JOURNAL OF BACTERIOLOGY, Oct. 1975, p. 232-242

Vol. 124, No. 1
Printed in U.S.A.

Neurospora crassa Conidial Germination: Role of Endogenous Amino Acid Pools

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Received for publication 9 June 1975

The levels of the endogenous amino acid pools in conidia, germinating conidia, and mycelia of wild-type *Neurospora crassa* were measured. Three different chromatographic procedures employing the amino acid analyzer were used to identify and quantitatively measure 28 different ninhydrin-positive compounds. All of the common amino acids were detected in conidial extracts except proline, methionine, and cystine. The levels of these three amino acid pools were also very low in mycelia. During the first hour of germination in minimal medium, the levels of most of the free amino acid pools decreased. The pool of glutamic acid, the predominant free amino acid in conidia, decreased 70% during the first hour. Very little glutamic acid or any other amino acid was excreted into the medium. During the first 20 min of germination, the decrease in the glutamic acid pool was nearly equivalent to the increase in the aspartic acid pool. The aspartic acid and γ-aminobutyric acid pools were the only amino acid pools that increased to maximum levels within the first 20 min of germination and then decreased. It is proposed that an important metabolic event that occurs during the early stages of conidial germination is the production of reduced pyridine nucleotides. The degradation of the large glutamic acid pool existing in the conidia (2.5% of the conidial dry weight) could produce these reduced coenzymes.

Many different compounds are efficient initiators of the germination of bacterial or fungal spores (9–11, 24). These include metabolizable compounds, i.e., amino acids, glucose, inosine, etc., and non-metabolizable compounds, i.e., inorganic salts, dipicolinic acid, furfural, etc. In addition, many of these same spores can be induced to germinate by a brief heat shock. After a heat shock, ascospores from *Neurospora crassa* germinate in deionized water (24). *Bacillus megaterium* endospores can be induced to germinate in salt solutions by a heat shock (20). Other spores, such as *N. crassa* conidia, initiate some of the metabolic events associated with germination as soon as they contact water (14; this paper). These results suggest that many spores contain endogenous storage compounds that are used for the initiation of germination. It has been proposed that the primary effect of many of the organic and inorganic initiators may be to activate the metabolism of these storage compounds (6). A heat shock may have the same effect.

High levels of readily metabolizable compounds such as glutamic acid (8, 16, 22), proline (19, 21), trehalose (2, 12), and polyols (1, 17) have been found in dormant spores. The function that these compounds might play in dormancy and spore germination is not clearly understood. This paper reports detailed studies on the changes in the amino acid pools that occur during *N. crassa* conidial germination. A role is proposed for the degradation of the large glutamic acid pool. Other studies on initial biochemical events that occur during conidial germination have been reported (5, 22).

MATERIALS AND METHODS

N. crassa strain. The wild-type strain of *N. crassa*, RL3-8A (FGSC no. 2218), was used in these studies and can be obtained from the Fungal Genetics Stock Center, Humboldt State College, Arcata, Calif.

Conidial preparation. Conidia were obtained from agar slant cultures containing 6 ml of Vogel minimal medium (25) with 2% glucose and 2% agar in test tubes (18 by 150 mm). The cultures were incubated at 30 C for 2 days and then at 22 C in constant light for an additional 5 to 8 days. Forty slants yielded about 160 mg (dry weight) of conidia.

Conidia were harvested with a sterile loop and either immediately extracted in ethanol (dry-harvested conidia) or suspended in 100 ml of cold (5 C) sterile water in a flask containing a magnetic stirring bar. In the latter case, the conidial suspension was vigorously agitated on a magnetic stirrer, and the

contaminating fragments of mycelia were removed by filtering through four layers of cheesecloth. The conidial preparation was then centrifuged and washed once with cold, sterile water. The washed conidia were either extracted in ethanol (wet-harvested conidia) or used immediately as inoculum. The total time required for preparing the conidial inoculum was about 30 min.

In the experiments with cycloheximide, the inhibitor (10 μg/ml) was present at all times during the preparation of the conidial inoculum.

Germination conditions. The standard germination medium consisted of 250 ml of Vogel minimal medium (25) with 2% glucose in 1-liter Erlenmeyer flasks. A nitrate minimal medium, which was used for determining the amount of free amino acids excreted into the medium during germination, was the same as the standard germination medium except that an equimolar amount of nitrate was substituted for ammonia, and the salts and sugar concentrations were one-tenth those of the standard medium. When cycloheximide was used, it was included at 10 μg/ml in the standard germination medium. This concentration of cycloheximide inhibited germ tube formation completely and inhibited phenylalamine incorporation into hot trichloroacetic acid-insoluble material by more than 97% (S. E. Hitchcock, and V. W. Cochrane, Neurospora Newslett. **15**:18–19, 1969). In all cases, germination was initiated by adding freshly prepared, wet-harvested conidia to each flask. The final concentration of conidia was about 5×10^6 spores/ml. All of these cultures were incubated at 22 C on a rotatory shaker at 125 rpm.

