

Regulation of the Acidic Amino-acid Permease of *Aspergillus nidulans*

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SUMMARY

Conidia of *Aspergillus nidulans* transported the acidic amino acids and some basic and neutral amino acids at very low rates. During germination the rates of transport of these amino acids increased. The rate of permease synthesis/litre of culture increased during germination, reaching a maximum soon after onset of exponential growth; during subsequent growth the rate of synthesis (per litre of culture) was constant.

The activity of the acidic amino acid permease, measured in germinated conidia, varied with the nitrogen source in the germination medium. An analysis of the intracellular pool of amino acids has shown no clear correlation between activity of the permease and the intracellular concentration of any amino acid.

The acidic amino-acid permease of *Aspergillus nidulans* is probably regulated by ammonia from within the organism and this regulation is achieved by both inhibition and repression.

INTRODUCTION

Previous investigations (Robinson, Anthony & Drabble, 1973) have established that germinated conidia of *Aspergillus nidulans* possess an energy-requiring transport system for the active uptake of the acidic amino acids L-glutamate, L-aspartate and L-cysteate. This acidic amino-acid permease has an extremely low activity in conidia, but the transport activity increases during germination. Preliminary work has established that the final activity of the permease in germinated conidia depends on the nitrogen source in the growth medium.

The formation of many amino-acid transport systems in fungi appears to depend on the availability of utilizable nitrogen (or carbon) sources in the growth medium. Nitrogen starvation produces a large increase in the activity of these permeases (Benko, Wood & Segel, 1967, 1969; Hackette, Skye, Burton & Segel, 1970; Magaña-Schwenke & Schwenke, 1969; Pall, 1969, 1970*b*; Tisdale & DeBusk, 1970; Grenson, Hou & Crabeel, 1970). It is postulated that the permeases are either derepressed or de-inhibited by the conditions of starvation. The intracellular effector substance which regulates permease activity has not been identified, but ammonia has been indirectly implicated as a regulatory metabolite in some cases. Many amino-acid permeases of fungi are reported to be regulated by 'feedback inhibition' by intracellular amino acids, or products of their metabolism (Grenson, 1966; Grenson, Mousset, Wiame & Bechet, 1966; Gits & Grenson, 1967; Crabeel & Grenson, 1970; Grenson *et al.* 1970; Grenson & Hou, 1972; Wiley & Matchett, 1968; Benko *et al.* 1967, 1969; Hunter & Segel, 1971; Pall, 1971; Robinson, 1973).

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The present paper shows that ammonia or a metabolite of ammonia is important in regulating the activity of the acidic amino-acid permease of *Aspergillus nidulans*. It is concluded that regulation is achieved both by repressing synthesis of a protein component of the permease and by inhibiting the activity of preformed permease.

Preliminary reports of some of this work have been published (Robinson, Anthony & Drabble, 1971, 1972).

METHODS

Chemicals. All chemicals were reagent grade. The following radioactive compounds were obtained from the Radiochemical Centre, Amersham, Buckinghamshire: L-[U-¹⁴C]arginine monohydrochloride (150 mCi/mmol); L-[U-¹⁴C]aspartic acid (227 mCi/mmol); L-[U-¹⁴C]-glutamic acid (14.7 mCi/mmol); [U-¹⁴C]glycine (11.3 mCi/mmol); L-[U-¹⁴C]leucine (150 mCi/mmol); L-[U-¹⁴C]lysine (312 mCi/mmol); L-[methyl-¹⁴C]methionine (25 mCi/mmol); L[U-¹⁴C]phenylalanine (200 mCi/mmol). Other chemicals were obtained from sources given in the preceding paper (Robinson *et al.* 1973), or from British Drug Houses Ltd, Poole, Dorset.

Organisms and growth media. The wild-type strain (bi-1) of *Aspergillus nidulans* and the growth media have been described in the previous paper (Robinson *et al.* 1973). The methylamine resistant mutant (originally designated bi-1, *mea*^R-8), was kindly supplied by Dr D. J. Cove of the Department of Genetics, University of Cambridge. This mutant is sometimes designated *meaA*-8.

The preparation of conidial suspensions. This is fully described in the previous paper (Robinson *et al.* 1973).

