filtration on Whatman no. 540 filter paper. The pad of mycelia was washed with distilled water and suspended in boiling water for 10 min to extract the amino acid pools. The boiled samples were centrifuged to remove precipitated protein. The precipitates were suspended in 0.5 N NaOH and used for protein estimation. The supernatants

were evaporated; amino acids were quantitated with a Beckman model 119C amino acid analyzer. All amino acid pools are expressed as nanomoles per milligram of protein.

Adenylate analysis and energy charge. Samples for analysis of adenylates (25 ml) were collected by filtration and suspended (without washing) in 3 ml of 95% ethanol. This suspension was boiled for 5 min. An additional 1.5 ml of 95% ethanol was added; each sample was then boiled for 5 additional min to extract the adenylate pools. Precipitated protein was collected by centrifugation and treated as above. Supernatants were frozen in an acetone-dry ice bath and lyophilized overnight. Dried samples were dissolved in 0.1 mM potassium phosphate, pH 3.5, and filtered (0.45-μm pore size) to remove undissolved particulates. The adenylates were separated on a Partisil-10 SAX column with an AS Pellionex SAX precolumn. Elution was performed with an exponential gradient (0 to 86%) of 0.7 M potassium phosphate (pH 4.5) and mixed with the equilibration buffer, 0.1 mM potassium phosphate, pH 3.35 (Waters Associates model 660 solvent programmer, gradient 8). The time of the program was 1 h, and the flow rate was 1.0 ml/min. Adenylates were detected and quantitated by their absorbance at 256 nm. The adenylate pools are expressed as nanomoles per milligram of protein. The energy charge (1) was calculated from the ratio (ATP + 1/2ADP)/(ATP + ADP + AMP).

Enzyme assays and protein estimation. Arginase activity was assayed as previously described (2). Protein was estimated by the method of Lowry et al. (11).

RESULTS

Effect of various inhibitors and an uncoupler of respiration on the sequestration of arginine in the vacuoles. If retention of arginine within the vacuoles of *N. crassa* requires energy, then depletion of the mycelia of metabolic energy should release arginine into the cytosol, where it would be degraded to ornithine and urea (Fig. 1). The following experiment was performed to test this possibility. The arginine pool of LA44 (*ure-1*) growing in minimal medium was labeled with [*guanido*-^{14}C]arginine by adding a small amount (2 μCi/100 ml) of highly radioactive arginine to the culture medium (radioactive arginine has been shown to equilibrate quickly with the vacuolar pool after uptake [10, 17]). The appearance of radioactivity in urea was measured at various times after exposure of the mycelia to an inhibitor or an uncoupler of respiration (Fig. 2).

Mycelia treated with an inhibitor (sodium azide or oligomycin) or an uncoupler (2,4-dinitrophenol) of the respiratory chain did not accumulate any urea (data not shown). Mycelia treated with an inhibitor (arsenate or 2-deoxyglucose) of glycolysis began producing urea shortly after the addition of the inhibitor. Addition of an inhibitor or uncoupler of respiration to such mycelia resulted in the immediate cessation