Harvesting and extraction. Conidia and germinating conidia were harvested by filtration on membrane filters (EHWPO4700, Millipore Corp.). Mycelia were harvested on Whatman no. 1 filter paper. The cells were washed quickly with water and then rapidly plunged into 20 ml of boiling 80% ethanol. The total time for harvesting was less than 1 min. Extracts of dry-harvested conidia were prepared by removing conidia from the slant cultures and plunging them directly into 20 ml of boiling 80% ethanol. The samples were boiled for 10 min, cooled, centrifuged, and filtered through EHWPO4700 membrane filters (Millipore Corp.). The alcohol-insoluble material was dried overnight at 100 C and weighed to determine the residual dry weight (RDW). The supernatant, containing the free amino acids, was flash evaporated to dryness. The dried samples were suspended in 3 ml of sodium citrate (pH 2.2) buffer and frozen at −15 C.

Free amino acids released into the medium during germination. After incubating the conidia for the desired period of time in either deionized water or in nitrate minimal medium, the cells were removed by filtration. Extracts containing the intracellular free amino acids were prepared as described above. The germination medium (water or nitrate minimal medium) was concentrated to dryness with a rotatory flash evaporator. The concentrated medium was dissolved in pH 2.2 sodium citrate buffer. The samples were clarified by centrifugation and stored frozen until analyzed for free amino acid content.

Amino acid analysis. The amino acid content was measured on a Beckman 120 C amino acid analyzer equipped with an Infotronics integrator. A summary of the three different procedures that were used is given in Table 1, and a discussion of the separation that was obtained is given in Results.

Amino acid standards and other chemicals. The amino acid calibration mixtures and the buffers for the amino acid analyzer were obtained from Pierce Chemical Co. Individual amino acids and other compounds that were used as standards were obtained from Sigma Chemical Co.

RESULTS

Separation of the free amino acids in extracts of N. crassa. Three different chromatographic procedures employing the amino acid analyzer were used to identify and quantitatively measure the levels of free amino acids in extracts of *N. crassa*. Procedure A (Table 1) was the standard two-column procedure designed to separate amino acids from hydrolyzed proteins (15). This procedure did not separate aspartic acid from glutathione, serine from the combined peak of glutamine plus asparagine, methionine from cystathionine, or ornithine from lysine. The second chromatographic procedure, B (Table 1), was adapted from the conditions for separating amino acids from physiological fluids (*Beckman Procedures Manual*, Beckman Instruments, Inc.). The only amino acids that were not separated by this procedure were cystine from valine and glutamine from asparagine. The third chromatographic procedure, C, was used to measure the glutamine and asparagine levels in dry-harvested conidia.

Unless otherwise indicated, the levels of the free amino acids presented in this paper were obtained using procedure B. All of the samples were also analyzed using procedure A to measure the level of free cystine. For those amino acids that were cleanly separated, identical levels were obtained by all three chromatographic procedures. The use of three different chromatographic procedures greatly enhanced the reliability of the identification of individual amino acids.

Typical chromatographs obtained with procedure B of extracts from dry-harvested conidia are given in Fig. 1. Thirty-six ninhydrin-positive compounds were detected. All of the amino acids and some of the other ninhydrin-positive compounds were identified by their cochromatography with known standards. Oxidized glutathione was identified as a broad peak

TABLE 1. *Summary of the three different chromatographic procedures used to separate amino acids and other ninhydrin-positive compounds*[a]

	Procedure	Column size (cm)	Beckman resin type	Height of resin column (cm)	Column flow rate (ml/h)	First buffer (pH)	Second buffer (pH)	Buffer change time (min)	First temp (C)	Second temp (C)	Temp change time (min)
A	(Sodium citrate) Basics	14 by 0.9	PA35	5.5	68	5.28 (0.38 N, Na^+)	NA[b]	NA	55	NA	NA
	Acidics, neutrals	69 by 0.9	UR30	55	68	3.25 (0.2 N, Na^+)	4.25 (0.2 N, Na^+)	84	55	NA	NA
B	(Sodium citrate) Basics	29 by 0.9	PA35	16	68	4.25 (0.2 N, Na^+)	5.28 (0.38 N, Na^+)	170	32.5	65	170
	Acidics, neutrals	69 by 0.9	UR30	55	68	3.25 (0.2 N, Na^+)	4.25 (0.2 N, Na^+)	170	32.5	65	90
C	(Lithium citrate) Acidics, neutrals	69 by 0.9	UR30	55	68	2.80 (0.3 N, Li^+)	4.16 (0.3 N, Li^+)	150	39	NA	NA

[a] A Beckman 120C amino acid analyzer was used.
[b] NA, Not applicable.

that appeared between glutamic acid and the combined peak of glutamine and asparagine (Fig. 1). An unknown compound also appeared in this region, but as a sharp peak. Even though more than 95% of the glutathione in either freshly harvested conidia or in mycelia was in its reduced form (5), very little reduce glutathione was usually detected in the extracts. Apparently, the reduced glutathione was oxidized to the disulfide (GSSG) during the preparation of the samples. The elution positions of some of the compounds that were used as standards but were not found in *Neurospora* extracts are also shown in Fig. 1.

