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Control of Arginine Metabolism in *Neurospora*: Flux Through the Biosynthetic Pathway

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The flux into the arginine biosynthetic pathway of *Neurospora crassa* was investigated using a mutant strain lacking the ornithine-degrading enzyme ornithine aminotransferase (EC 2.6.1.13). Flux was measured by the increase in the sum of the radioactivity (derived from [^{14}C]glutamic acid) in the ornithine pool, the arginine pool, and arginine incorporated into proteins. Complete cessation of flux occurred immediately upon the addition of arginine to the growth medium. This response occurred prior to expansion of the arginine pool. After short-term exposure to arginine (80 min), flux resumed quickly upon exhaustion of arginine from the medium. This took place despite the presence of an expanded arginine pool. Initiation of flux required approximately 80 min when the mycelia were grown in arginine-supplemented medium for several generations before exhaustion of the exogenous arginine. The arginine pool of such mycelia was similar to that found in mycelia exposed to exogenous arginine for only 80 min. The results are consistent with rapid onset and release of feedback inhibition of arginine biosynthesis in response to brief exposure to exogenous arginine. The insensitivity of flux to the size of the arginine pool is consistent with a role for compartmentation in this regulatory process. The lag in initiation of flux after long-term growth in the presence of exogenous arginine suggests the existence of an additional regulatory mechanism(s). Several possibilities are discussed.

The control of amino acid metabolism requires elegant regulatory mechanisms to insure efficient utilization of cellular resources. In procaryotes, feedback inhibition combined with enzyme induction and repression often appear to be sufficient to provide for the necessary metabolic flexibility. In contrast, eucaryotes often exhibit characteristics which suggest additional levels of regulatory complexity. One example of such complexity is the compartmentation of enzymes and metabolites in subcellular organelles (reviewed in references 8, 12, and 13). Arginine metabolism in *Neurospora crassa* is typical of such complex systems. Its control involves the interrelationship between simple regulatory mechanisms and a variety of complex organizational features (8).

Arginine is synthesized from carbamyl phosphate and glutamic acid (via ornithine). The synthesis of carbamyl phosphate and ornithine and their condensation to yield citrulline take place within the mitochondrion (2, 20). The final two reactions of arginine biosynthesis are cytosolic (20). Arginine can be degraded to glutamic acid by a sequence of three cytosolic enzymes (Fig. 1). During growth in medium free of arginine, over 95% of the arginine is sequestered in the vacuole (11, 17). These metabolic and organizational features are shown in Fig. 1.

The vacuolar compartmentation of arginine results in a low concentration in the cytosol, the site of its degradation. Consequently, degradation occurs only to a small extent during growth in minimal medium (18, 21). The cytosolic concentration of arginine is rapidly elevated when arginine enters the mycelia from the medium (18). The elevated cytosolic concentration initiates catabolism within minutes (21). In addition, production of the catabolic enzymes is quickly accelerated (19, 21). Depletion or removal of arginine from the growth medium rapidly reverses these effects (21). The effectiveness of cytosolic arginine in mediating these metabolic and regulatory changes is easily understood since the sites of action are readily accessible to the cytosol (Fig. 1).

Biosynthesis of carbamyl phosphate and ornithine is separated from the cytosol by the mitochondrial membrane. Carbamyl phosphate synthetase-A (Fig. 1) is controlled solely by repression (7), mediated by cytosolic arginine in the nucleus. In contrast, ornithine synthesis appears to be controlled solely by feedback inhibition (5, 6). This requires the transmission of a regulatory signal (cytosolic arginine) across the mitochondrial membrane to the enzyme acetylglutamate kinase. The effectiveness of this regulatory process in vivo has been examined.