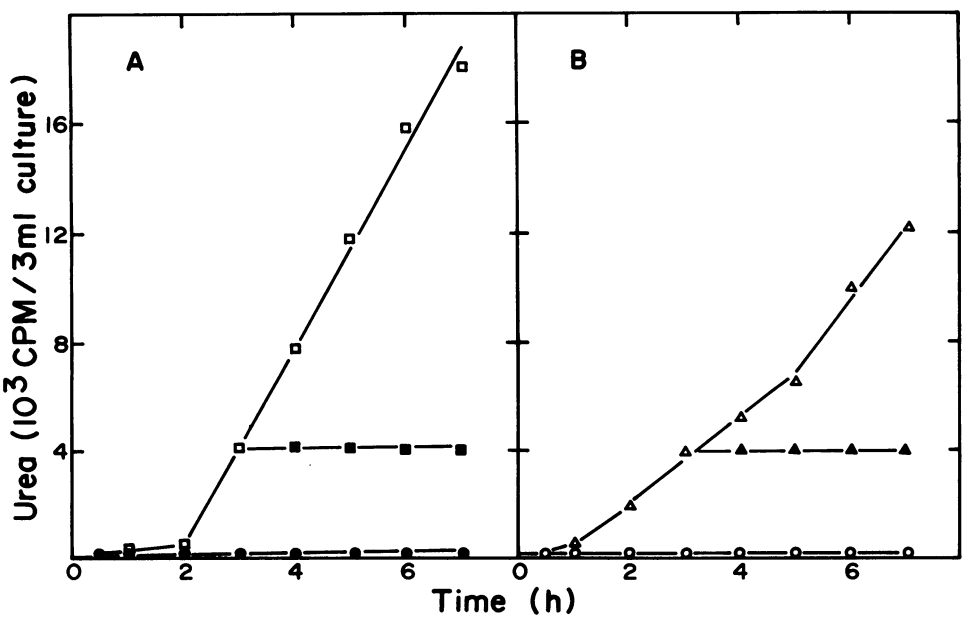

FIG. 2. Effect of various inhibitors on urea production (arginine degradation) by *N. crassa*. Mycelia (strain LA44) growing in minimal medium were pulsed with [*guanido*-^{14}C]arginine and then transferred to fresh minimal medium containing various inhibitors. At various times samples were withdrawn and analyzed for radioactivity in urea. Symbols: (A) ●, control (no inhibitor); □, arsenate (20 mM); ■, arsenate (20 mM) plus sodium azide (10 μg/ml) at 2 h. (B) ○, Cycloheximide (20 μg/ml); △, 2-deoxyglucose (50 mM); ▲, 2-deoxyglucose (50 mM) plus sodium azide (10 μg/ml) at 2 h.

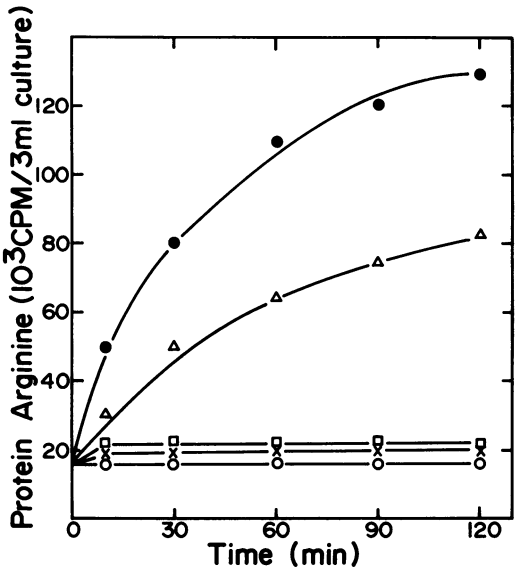

FIG. 3. Effect of various inhibitors on the incorporation of radioactive arginine into protein. Mycelia (strain LA44) growing in minimal medium were pulsed (2 min) with [*guanido*-^{14}C]arginine and then treated with the indicated inhibitors. At various times samples were withdrawn and analyzed for radioactivity in protein: ●, control (no inhibitor); ×, sodium azide (10 μg/ml); □, arsenate (20 mM); △, 2-deoxyglucose (50 mM); ○, cycloheximide (20 μg/ml).

of urea production (Fig. 2). The inhibitors and uncoupler all inhibited protein synthesis (Fig. 3). Treatment of mycelia with cycloheximide, a more specific inhibitor of protein synthesis, or a mixture of all the inhibitors did not result in the production of urea (data not shown). These mycelia maintained their original arginine pools.

Effect of various inhibitors and an uncoupler of respiration on the energy status, level, and activity of arginase and amino acid pools of treated mycelia. The adenylate pools and the energy charge of LA44 (*ure-1*) exposed for 3 h to an inhibitor or uncoupler are shown in Table 1. All of the treatments resulted in a decrease in the pool of ATP and total adenylates. Mycelia treated with azide, oligomycin, arsenate, or deoxyglucose had similar ATP pools. Cycloheximide had a smaller effect, whereas dinitrophenol resulted in almost complete loss of ATP. Dinitrophenol and the combination of inhibitors had the greatest effect. No ATP was detectable in mycelia treated with a mixture of all of the inhibitors.