Free amino acid pools of dormant conidia. The average levels of individual free amino acids and other ninhydrin-positive compounds from extracts of dry-harvested conidia are given in Table 2. All of the common amino acids except proline, cystine, and methionine were detected in dormant conidia. Previous estimates of the endogenous proline (28), cystine, and methionine (27) pools also indicated that these pools were very small. The predominant free amino acids were glutamic acid, alanine, and glutamine. Together these three pools accounted for nearly 70% of the total free amino acid pool of conidia (Table 2).

The levels of the individual amino acid pools from different preparations of conidia were fairly reproducible, with the exception of the glutamine pool (Table 2). In different conidial preparations the glutamine pool varied from 18 to more than 104 μmol/g of RDW. However, the sum of the glutamine plus glutamic acid pools was very reproducible, 230 ± 4.2 μmol/g of RDW. The large variation in the glutamine pool appears to be the result of a change in the relative proportions of free glutamine and glutamic acid in the different batches of conidia.

The amino acid pools of conidia reported here are reasonably similar to those reported by DeBusk and DeBusk (Neurospora Newslett. 11:3, 1967). The differences that are seen are probably due to the use of different harvesting and chromatographic procedures.

Conidial germination. Germ tubes, the first visible signs of germination, began to appear about 3 h after inoculation (Fig. 2). Germ tubes were formed asynchronously with the larger conidia producing germ tubes first. By 5 h, more than 80% of the conidia had germinated. The

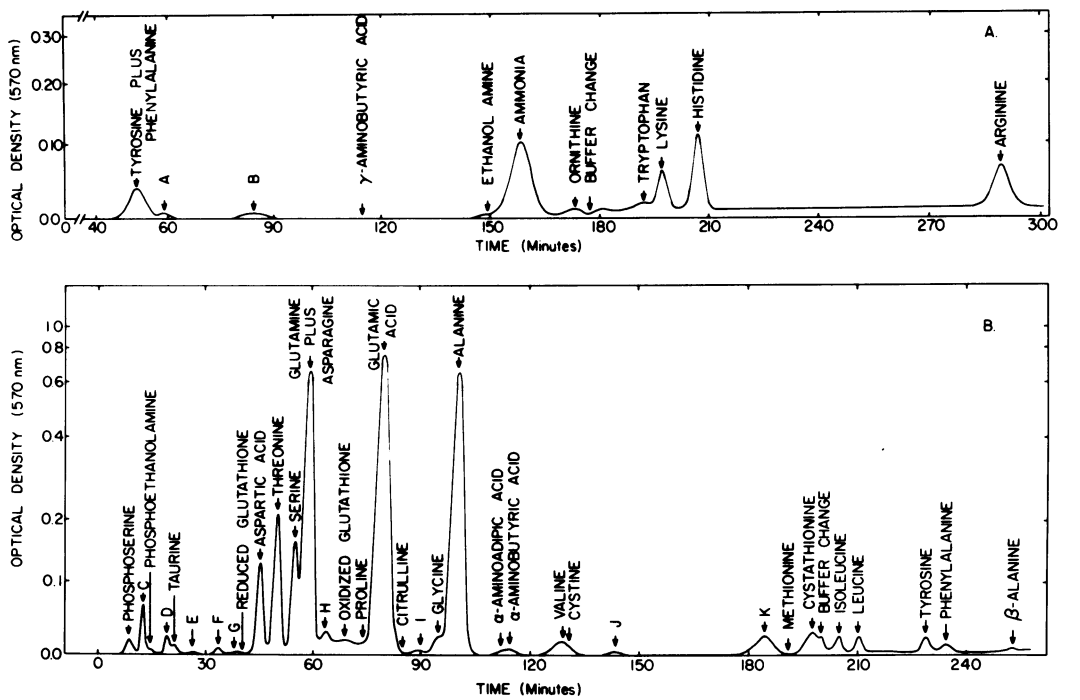

FIG. 1. *Elution profiles of ninhydrin-positive compounds in extracts of dry-harvested conidia. (A) Basic amino acids. The sample applied to the column was equivalent to 4.8 mg of RDW. (B) Acidic and neutral amino acids. The sample applied to the column was equivalent to 1.6 mg of RDW. The chromatographic conditions were as described for procedure B in Table 1.*

TABLE 2. *Levels of the free amino acids and other ninhydrin-positive compounds from extracts of dry-harvested conidia*[a]