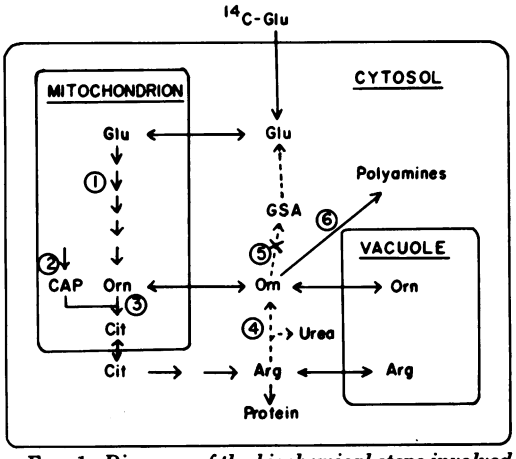

FIG. 1. *Diagram of the biochemical steps involved in arginine metabolism and their subcellular localization in N. crassa. The metabolites are: Glu, glutamate; CAP, carbamyl phosphate; Orn, ornithine; Cit, citrulline; Arg, arginine; GSA, glutamic-γ-semialdehyde. The enzymes indicated are: (1) N-acetylglutamate kinase (EC 2.7.2.8); (2) carbamyl phosphate synthetase-A, arginine specific (EC 2.7.2.5); (3) ornithine transcarbamylase (EC 2.1.3.3); (4) arginase (EC 3.5.3.1); (5) ornithine aminotransferase (EC 2.6.1.13); (6) ornithine decarboxylase (EC 4.1.1.17); (6) ornithine transcarbamylase (EC 2.1.3.3). In these experiments a strain lacking ornithine aminotransferase was used.*

A method of measuring arginine biosynthesis in intact cells has been developed. The technique involves measuring the appearance of radioactivity derived from glutamic acid in the ornithine pool, the arginine pool, and arginine in protein (Fig. 1). Radioactivity in other intermediates of the pathway is neglected since such compounds are only present in small quantities (3). Polyamines (Fig. 1) are included in this category because of the small and constant rate of flux associated with their synthesis (3). Loss of radioactivity from the pathway as a result of degradation (Fig. 1) is avoided by using a mutant strain (*ota*) lacking ornithine aminotransferase (Fig. 1). The carbon atom lost as urea in the degradation of arginine is not labeled by glutamic acid and is therefore neglected. The sum of radioactivity in the ornithine pool, the arginine pool, and arginine in protein represents the net utilization of glutamic acid in the arginine biosynthetic pathway. This is referred to as the pathway label. The rate of accumulation of pathway label is indicative of the flux into the pathway. This technique provided a means of examining the regulatory response of arginine biosynthesis to environmental changes.

The results presented here substantiate the importance and rapidity of feedback inhibition, reaffirm a role for compartmentation in the control of arginine biosynthesis, and suggest the possible existence of additional regulatory system(s) in the control of ornithine synthesis.

MATERIALS AND METHODS

Strains, media, and chemicals. The strain used was a mutant obtained from R. H. Davis which lacked ornithine aminotransferase (*ota*, Fig. 1). It was grown in Vogel minimal medium N with 1.5% sucrose (16) and was supplemented with 2 mM arginine when indicated. All chemicals were reagent grade or equivalent. L-[*U*-^{14}C]glutamic acid was obtained from New England Nuclear Corp. and was purified by ion-exchange chromatography before use. Analytical-grade AG 50W-X8 ion-exchange resin was purchased from Bio-Rad Laboratories. Triton X-100 was obtained from Research Products International. Scintillation fluid contained 0.5% (wt/vol) 2,5-diphenyloxazole in 2:1 toluene–Triton X-100 (vol/vol).

Growth and sampling. Conidia (approximately 5 × 10^6 per ml) were germinated overnight at 16°C as previously described (1). Experiments were initiated 2 to 4 h after raising the temperature to 30°C. Germinated conidia (mycelia) exhibited exponential growth for an additional 6 h. Transfer of mycelia from one medium to another was accomplished by collecting on Whatman no. 540 paper, washing with water, and suspending in prewarmed fresh medium. Samples for analysis of radioactivity in proteins were obtained by mixing 1 ml of culture with 1 ml of 10% trichloroacetic acid. Precipitation was allowed to proceed overnight. The precipitate was collected on Whatman no. 540 filter paper and washed with 5% trichloroacetic acid. The trichloroacetic acid was extracted by washing several times with a 1:1 mixture of absolute ethanol and ethyl ether. The sample was dried by washing with ethyl ether. Radioactivity in protein was determined after emersion of the dried filter paper in Triton-free toluene scintillation fluid. Intracellular metabolites were extracted as follows. Cells were collected by filtration using Whatman no. 540 filter paper, washed, and extracted in 3 ml of water at 100°C for 15 min. Precipitated protein was collected by centrifugation and was used to analyze for arginine in protein. Arginine and ornithine in the supernatant were purified and assayed for radioactivity and amount as described below. In some experiments the culture filtrates were assayed for [^{14}C]glutamic acid, nonlabeled arginine, or both.