Germination of conidia in aerated cultures. Conidia (approx. 10⁷/ml medium) were incubated in the growth medium with 25 mM nitrogen source (unless otherwise stated). Growth was at 30 °C in conical rimless flasks (with aluminium caps and with invaginations in the sides to increase aeration). Unless otherwise stated the conidia were germinated until germ tubes were just visible in more than 80 % of conidia (usually 10 to 12 h after inoculation).

Extraction of intracellular amino acids. Germinated conidia were extracted for 18 h at 2 °C with 5 % trichloroacetic acid (approx. 2 mg dry wt of germinated conidia/ml acid). The residue was sedimented by centrifugation and the supernatant fluid assayed for extracted amino acids and other nitrogen-containing compounds. Extracts were stored at -22 °C and were analysed within 6 days. The intracellular concentration of extracted compounds was calculated by assuming that the weight of intracellular water of germinated conidia is three times their dry weight (Pall, 1970a).

Measurement of extracted amino acids and ammonia. Extracts were analysed with a Jeol JLC-5AH amino-acid analyser. Equal volumes (1 ml) of a 5 % trichloroacetic acid extract of germinated conidia were injected on to the basic and the acid-neutral columns of the amino-acid analyser. A known quantity of L-norleucine was added to the extract as an internal standard. Columns were calibrated with a standard solution containing 19 amino acids (including glutamine) and ammonia. The amino acids were identified by the sequence of elution from the columns. The amount of each amino acid was determined from the extinction at 570 nm of the coloured products obtained by reaction with ninhydrin.

Measurement of transport of [¹⁴C]amino acids by Aspergillus nidulans. Measurement of amino-acid transport is fully described in the previous paper (Robinson *et al.* 1973); identical procedures were employed. Initial rates of uptake were expressed as μmol substrate transported /g dry wt/min. The specific activity of a permease was expressed in units/g dry wt.

One unit of permease activity is defined as a rate of transport of 1 μmol substrate/min at 30 °C and pH 6.5 (1 milliunit = 10^{-3} unit).

Incorporation of radioactive leucine into protein. Conidia were germinated for 10 h at 30 °C in an ammonia (12.5 mM) medium. The medium was then divided into two equal portions and the germinated conidia harvested by centrifugation and washed with 0.1 M phosphate buffer. One portion of conidia was resuspended in glutamate (25 mM) medium containing cycloheximide (final concn. 10 $\mu\text{g}/\text{ml}$); the other portion was resuspended in glutamate medium without cycloheximide. After 10 min incubation at 30 °C, L-[U- ^{14}C]leucine (0.1 mM, 0.02 $\mu\text{Ci}/\mu\text{mol}$) was added to both media and the germinated conidia incubated for a further 3 h. The germinated conidia were harvested by filtration through a 47 mm diameter Millipore filter (type HAWP, 0.45 μm) washed twice with 100 ml volumes of distilled water (4 °C), then scraped off the filter and weighed. A large sample of the germinated conidia was weighed and dried at 105 °C to constant weight. From the wet-weight to dry-weight ratio of this sample, the dry weights of the samples used for determination of radioactivity were calculated. Weighed samples were extracted twice, for 20 min each time, with boiling trichloroacetic acid (5%, containing 10 mM-non-radioactive leucine). The extracts were centrifuged in an MSE Minor centrifuge at 3000 rev./min for 5 min and the supernatants discarded. The pellets were extracted twice, for 15 min each time, with methanol:chloroform (3:1, containing 10 mM-non-radioactive leucine) at 40 °C. This procedure extracts the lipid precipitated by trichloroacetic acid. The residues were filtered on to a 13 mm diameter Millipore filter (type HAWP, 0.45 μm) and the filter added to 10 ml butyl-PBD scintillation fluid for determination of radioactivity.

RESULTS

Activity of the acidic amino-acid permease (aaap) during germination. Conidia, produced during sporulation of *Aspergillus nidulans* on pearl barley with added glucose + glutamate, glucose + ammonia, or glycerol + asparagine, transported glutamate at very low rates (approx. 0.01 $\mu\text{mol}/\text{g}$ dry wt/min.). During subsequent germination of conidia in a glutamate medium (25 mM) the specific activity of the permease increased over a hundred-fold (Fig. 1). The specific activity was at a maximum when germ tubes were just emerging from the conidia (8 to 10 h) and subsequently the activity declined to a level of between 20 and 30 % of the maximum level attained.