Compound	Concn[b] (μmol/g of RDW)
γ-Aminobutyrate	<0.1
Ethanolamine	<0.6
Ornithine	1.1 ± 0.4
Tryptophan	1.7 ± 0.2
Lysine	4.3 ± 0.3
Histidine	8.5 ± 0.8
Arginine	8.0 ± 1.1
Aspartate	20.3 ± 1.1
Threonine	24.6 ± 2.1
Serine	20.2 ± 2.0
Asparagine	33.8 ± 6.0
Glutamine	64.8 ± 20.5
Half-glutathione (oxidized)	19.7 ± 2.7
Proline	<0.2
Glutamate	177.7 ± 10.8
Citrulline	<0.2
Glycine	5.4 ± 1.2
Alanine	92.0 ± 11.8
α-Aminobutyrate	1.8 ± 0.3
Half-cystine	<0.2
Valine	5.7 ± 0.7
Methionine	<0.2
Cystathionine	3.9 ± 1.7
Isoleucine	2.5 ± 0.3
Leucine	2.4 ± 0.3
Tyrosine	3.2 ± 0.4
Phenylalanine	1.6 ± 0.1
β-Alanine	0.7 ± 0.2

[a] Extracts were prepared as described in Materials and Methods.

[b] The levels given are the average concentrations from six different preparations of conidia except for the levels of asparagine and glutamine, which were obtained from three preparations. The level of cystine was measured using procedure A (Table 1). Asparagine and glutamine were measured using procedure C, and the remaining compounds were measured using procedure B (Table 1). The range is in standard error.

residual dry weight increased exponentially throughout germination, with a doubling time of 3.2 h (Fig. 2). As indicated by the continuous increase in the residual dry weight from time 0, a considerable amount of metabolic activity must have occurred prior to the first appearance of germ tubes. By 24 h, the cultures had reached early stationary phase.

Changes in free amino acid pools during conidial germination. The levels of the free amino acid pools were measured at various times during conidial germination and vegetative growth in liquid shake cultures. The results obtained for one such experiment are given in Table 3. Duplicate experiments gave essentially the same pattern of changes in the free amino acid pools, although the initial pool levels varied somewhat (Table 2).

A comparison of the free amino acid pools obtained from the same batch of conidia harvested dry ($T_{0\text{-dry}}$) or in cold water ($T_{0\text{-wet}}$) is given in Table 3. Wet-harvested conidia were extracted about 30 min after the conidia had been suspended in cold water. Most of the free amino pools did not change significantly during the preparation of the conidial inoculum. The only significant difference in these two extracts was the appearance of γ-aminobutyric acid, which could not be detected in dry-harvested conidia.

During the first hour of germination in minimal medium, most of the amino acid pools decreased (Table 3). Only 30% of the glutamic acid pool, the predominant amino acid pool in conidia, remained after 1 h. A decrease was also observed during these early stages of germination in the levels of the unidentified ninhydrin-positive compounds that were detected in conidia (Fig. 1).

The only amino acid pools that increased prior to the appearance of germ tubes were the γ-aminobutyric acid, ornithine, aspartic acid, and glycine pools (Table 3). The ornithine and glycine pools increased continuously during germination. Only the γ-aminobutyric acid and aspartic acid pools increased temporarily, prior to the first appearance of germ tubes. The highest level of γ-aminobutyric acid (2.7 μmol/g of RDW) was in the $T_{0\text{-wet}}$ sample (Table 3). By

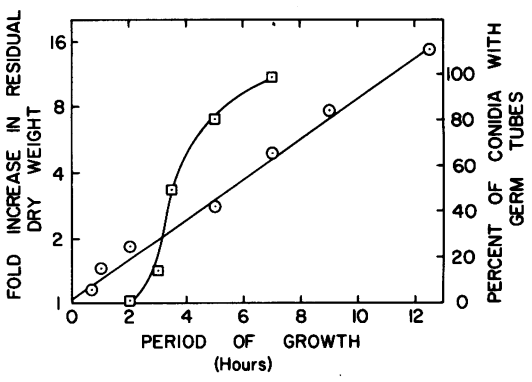

FIG. 2. *The rate of germ tube formation and the fold increase in residual dry weight. Conidia were considered germinated when their germ tubes were one-half the diameter of the conidium. The RDW includes all of the material that is insoluble in boiling 80% ethanol. Symbols:* ○, *residual dry weight;* □, *percentage of the conidia with germ tubes.*

TABLE 3. *Free amino acid pools of germinating conidia*[a]