Arsenate, deoxyglucose, and cycloheximide did not reduce the energy charge. Only the respiratory chain inhibitors and the uncoupler caused a decrease in the energy charge; dinitrophenol and the mixture of inhibitors were the most effective. Despite the depletion of the adenylate pools and the collapse of the energy charge, mycelia treated with an inhibitor or uncoupler of respiration (or with a mixture of inhibitors) failed to degrade their arginine pool (Fig. 2).

Mycelia of LA44 (*ure-1*) were exposed to an inhibitor or uncoupler for 3 h, washed, permeabilized (2), and assayed for arginase activity. No effect was observed on the level of the enzyme (data not shown). None of the inhibitors at the concentrations used had any effect on the activity of the enzyme assayed in vitro (data not shown).

The effect of the inhibitors and uncoupler on the amino acid pools of LA44 (*ure-1*) is summarized in Table 2. Only amino acids related to arginine metabolism or which exhibited major changes in response to the various treatments are shown. Other amino acid pools did not change significantly and, for clarity, are not included in the table. The arginine pool of mycelia treated with arsenate or deoxyglucose de-

TABLE 1. Effect of inhibitors and an uncoupler of respiration on adenylate pools and energy charge of *N. crassa* grown in minimal medium[a]

Inhibitor	Concn (μg/ml)	Adenylate pool (nmol/mg of protein)				Energy charge
		ATP	ADP	AMP	Total	
None		17.7	7.1	1.2	26.0	0.82
Azide	10	5.5	5.7	2.5	13.7	0.60
Oligomycin	1	7.7	5.6	1.0	14.3	0.73
Dinitrophenol	100	0.6	1.9	1.8	4.3	0.36
Arsenate	20 mM	5.8	2.4	0.3	8.5	0.82
Deoxyglucose	50 mM	6.9	3.4	0.4	10.7	0.80
All (−dinitrophenol)			2.0	6.0	8.0	0.13
Cycloheximide	20	11.6	4.5	0.6	16.7	0.83

[a] The ureaseless strain LA44, growing in minimal medium, was transferred to fresh minimal medium containing the inhibitor or uncoupler at time zero. Samples were taken 3 h after exposure to the inhibitor or uncoupler. Adenylates were determined by high-performance liquid chromatography as described in the text. The adenylate pool values represent the mean of three repeats. The limits of confidence were within ±0.5.

TABLE 2. Effect of various inhibitors and an uncoupler of respiration on amino acid pools of *N. crassa* grown in minimal medium[a]

Inhibitor	Concn (μg/ml)	Amino acid pools (nmol/mg of protein)					
		Glu	Gln	Ala	Orn	Lys	Arg
None		190	396	168	40	20	142
Azide	10	69	21	1,496	30	10	148
Oligomycin	1	75	780	2,120	45	22	145
Dinitrophenol	100	20	18	190	30	6	147
Arsenate	20 mM	28	250	2,115	40	30	105
Deoxyglucose	50 mM	48	255	32	58	15	110
All (−dinitrophenol)			60	161	31	11	149
Cycloheximide	20	160	1,113	820	110	64	280

[a] Strain LA44 (*ure-1*) was grown in minimal medium and transferred to fresh medium containing the inhibitor or uncoupler at zero time. Samples were taken 3 h after the final transfer. Amino acids were extracted with boiling water and analyzed as described in the text. The values represent the mean of three repeats. The limits of confidence were within ±10, except for values below 10, which had confidence limits within ±3.

creased approximately 20%. Mycelia treated with an inhibitor or uncoupler of respiration had arginine pools similar to the untreated mycelia. This is consistent with the observed accumulation of urea (Fig. 2). Mycelia treated with cycloheximide had an increased arginine pool. The ornithine pool was not significantly affected except in mycelia treated with cycloheximide. The glutamate pool was lower in all treated mycelia. No apparent pattern was discernible in the variations in the pools of glutamine or alanine between mycelia treated with a glycolytic or a respiratory chain inhibitor (or uncoupler).