Fractionation and chromatography. Arginine in protein was solubilized by hydrolysis in 6 N HCl for 24 h at 110°C. Ornithine and arginine were purified using ion-exchange chromatography as previously described (1). Control experiments confirmed the radioisotopic purity of the purified ornithine and arginine. Glutamic acid and arginine in the medium were separated on AG 50W-X8 (H^+ form, 0.7 by 15 cm). Amino acids were eluted with successive additions of 10 ml of 0.1 N HCl, 20 ml of 1.5 N HCl, and 10 ml of 6 N HCl. Glutamic acid emerged in the first 10 ml of 1.5 N HCl, and arginine emerged in the 10 ml of 6 N HCl. Fractions were evaporated, redissolved in water, and counted in toluene-Triton scintillation fluid. Arginine was assayed colorimetrically by the method of Van Pilsum et al. (15).

Calculations. Radioactivity in the ornithine pool, in the arginine pool, and in arginine in protein was determined as described above. The best-fit curves derived from these individually measured values were used to calculate the total radioactivity in the arginine biosynthetic pathway (pathway label).

RESULTS

Entry of glutamic acid into the arginine biosynthetic pathway. The increase in radioactivity in metabolites of the arginine biosynthetic pathway after exposure of *N. crassa* to [^{14}C]glutamic acid is shown in Fig. 2. Radioactivity accumulated rapidly in the ornithine and arginine pools after a short lag period (Fig. 2A and B). The rates of accumulation declined at later times (Fig. 2A and B). A similar curve was observed for arginine in protein except that the rate of accumulation increased continually throughout the experimental period (Fig. 2C). Pathway label (Fig. 2D) increased approximately linearly after an initial lag period.

The effect of adding arginine (final concentration of 2 mM) to the growth medium on the accumulation of radioactivity in the arginine biosynthetic pathway was immediate and substantial. Radioactivity in the ornithine pool declined rapidly (Fig. 2A). Radioactivity continued to increase in the arginine pool and in arginine in protein, but at a substantially reduced rate (Fig. 2B and C). The pathway label declined slowly after the addition of arginine (Fig. 2D). This slow loss of radioactivity could be accounted for by the conversion of a small amount of ornithine to polyamines.

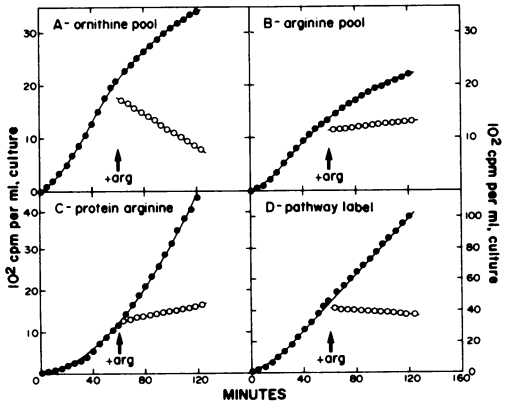

FIG. 2. *Accumulation of radioactivity in the arginine biosynthetic pathway after exposure of N. crassa to [^{14}C]glutamic acid. An exponential culture of N. crassa was supplemented with [^{14}C]glutamic acid (1 mM, 0.16 mCi/mmol) at time zero. After 60 min the culture was split; one half received no additions (●), and the other (○) was supplemented with unlabeled arginine (2 mM). Radioactivity in the arginine biosynthetic pathway was determined as described in the text. (A) Ornithine pool; (B) arginine pool; (C) arginine in protein; (D) pathway label (sum of A + B + C).*

Inhibition of [^{14}C]glutamic acid uptake and utilization. The addition of arginine to the growth medium inhibited the uptake of [^{14}C]-glutamic acid and the accumulation of radioactivity in trichloroacetic acid-insoluble material (total protein, Fig. 3). To test whether this inhibition could account for the cessation of flux into the arginine biosynthetic pathway, unlabeled glutamic acid was added to the growth medium in place of arginine (Fig. 4). A 55% decline was observed in the rate of accumulation of radioactivity in total protein. Flux into the arginine biosynthetic pathway declined to 48% of that observed in the absence of unlabeled glutamic acid. In contrast, the addition of arginine resulted in a 59% decline in incorporation into total protein, but complete inhibition of flux into arginine biosynthesis (Fig. 3).