Amino acid	Free amino acid concn[b] (μmol/g of RDW)							
	$T_{0\text{-dry}}$[c]	$T_{0\text{-wet}}$	$T_{0.3}$	T_1	T_3	T_6	T_{12}	T_{24}
γ-Aminobutyrate	ND[d]	2.7	2.1	0.5	<0.2	0.4	1.5	—[e]
Ornithine	0.9	1.5	2.3	3.2	5.6	17.4	20.8	—
Tryptophan	2.2	0.7	ND	0.4	ND	ND	1.3	—
Lysine	5.2	5.4	4.3	2.9	2.8	8.1	7.7	—
Histidine	10.9	9.9	11.9	8.2	3.2	8.3	6.7	1.9
Arginine	10.6	13.1	10.5	5.6	25.9	41.3	39.1	46.9
Aspartate	18.2	17.1	89.6	34.1	20.0	16.0	3.9	5.0
Threonine	23.5	20.7	20.5	6.2	7.5	9.5	7.2	2.4
Serine	21.2	24.7	17.1	13.1	14.8	16.0	16.5	—
Glutamine plus asparagine	32.8	30.0	25.5	4.7	23.0	32.0	5.4	—
Proline	ND	ND	ND	ND	ND	ND	ND	ND
Glutamate	207.1	214.2	141.7	56.4	126.0	124.9	97.4	60.0
Citrulline	ND	ND	ND	2.1	3.9	7.0	6.7	11.0
Glycine	3.1	2.4	6.1	6.8	11.6	10.7	10.8	5.6
Alanine	86.7	91.1	79.3	20.0	52.7	69.8	298.3	193.8
Half-cystine	ND	ND	ND	ND	ND	ND	ND	ND
Valine	3.1	3.7	4.4	1.6	4.9	5.6	17.1	6.7
Methionine	ND	ND	0.8	ND	ND	ND	ND	ND
Cystathionine	0.7	1.1	1.6	1.6	0.8	1.4	0.2	—
Isoleucine	2.4	2.5	2.7	0.3	0.8	1.4	1.8	1.2
Leucine	2.3	2.2	2.1	0.9	1.0	1.1	1.5	0.7
Tyrosine	4.2	3.4	3.1	0.1	0.4	0.6	0.5	2.9
Phenylalanine	1.4	1.2	0.9	0.1	0.4	0.7	0.5	2.4

[a] Conidia were germinated in minimal medium at 22 C in shake cultures. The rate of germ tube formation and the increase in RDW (alcohol-insoluble material) for this experiment are given in Fig. 2.

[b] All of the amino acids were quantitatively measured using procedure B except cystine, which was measured using procedure A (Table 1).

[c] The time of growth, T, is in hours.

[d] ND, Not detectable.

[e] —, Indicates that the concentration of this amino acid in this sample was not determined.

1 h, this pool had decreased to less than one-fifth of this level.

During the first 20 min of germination, the aspartic acid pool increased from 18 to about 90 μmol/g of RDW (Table 3; Fig. 3). The free aspartic acid pool then decreased, reaching levels that were comparable to those in dry conidia by the second hour (Fig. 3). During the first 20 min the increase in the aspartic acid pool was nearly equivalent to the decrease in the glutamic acid pool (Table 3). This suggests that glutamic acid was being converted to aspartic acid during germination.

Within 3 to 5 h after $T_{0\text{-wet}}$, most of the amino acid pool levels had increased (Table 3). Exceptions were tryptophan and threonine pools, which remained at their low levels (Table 3). Very large increases occurred in the ornithine, arginine, and citrulline pools coincident with germ tube formation. Considered together, these three amino acid pools accounted for less than 2% of the total amino acid pool in conidia (Table 2). By 5 h, these three pools had increased to 19% of the total pool (Table 3). Large ornithine and arginine pools in *N. crassa* mycelia have been reported previously (3, 23, 26).

The levels of the histidine, serine, glutamic acid, isoleucine, leucine, tyrosine, and phenylalanine pools increased during germ tube formation but did not reach levels that were as high as those in conidia. The lysine, alanine, and valine pools reached levels by 5 h that were higher than those found in conidia. In mid-log-phase cultures, alanine was the predominant free amino acid (T_{12}, Table 3).

Changes in the free amino acid pools during incubation in deionized water. To determine the effect of the absence of both exogenous carbon and nitrogen sources on the amino acid pool levels, the amino acid content of the free pools of conidia were measured during incubation in deionized water (Table 4). A duplicate experiment gave essentially the same results as given in Table 4. The conidia did not produce

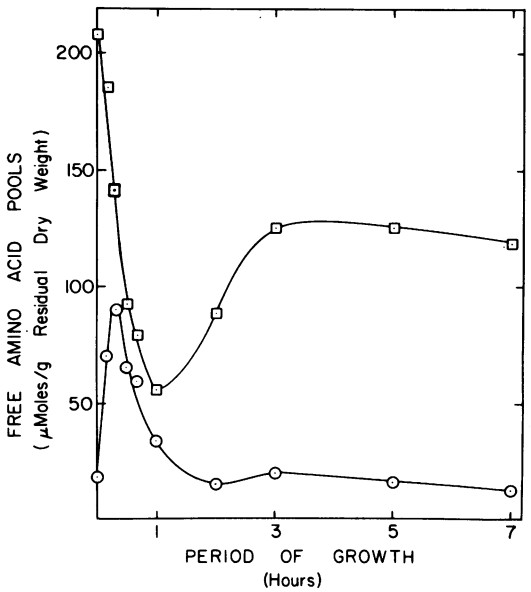

FIG. 3. *Changes in the free pools of glutamate and aspartate during conidial germination. The data in this figure and Table 3 were obtained from the same experiment. More time points are included in the figure than in Table 3. Symbols:* □, *glutamic acid;* ○, *aspartic acid.*

germ tubes in deionized water, and the residual dry weight remained constant.