The inhibitors and the uncoupler of respiration reduced the energy status of treated mycelia without provoking arginine degradation. The absence of degradation could not be accounted for by indirect effects on the level or activity of arginase or by accumulation of inhibitors of the enzyme (ornithine and lysine). In contrast, inhibitors of glycolysis reduced the adenylate levels without concomitant reduction of the energy charge; degradation of arginine occurred. The latter could not be accounted for by depletion of inhibitors of arginase or of glutamine.

Effect of inhibitors and an uncoupler of respiration on uptake of arginine into the vacuoles. If uptake of arginine into the vacuoles requires energy, then depletion of metabolic energy from mycelia degrading arginine should result in degradation of only that fraction of the arginine pool present in the cytosol at the time of energy deprivation. The following experiment tested this possibility. The effect of respiratory chain inhibitors on the accumulation of urea and on the arginine pool of LA401 (*arg-10 aln ure-1*) transferred from arginine-supplemented to minimal medium is shown in Fig. 4. Mycelia were pulsed for 30 min before the transfer with [*guanido*-^{14}C]arginine to label the arginine pool. The radioactive arginine rapidly equilibrated between the cytosolic and vacuolar compartments (10).

Cultures containing an inhibitor of the respiratory chain rapidly produced radioactive urea. Production ceased approximately 1 h after the transfer to minimal medium (Fig. 4A). Mycelia transferred to medium without an inhibitor incorporated significantly lower amounts of radioactivity into urea. This incorporation persisted throughout the 3-h period of the experiment. Incorporation of radioactivity into the urea was delayed approximately 30 min in cultures containing cycloheximide. This incorporation was more rapid than that observed in control cultures and continued at a significant rate for several hours (Fig. 4A; data not shown). The effect of inhibitors of glycolysis was not studied in these experiments as such inhibitors promote catabolism of vacuolar arginine (see above).

The arginine pool of the culture without any inhibitors decreased rapidly after the transfer to unsupplemented medium (Fig. 4B). This decrease was accounted for by the incorporation into protein (Fig. 5) and degradation to urea (Fig. 4A). Arginine provided by the arginine pool supported the growth of the arginine auxotrophic strain during the time of the experiment. After 3 h the growth slowed and stopped as the mycelia exhausted all of their arginine pool (Fig. 6). The inhibitors and uncoupler of the respiratory chain inhibited the incorporation of radioactive arginine into protein after a short lag; cycloheximide exhibited a similar but more immediate effect (Fig. 5).

The arginine pool of mycelia treated with an inhibitor or uncoupler of the respiratory chain decreased rapidly to approximately 80% of the initial value during the first hour after the transfer. It remained at this value for the remainder of the experiment (Fig. 4B) and persisted at this level for up to 7 h (data not shown). The arginine