Initiation of flux upon exhaustion of medium arginine. The effect of short- and long-term exposure to arginine on the reinitiation of flux from glutamic acid (upon exhaustion of exogenous arginine) was examined. In the first experiment, a small amount of arginine was added to the culture along with the radioactive glutamic acid. Uptake of the exogenous arginine was complete at approximately 78 min (Fig. 5A, arrow). Despite continuous [^{14}C]glutamic acid uptake and incorporation into total protein throughout this period, very little radioactivity was observed in the arginine pathway (Fig. 5B). Upon exhaustion of the exogenous supply of arginine, radioactivity in the pathway increased rapidly for the remaining 100 min of the experiment (Fig. 5A). The arginine pool expanded from 150 nmol/mg of protein at the time of arginine addition to 800 nmol/mg of protein at

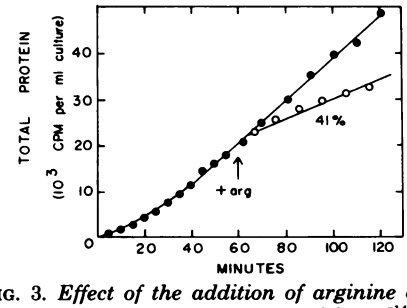

FIG. 3. *Effect of the addition of arginine on the incorporation of radioactivity derived from [^{14}C]glutamic acid into trichloroacetic acid-insoluble material (total protein). These results are from the same experiment shown in Fig. 2. Radioactivity in protein was determined as described in the text. Figure represents percent of rate of control culture.*

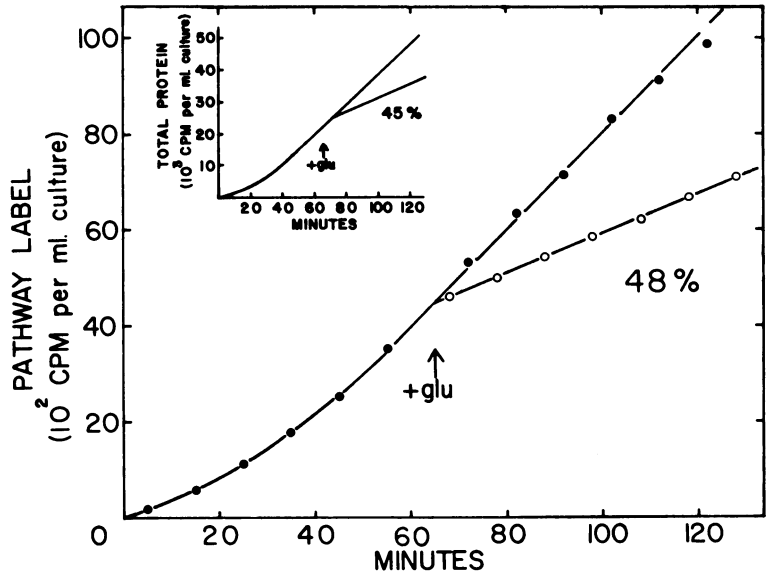

FIG. 4. *Effect of dilution of exogenous [^{14}C]glutamic acid on the appearance of radioactivity in the arginine biosynthetic pathway and total protein. Addition of radioactive glutamic acid was initiated as in Fig. 2. At 65 min the culture was split. One half received no additions (●), and the other (○) was supplemented with additional unlabeled glutamic acid to a final concentration of 2.75 mM. Pathway label and radioactivity in protein were determined as described in the text. Inset: Incorporation into trichloroacetic acid-insoluble material (total protein). Figures represent percent of rate of incorporation in control culture.*

80 min and declined to 400 nmol/mg of protein at the end of the experimental period.

Figure 6 shows the results of a similar experiment in which conidia were germinated and grown in arginine-supplemented medium. At time zero, the mycelia were collected, washed, and transferred to unsupplemented medium containing [^{14}C]glutamic acid. No radioactivity entered the arginine biosynthetic pathway until approximately 80 min after the removal of arginine from the medium (Fig. 6D). Shortly thereafter, radioactivity began to enter the pathway at a rapidly increasing rate (Fig. 6D). Radioactive glutamic acid was incorporated into trichloroacetic acid-precipitable material throughout the experimental period (Fig. 6D). Radioactivity appeared in the ornithine pool, in the arginine pool, and in arginine in protein essentially simultaneously (Fig. 6A-C). Throughout the 180 min of this experiment the arginine pool was continuously declining from the expanded state (720 nmol/mg of protein) produced by growth in arginine-supplemented medium (Fig. 6B). Flux only began at the later stages of this decline, when the arginine pool was similar to that of mycelia grown in unsupplemented medium (150 nmol/mg of protein, Fig. 6B). No flux was detected after 3 h if inhibitors of RNA or protein synthesis were added to the cultures at the time of arginine removal (Fig. 7).