After 3 h of incubation in deionized water, the total amino acid pool had decreased to about one-half the conidial level (Table 4). In comparison, in minimal medium the total amino acid pool had decreased to one-third the conidial level within the first hour (Table 3). Most of the decrease in the total amino acid pool during incubation in deionized water was the result of substantial decreases in the levels of the glutamic acid, alanine, and glutamine plus asparagine pools (Table 4). These three pools did not increase during the 5 h of this experiment. Also, the ornithine and citrulline pools did not increase during incubation in deionized water.

The levels of the lysine, histidine, arginine, serine, glycine, valine, isoleucine, leucine, tyrosine, and phenylalanine pools decreased for the first hour of incubation in deionized water and then increased (Table 4). This was the same pattern of changes in these amino acid pools that was observed during germination in minimal medium (Table 3). The final levels of four of these amino acid pools, lysine, histidine, isoleucine, and leucine, were higher after 5 h in deionized water than at any time during germination and growth in minimal medium.

The temporary increase in the γ-aminobutyric acid and the aspartic acid pools that occurred during germination in minimal medium also occurred in deionized water (Table 4). However, the aspartic acid pool remained at its high level for a longer period of time in deionized water.

Effect of cycloheximide on free amino acid pools. To determine the effect of the inhibition of protein synthesis on the endogenous amino acid pools, conidia were incubated in minimal glucose medium with 10 μg of cycloheximide per ml. Even after 24 h, no germ tubes had appeared. In general, the levels of the amino acid pools either stayed constant or increased when protein synthesis was inhibited by cycloheximide (Table 5). The pools of the basic amino acids, ornithine, lysine, histidine, and arginine, as well as aspartic acid, glycine, alanine, and valine, increased significantly. Apparently, protein synthesis was not required for the formation of these free amino acids. The only amino acid pools that decreased were the glutamic acid and glutamine plus asparagine pools.

Excretion of free amino acids into the medium. One possible explanation for the rapid decrease in the total free amino acid pool during conidial germination in minimal medium (Table 3) or during incubation in deionized water (Table 4) could be that these amino acids were being excreted into the medium. Since the standard minimal medium contained ammonia, which overloaded the columns of the amino acid analyzer, a minimal medium with nitrate substituted for ammonia was used. Conidial germination was not retarded in this medium.

After incubating for 1.5 h in nitrate minimal medium, the free intracellular acidic and neutral amino acids (basic amino acids were not measured) had decreased by more than 300 μmol/g of RDW. Only 7.3 μmol of free amino acids per g was recovered in the medium (Table 6).

The free intracellular and extracellular amino acids were also measured after incubating the conidia in deionized water for 40 min, 1 h, 3 h, and 6 h. The results after 3 h, when the total internal pool had decreased to its lowest level, are given in Table 6. By this time the free intracellular acidic and neutral amino acids had decreased by 250 μmol/g of RDW, but only 7.3 μmol of free amino acid per g was recovered from the water. Thus, it was concluded that the large decrease in the amino acid pools that occurred during germination in minimal medium and during incubation in deionized water was not due to excretion of the free amino acids.

TABLE 4. *Changes in the free amino acid pools during incubation in deionized water*[a]

Amino acid	Free amino acid concn (μmol/g of RDW)				
	$T_{0\text{-wet}}$	$T_{0.3}$	T_1	T_3	T_5
γ-Aminobutyrate	2.7	1.8	—	—	0.5
Ornithine	1.7	1.8	1.8	0.6	0.9
Tryptophan	1.5	4.3	0.7	0.9	0.9
Lysine	5.3	4.0	3.7	10.7	20.4
Histidine	10.9	8.9	9.3	10.5	14.7
Arginine	12.0	9.9	7.7	12.0	18.6
Aspartate	23.3	48.7	43.5	18.1	11.7
Threonine	31.0	26.2	21.9	18.1	19.8
Serine	28.8	19.5	14.8	19.7	27.9
Glutamine plus asparagine	123.5	79.7	51.9	47.0	55.2
Proline	ND	ND	ND	ND	ND
Glutamate	146.6	134.4	127.8	85.4	79.3
Citrulline	ND	ND	ND	ND	ND
Glycine	5.4	5.6	4.1	6.5	11.3
Alanine	122.8	96.2	54.4	15.3	15.8
Half-cystine	ND	ND	ND	ND	ND
Valine	6.5	5.7	3.4	7.1	6.7
Methionine	ND	ND	ND	ND	ND
Cystathionine	6.6	6.9	6.3	6.1	7.4
Isoleucine	2.3	2.2	1.5	4.0	3.4
Leucine	2.5	2.0	1.2	4.5	4.0
Tyrosine	3.3	2.4	1.1	1.5	1.5
Phenylalanine	1.8	1.1	0.4	1.3	1.2

[a] See footnotes to Table 3.