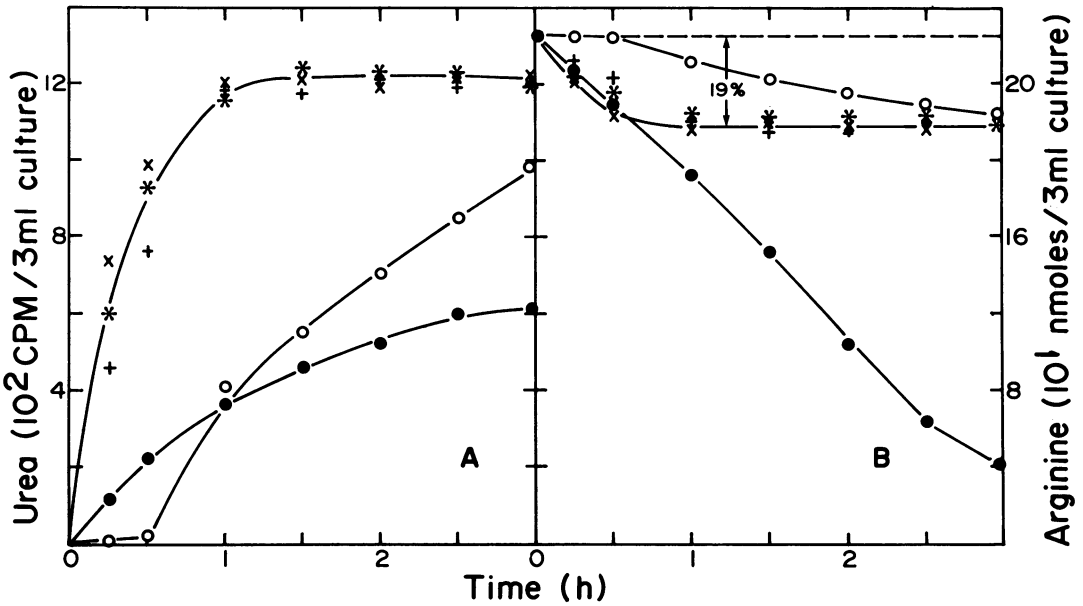

FIG. 4. Effect of various inhibitors on urea production and the arginine pool of *N. crassa* growing in arginine-supplemented medium. Mycelia (strain LA401) growing in arginine-supplemented medium were pulsed with [*guanido*-^{14}C]arginine (30 min) and then transferred to minimal medium containing various inhibitors. At various times samples were withdrawn and analyzed for radioactivity in urea and in the arginine pool: ●, control (no inhibitor); ○, cycloheximide (20 μg/ml); ×, sodium azide (10 μg/ml); +, oligomycin (1 μg/ml); ⁎, dinitrophenol (100 μg/ml).

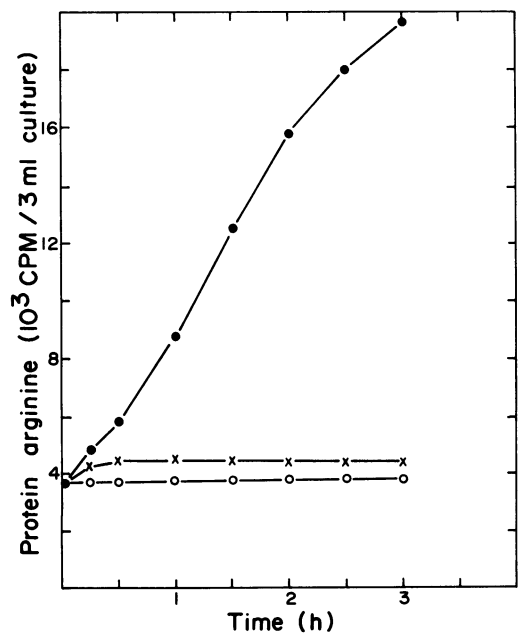

FIG. 5. Effect of various inhibitors on the incorporation of arginine into protein. Mycelia (strain LA401) treated as in the legend to Fig. 4 were assayed for radioactivity in protein at various times after exposure to an inhibitor: ●, control (no inhibitor); ○, cycloheximide (20 μg/ml); ×, sodium azide (10 μg/ml).

pool of mycelia treated with cycloheximide began to decline 30 min after exposure to the inhibitor (Fig. 3B) and continued to decline slowly for several hours (Fig. 4B; data not shown). These results correlate with the incorporation of radioactivity into urea in the same cultures (Fig. 4A). The decrease in the arginine pool of the energy-starved mycelia is approximately equal to the cytosolic fraction (20%) of the arginine pool of mycelia growing in arginine-supplemented medium (20).

Effect of inhibitors and an uncoupler of respiration on the energy status and on the pools of amino acids involved in nitrogen metabolism. The adenylate pools and energy charge of strain LA401 (*arg-10 aln ure-1*) treated with an inhibitor or uncoupler are shown in Table 3. Mycelia treated with a respiratory chain inhibitor or uncoupler had considerably decreased pools of total adenylates and ATP and had a reduced energy charge. Cycloheximide reduced the level of adenylates and ATP to a smaller extent but had no effect on the energy charge.