DISCUSSION

The radioactive labeling technique used here provided a measure of the amount of arginine biosynthesis (from glutamic acid) in intact cells under a variety of experimental conditions. The results indicate that the availability of exogenous arginine is sensed quickly; the accumulation of radioactivity in the pathway ceased abruptly upon supplementation of the growth medium with arginine (Fig. 2D). This feedback control was exerted long before complete expansion of the arginine pool (60 min; 19).

Radioactivity was rapidly lost from the ornithine pool (Fig. 2A) but continued to increase (at a reduced rate) in the arginine pool and in arginine in protein (Fig. 2B and C) upon exposure of mycelia to exogenous arginine. This is consistent with continued conversion of ornithine to arginine. This continued metabolism of ornithine in the presence of exogenous arginine has previously been shown to persist until carbamyl phosphate synthetase-A has been completely repressed (3).

Biosynthetic flux recommenced quickly upon exhaustion of a small amount of exogenous arginine (Fig. 5). This occurred despite an arginine pool considerably larger than that observed in mycelia grown in minimal medium or in mycelia exposed to exogenous arginine and no longer

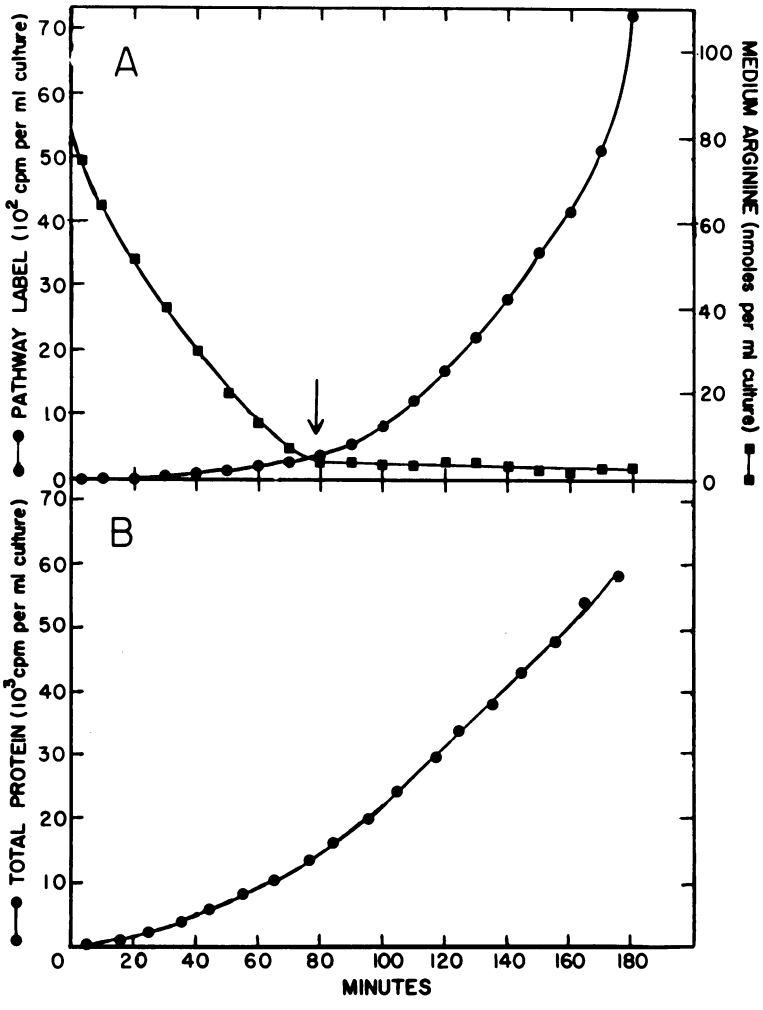

FIG. 5. *Response of arginine biosynthesis to short-term exposure to exogenous arginine. An exponential culture was supplemented with [^{14}C]glutamic acid (1 mM, 0.17 mCi/mmol) and a limited supply of arginine (0.8 mM) at time zero. At 10-min intervals, samples were withdrawn and analyzed for medium arginine, pathway label, and trichloroacetic acid-insoluble material (total protein) as described in the text. The arrow indicates the time of exhaustion of arginine from the medium. (A) Medium arginine and pathway label; (B) total protein.*