DISCUSSION

Amino acids of the urea cycle. The levels of the free pools of ornithine, arginine, and citrulline were lower in conidia than in mycelia from mid-log-phase cultures. All three of these amino acid pools increased coincident with germ tube formation. The accumulation of citrulline, but not ornithine and arginine, was inhibited by cycloheximide. Thus, the enzymes required for ornithine and arginine synthesis are presumably packaged into the conidia during conidiation. One of these enzymes, ornithine transcarbamylase, has been assayed in conidial extracts (13). The activity of this enzyme increased during conidial germination.

In *N. crassa* mycelia more than 90% of the free arginine and ornithine is located in discrete, membrane-enclosed vesicles (23, 26). Since arginine and ornithine accumulated during germ tube formation, either the membrane-enclosed vesicles were being formed at that time or the vesicles were already in the conidia and were simply being refilled.

Degradation of glutamic acid during conidial germination. The glutamic acid pool was the largest amino acid pool in conidia. In some conidial preparations, the glutamine pool was also large, but the level of this pool varied considerably. However, the sum of the glutamine and glutamic acid pools was always very consistent. Perhaps small changes in the culture conditions, such as humidity, could change the relative proportions of these two amino acid pools. Together, these two pools accounted for nearly 50% of the total free amino acids in the conidia and for about 3.0% of the conidial dry weight. If these two amino acids were uniformly distributed in the conidium, their intracellular concentration would be nearly 0.1 M (1 g of RDW was obtained from 3 g of dry-harvested conidia).

During the first 20 min of conidial germination, aspartic acid is accumulated. It is proposed that aspartic acid is one of the products of glutamic acid degradation. Other potential sources of aspartic acid could be from stored tricarboxylic acid cycle intermediates, from degradation of asparagine, or from proteolysis of conidial proteins. However, none of these possibilities are very likely. First, conidia have only low levels of tricarboxylic acid cycle intermediates (18). Second, the asparagine level in conidia (Table 2) was not sufficient to account for all of the free aspartic acid that was formed during germination (Table 3). Finally, since

most of the amino acid pools decreased during the early stages of germination (Table 3), general proteolysis would be an unlikely source of the rapid increase in the free aspartic acid pool.

TABLE 5. *Effect of cycloheximide on the free amino acid pools*[a]

Amino acid	Free amino acid concn (μmol/g of RDW)		
	$T_{0\text{-wet}}$	T_1	T_3
γ-Aminobutyrate	1.6	ND	ND
Ornithine	2.8	1.2	10.5
Tryptophan	0.9	ND	ND
Lysine	3.5	7.4	18.8
Histidine	10.9	13.6	18.8
Arginine	14.6	21.7	35.6
Aspartate	18.1	100.5	99.9
Threonine	33.4	38.6	37.6
Serine	27.1	23.5	34.9
Glutamine plus asparagine	47.7	17.2	19.3
Proline	ND	ND	ND
Glutamate	278.6	284.0	193.5
Citrulline	ND	ND	ND
Glycine	6.0	19.0	12.1
Alanine	195.3	201.0	244.0
Half-cystine	ND	ND	ND
Valine	7.8	15.8	16.5
Methionine	ND	ND	ND
Cystathionine	6.6	6.5	8.9
Isoleucine	2.7	3.4	4.1
Leucine	3.1	4.1	3.5
Tyrosine	3.6	3.6	3.3
Phenylalanine	1.9	1.8	1.0

[a] See footnotes to Table 3.

Aspartic acid could be synthesized from glutamic acid by one of the following two pathways. In the first pathway (22), aspartic acid would be synthesized from glutamic acid by transamination with oxaloacetic acid. The α-ketoglutarate formed by the transaminase would be degraded to oxaloacetic acid via the enzymes of the tricarboxylic acid cycle. The oxaloacetic acid would then be used to make a new molecule of aspartic acid and, thus, complete the cycle. In summary: glutamic acid + 2 nicotinamide adenine dinucleotide (NAD) + flavine adenine dinucleotide (FAD) + guanosine 5′-diphosphate (GDP) + P_i → aspartic acid + CO_2 + 2 reduced NAD (NADH) + reduced FAD ($FADH_2$) + guanosine 5′-triphosphate (GTP).

The second pathway is adapted from that proposed by Dover and Halpern for the degradation of γ-aminobutyric acid in *Escherichia coli* (4). In the first step (Fig. 4), glutamic acid would be decarboxylated to form γ-aminobutyric acid. A transaminase would then convert γ-aminobutyric acid to succinyl semialdehyde. The succinyl semialdehyde would be oxidized to succinate, which would would then be degraded to oxaloacetic acid by the enzymes of the tricarboxylic acid cycle. In summary: glutamic acid + NAD phosphate (NADP) + NAD + FAD → aspartic acid + CO_2 + reduced NADP (NADPH) + NADH + $FADH_2$.