The effect of the inhibitors and the uncoupler on the level of amino acids involved in nitrogen metabolism in *N. crassa* is shown in Table 4. The pools of glutamate, ornithine, and lysine were reduced in LA401 (*arg-10 aln ure-1*) treated with azide, oligomycin, or dinitrophenol but were normal or elevated in cultures treated with

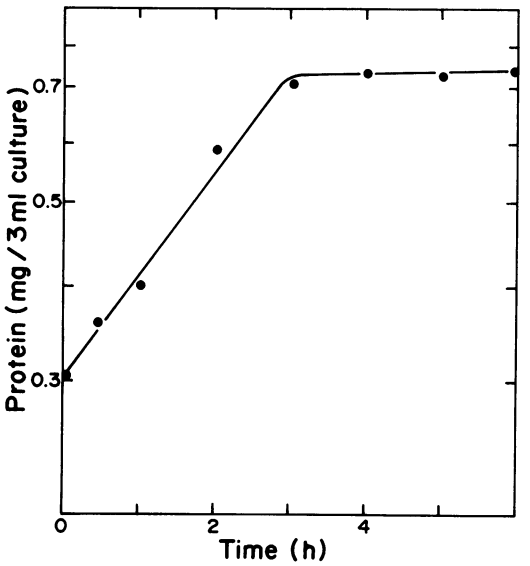

FIG. 6. Growth of the arginine auxotrophic strain LA401 after transfer from arginine-supplemented medium to minimal medium.

cycloheximide. Glutamine was reduced in mycelia treated with azide or dinitrophenol but elevated in mycelia treated with oligomycin or cycloheximide. All treatments resulted in the accumulation of alanine. The arginine pool was smaller in all treated mycelia, but considerably greater than that of the untreated mycelia. These results correlate well with the results of urea accumulation and arginine disappearance (Fig. 4).

TABLE 3. Effect of various inhibitors and an uncoupler of respiration on adenylate pools and energy charge of *N. crassa* grown in arginine-supplemented medium[a]

Inhibitor	Concn (μg/ml)	Adenylate pool (nmol/mg of protein)			Energy charge
		ATP	ADP	AMP	
None		18.2	7.4	0.9	0.82
Azide	10	2.4	2.8	1.5	0.56
Oligomycin	1	7.1	4.3	1.1	0.73
Dinitrophenol	100	0.5	1.3	1.5	0.35
Cycloheximide	20	12.8	5.2	0.4	0.84

[a] Strain LA401 (*arg-10 aln ure-1*) was grown in arginine-supplemented (1 mg/ml) medium, transferred to low arginine-supplemented (0.1 mg/ml) medium for 30 min, and then transferred to unsupplemented medium containing the indicated concentrations of the inhibitors. Samples were removed after 3 h and analyzed for adenylates as described in the text. The results represent the means of three independent experiments. The maximum deviation from the mean was ±0.5 nmol/mg of protein.

TABLE 4. Effect of inhibitors and an uncoupler of respiration on amino acid pools of *N. crassa* grown in arginine-supplemented medium[a]

Inhibitor	Concn (μg/ml)	Amino acid pool (nmol/mg of protein)					
		Glu	Gln	Ala	Orn	Lys	Arg
None (time zero)		122	376	132	75	69	769
None		126	351	145	82	78	13
Azide	10	35	55	1,568	54	17	635
Oligomycin	1	70	762	2,421	54	21	612
Dinitrophenol	100	3	15	250	50	7	618
Cycloheximide	20	130	973	666	194	70	402

[a] Strain LA401 (*arg-10 aln ure-1*) was grown in minimal medium supplemented with 1 mg of arginine per ml, transferred to medium supplemented with 0.1 mg of arginine per ml for 30 min, and then transferred to fresh minimal medium. Samples were taken 5 h after the final transfer. Amino acids were extracted with boiling water and analyzed as described in the text. All treatments were initiated at the time of the final transfer. The values represent the mean of three repeats. The limits of confidence were within ±10, except for the values below 10, which had confidence limits within ±3.

The inhibitors and the uncoupler reduced the energy status of treated mycelia; approximately 20% of the arginine pool was subsequently degraded. Cessation of degradation could not be accounted for by changes in the activity or level of arginase, increased concentrations of inhibitors of its activity, or by accumulation of glutamine.