exhibiting biosynthetic flux (Fig. 2D). The onset and cessation of ornithine and arginine degradation have been shown to respond similarly to the addition and exhaustion of exogenous arginine (21). The synthesis of enzymes of arginine degradation also exhibits similar kinetics in response to arginine availability (18, 19). A summary of these regulatory features is shown in Fig. 8. It has been suggested that these responses are a consequence of variations in the cytosolic arginine concentration, which is only indirectly related to the size of the arginine pool.

Vacuolar compartmentation and the initial entry of exogenous amino acids directly into the cytosol allow for rapid expansion and contraction of cytosolic arginine concentrations in response to alterations in the availabliity of exogenous arginine. These rapid variations in the cytosolic arginine concentration appear to effect the efficient controls on the operation and production of the arginine catabolic enzymes described above. The sites of action of these controls are readily accessible to cytosolic arginine (Fig. 8).

Feedback control of arginine biosynthesis in *N. crassa* involves an organizational problem not encountered in controlling arginine degradation. The early steps of arginine biosynthesis

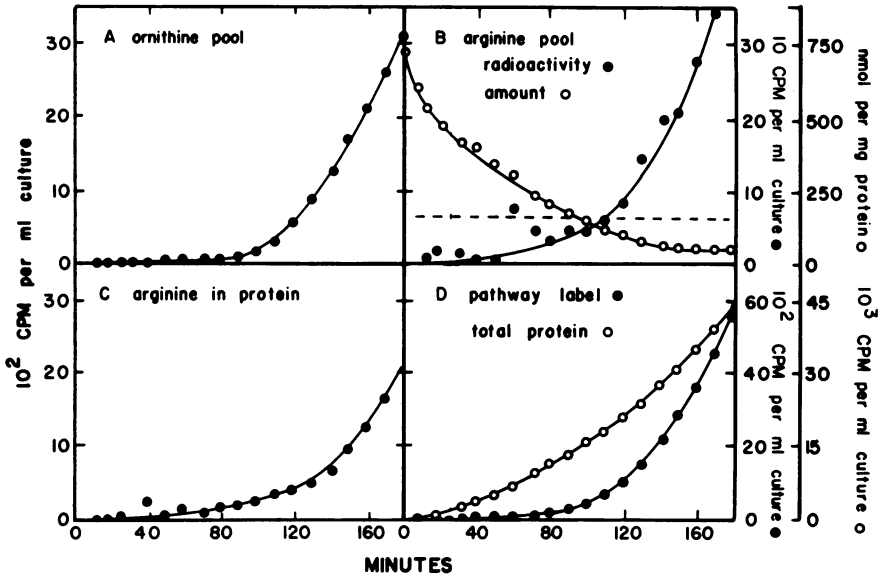

FIG. 6. *Response of arginine biosynthesis to long-term exposure to exogenous arginine. Mycelia were grown for four generations in arginine-supplemented (1 mg/ml) medium. At time zero, the mycelia were collected on filter paper, washed, and suspended in fresh medium containing 1 mM [^{14}C]glutamic acid (0.20 mCi/mmol). At 10-min intervals, samples were withdrawn and analyzed for intracellular arginine, pathway label, and radioactivity in trichloroacetic acid-insoluble material (total protein) as described in the text. The dotted line indicates the size of the arginine pool in mycelia grown in minimal medium.*

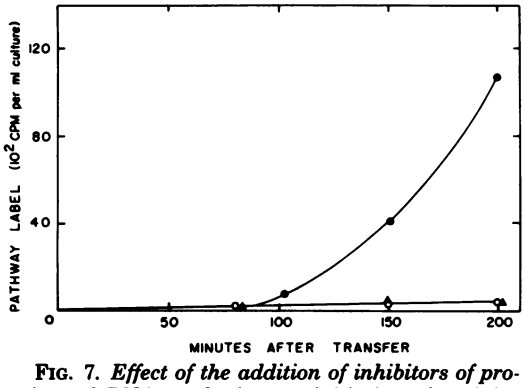

FIG. 7. *Effect of the addition of inhibitors of protein and RNA synthesis on reinitiation of arginine biosynthetic flux after long-term exposure to exogenous arginine. The experiment was similar to the one in Fig. 6 except that after resuspension in medium containing [^{14}C]glutamic acid the culture was divided into thirds. One part received no additions (●), and the others were supplemented with 10 μg of cycloheximide per ml (○) or 6 methylpurine to 1 mM (△). Samples were withdrawn and analyzed for pathway label as described in the text.*