For several reasons, the second pathway (Fig. 4) is currently favored. During the first few minutes of germination, there was a 20-fold increase in the level of the γ-aminobutyric acid

TABLE 6. *Amino acids released into the medium during conidial germination*

Amino acid[a]	Free amino acids (μmol/g of RDW)				
	Conidial pools[b]	Incubated 1.5 h in nitrate minimal medium		Incubated 3 h in deionized water	
		Intracellular	Extracellular	Intracellular	Extracellular
Aspartate	17.1	22.0	0.8	18.1	1.0
Threonine	31.0	8.1	0.6	18.1	0.4
Serine	28.8	10.7	0.7	19.7	0.4
Glutamine plus asparagine	103.6	24.9	1.0	47.0	0.1
Glutamate	156.4	62.6	3.2	85.4	4.3
Glycine	5.4	5.4	0.3	6.5	0.2
Alanine	122.8	22.8	0.6	15.3	0.3
Valine	6.5	2.4	Trace	7.1	0.4
Isoleucine	2.3	0.7	0.04	4.0	0.08
Leucine	2.5	1.0	0.05	4.5	0.08
Tyrosine	3.3	0.3	Trace	1.5	Trace
Phenylalanine	1.8	0.3	Trace	1.3	Trace

[a] Only the levels of the acidic and neutral amino acid pools were measured.

[b] Conidial pools were obtained from extracts of cold, washed conidia, which were prepared just prior to the start of incubation in either nitrate minimal medium or in deionized water.

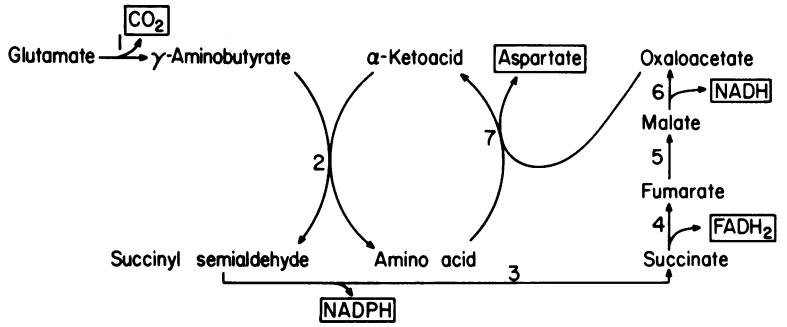

FIG. 4. *Possible pathway for the degradation of glutamic acid during conidial germination. The pathway given is adapted in part from that proposed by Dover and Halpern (4). 1, Glutamate decarboxylase; 2, γ-aminobutyrate-α-ketoglutarate transaminase; 3, succinyl semialdehyde dehydrogenase; 4, succinate dehydrogenase; 5, fumarase; 6, malate dehydrogenase; 7, glutamate-oxaloacetate transaminase. The boxes contain the products of glutamic acid degradation via this pathway.*

pool (Table 3). The γ-aminobutyric acid pool was never large and followed the same pattern of changes during germination as the aspartic acid pool. This would be consistent with γ-aminobutyric acid being an intermediate in the conversion of glutamic acid to aspartic acid. In addition, free glutamic acid is degraded to γ-aminobutyric acid during *Bacillus megaterium* endospore germination (8). A mutant strain of *B. megaterium* has been isolated that requires γ-aminobutyric acid for germination (7) and has low levels of glutamic acid decarboxylase (6). Thus, both bacteria and *N. crassa* may use similar pathways for degrading their large endogenous glutamic acid pools during spore germination. Additional studies, including radioactive labeling experiments and specific enzyme assays, will be necessary to determine if this pathway is actually used for the degradation of glutamic acid during conidial germination.

During the first 11 min of conidial germination, the levels of both NADH and NADPH increased two- to threefold (22). By 22 min, the levels of these reduced pyridine nucleotides had decreased to approximately the concentration found in dry conidia. It is proposed that the transient increases observed in the levels of NADH and NADPH may be due to glutamic acid degradation. For each molecule of glutamic acid that is degraded by either of the pathways given above, two molecules of reduced pyridine nucleotides would be produced. Thus, glutamic acid may be stored in conidia as a reservoir for the production of reduced coenzymes during germination.

ACKNOWLEDGMENTS

The excellent technical assistance of Stan Martins is gratefully acknowledged.

This investigation was supported by National Science Foundation grant GB 21227 and by Public Health Service grant GM 19308 from the National Institute of General Medical Sciences. One of us (J.C.S.) was supported by Public Health Service postdoctoral fellowship 1-F02-GM-50, 529-02 from the National Institute of General Medical Sciences.

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