DISCUSSION

The majority of the arginine pool of *N. crassa* is sequestered in the vacuoles (19). Vacuolar arginine is mobilized (effluxes from the vacuole) and is degraded to urea and ornithine upon nitrogen starvation (10). These findings suggest that vacuoles sequester nitrogen metabolite reserves and release their contents upon specific nutritional stress (10). The metabolite signal mediating the response to nitrogen starvation appears to be glutamine: reduction of the intracellular glutamine pool results in mobilization of vacuolar arginine (T. L. Legerton and R. L. Weiss, manuscript in preparation).

The results presented here suggest that metabolic energy is not required for the retention of arginine within the vacuoles. Mycelia treated with an inhibitor (oligomycin or sodium azide) or an uncoupler (dinitrophenol) of the respiratory chain did not produce urea (degrade arginine), but maintained their original large arginine pool. There was no indication that catabolism of arginine was prevented by an indirect inhibition of arginase.

Mycelia treated with a glycolytic inhibitor

(arsenate or 2-deoxyglucose) failed to retain their vacuolar arginine and produced urea (Fig. 3). Addition of an inhibitor of the respiratory chain to mycelia treated with an inhibitor of glycolysis stopped further mobilization and resulted in a decrease in the energy charge. These results suggest that a product of glycolysis may be required for continued vacuolar compartmentation even in the presence of glutamine and that mobilization may require an appropriate energy status, possibly related to energy charge.

The experiments with strain LA401 growing in arginine-supplemented medium suggest that transport of arginine into the vacuoles requires energy. The observations suggest that mycelia starved for metabolic energy fail to transport their cytosolic arginine into the vacuoles upon transfer to unsupplemented medium. This pool (20% of the total; 20) remains in the cytosol and is catabolized. Catabolism proceeds rapidly and ceases with the depletion of the cytosolic arginine pool. Mycelia transferred to unsupplemented medium in the absence of an inhibitor or uncoupler of the respiratory chain rapidly sequester much of their cytosolic arginine pool in the vacuoles. This results in a lower cytosolic arginine concentration and a decreased rate of urea production. Catabolism does not cease, but continues slowly, supported by the vacuolar arginine pool. The latter also supplies arginine for protein synthesis. Urea is produced at a more rapid rate in the presence of cycloheximide since cytosolic arginine is not being used for protein synthesis. In addition, accumulation of other basic amino acids may displace arginine from the vacuole.

A simple model consistent with the above results is that metabolic energy is required for the accumulation of arginine into the vacuoles but not for its retention: ATP hydrolysis establishes a proton gradient across the vacuolar membrane (the latter has been shown to promote accumulation of arginine in vesicles derived from fragments of vacuolar membranes of *S. cerevisiae* [15]); once inside the vacuole, arginine is retained even in the absence of metabolic energy, possibly by association with a polyanion. The stability of the vacuolar arginine pool in energy-starved mycelia suggests an energy requirement for efflux. It is not clear whether the ATP concentration or the energy charge is responsible for the magnitude of the proton gradient and thus arginine transport: both of these values are low in the presence of inhibitors or an uncoupler of the respiratory chain. Inhibitors of glycolysis which reduce only the ATP pool but not the energy charge (Tables 1 and 3) might distinguish between these possibilities, but they promote mobilization of vacuolar arginine.

The results described here strongly support the direct participation of metabolic energy in the mechanism(s) that concentrates arginine in the vacuoles of *N. crassa* and suggest the existence of an active transport mechanism. In the absence of sufficient metabolic energy, the translocating mechanism(s) (putative transport system) does not function and arginine is retained in the vacuoles. The mechanism of this transport and its relationship (if any) to plasma membrane transport systems is as yet poorly understood. Experiments are in progress to identify and characterize this putative transport mechanism and distinguish the form of energy which is required for the operation of this system. Furthermore, the availability of mutants with altered intracellular distribution of arginine (Drainas and Weiss, unpublished data) may help to characterize this complicated system.

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