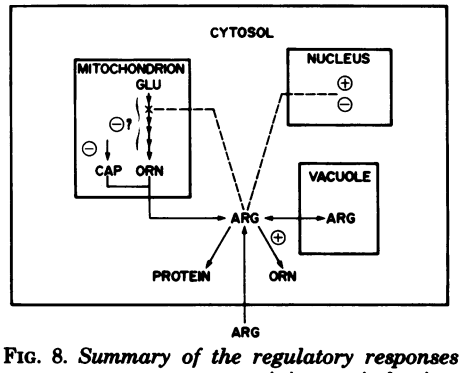

FIG. 8. *Summary of the regulatory responses of Neurospora to exogenous arginine: +, induction; −, repression; ×, feedback inhibition. The solid lines indicate chemical reactions, and the dashed lines represent the transmission of regulatory signals.*

are carried out by mitochondrial enzymes (5, 20). Acetylglutamate kinase, the feedback-inhibitable enzyme, was originally reported to be uniquely cytosolic (5). Recent experiments have identified the mitochondrion as the subcellular location of this arginine biosynthetic enzyme (E. Wolf and R. L. Weiss, manuscript in preparation). Consequently, for biosynthesis to be regulated by the cytosolic arginine concentration, some form of transmembrane communication must exist to convey the signal from the cytosol to the feedback-sensitive enzyme within the mitochondrion (Fig. 8).

In contrast to the behavior observed upon exhaustion of a small quantity of exogenous arginine, radioactivity only entered the arginine

biosynthetic pathway after an 80-min lag when conidia were germinated and the mycelia were grown in arginine-supplemented medium (Fig. 6). When inhibition of flux was ultimately relieved, radioactivity appeared quickly in all the intermediates and products (Fig. 6). This suggests that the rate-limiting step is prior to ornithine synthesis and that any regulatory restraints subsequent to ornithine (e.g., repression of carbamyl phosphate synthetase-A) were relieved during the 80-min lag period. The delayed response occurred despite the presence of an arginine pool which was similar in size to that following short-term exposure to exogenous arginine (720 versus 800 nmol/mg of protein) and which declined more rapidly (720 to 150 versus 800 to 400 nmol/mg of protein in 100 min) after the removal of exogenous arginine. This latter difference is partially the result of an induced level of arginase in mycelia grown for several generations in arginine-supplemented medium.

The following possibilities might account for the observed lag in the initiation of flux after long-term exposure to exogenous arginine: (i) the cytosolic arginine concentration might persist at an elevated level due to an alteration in the ability of the vacuole to sequester arginine; (ii) repression of an ornithine biosynthetic enzyme (no such repression has been reported [6]); (iii) a requirement for carbamyl phosphate (carbamyl phosphate synthetase-A is repressed under these conditions) as a positive effector of an early biosynthetic enzyme; or (iv) the persistence of an expanded mitochondrial arginine pool within these mycelia. Alternatives (ii) and (iii) would be consistent with the observation that initiation of biosynthetic flux required both protein and RNA synthesis. However, the effects of the inhibitors may be the result of the large arginine pool which persists in the absence of protein synthesis rather than a result of a requirement for macromolecular synthesis. Experiments are being performed to distinguish between these alternatives.

Although the mechanisms involved in the control of arginine metabolism in *N. crassa* are still not fully understood, it is clear that the organism responds efficiently to fluctuations in the availability of exogenous arginine. Biosynthesis ceases and catabolism is initiated when arginine is available in the medium. Appropriate responses occur upon depletion of the exogenous arginine. The responses are rapid and do not always require macromolecular synthesis. In this sense, *N. crassa* appears to be more efficient than procaryotic microorganisms. The cost of this efficiency, however, is an energetically expensive compartmentation of arginine within the vacuole. The necessity for such large stores of arginine (and ornithine) is not completely clear, but their use as a nitrogen reserve appears to be one possibility (10). Additional functions for this compartmentation phenomenon are being sought.

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