Synthesis of the acidic amino-acid permease (aaap) during germination with glutamate as sole nitrogen source. It has been shown that during germination the specific activity of *aaap* increases until germ-tube emergence and then declines (Fig. 1). This section deals with the kinetics of appearance of *aaap* activity during the first 12 h of germination.

The specific activity of *aaap* increased markedly during the first 9 h of germination (Fig. 2). Soon after exponential growth commenced a decline in the specific activity of *aaap* occurred. The total activity of *aaap* (expressed as milliunits/l of culture) showed no such decline, but increased in a linear fashion during exponential growth (Fig. 3). The rate of synthesis of the permease/litre of culture (Fig. 4) was calculated by differentiating the equation of the curve in Fig. 3 with respect to time. The rate of synthesis reached a maximum soon after the onset of exponential growth. Thereafter, the rate of permease synthesis/litre of culture was constant despite the exponential increase in the mass of the germinating conidia. The results in Fig. 5 show, by contrast, that the rate of synthesis of *aaap/g* dry wt of conidia increased in a linear fashion during the initial 7 h of germination but after onset of exponential growth this rate decreased. These unusual kinetics of permease synthesis are

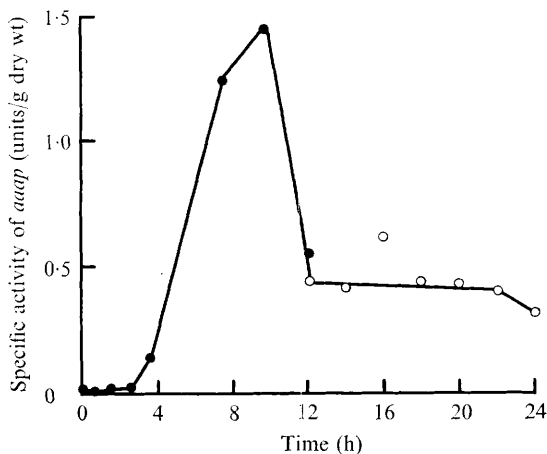


Fig. 1

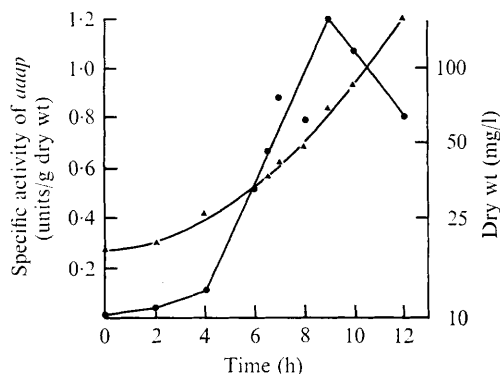


Fig. 2

Fig. 1. Development of the acidic amino-acid permease (*aaap*) during germination. Conidia ($10^7/\text{ml}$) were grown in a glutamate medium (25 mM) at 30°C on a gyrotary shaker (240 rev./min). Samples were withdrawn during growth and transport of L- ^{14}C glutamate (0.1 mM) measured as described in Methods. For convenience, two cultures were used: ●, flask 1; ○, flask 2. The data are from one typical experiment. The morphology of germinating conidia was observed under phase-contrast microscopy at intervals during germination. For the first 2 h the diameter of the conidia was $3.1\ \mu\text{m}$. After this time the diameter increased until at 8 h more than 90 % of the germinating conidia had a diameter of $5.0\ \mu\text{m}$. At 9 h conidia were seen to darken and by 10 h more than 90 % showed visible germ tubes.

Fig. 2. Variation in the specific activity of the acidic amino-acid permease (*aaap*) during germination in a glutamate medium. Conidia ($3.5 \times 10^8/\text{ml}$) were germinated in a glutamate (25 mM) medium at 30°C . Samples were withdrawn at various times during germination and transport of L- ^{14}C glutamate (0.1 mM) measured. ●, Specific activity of *aaap*; ▲, dry weight.

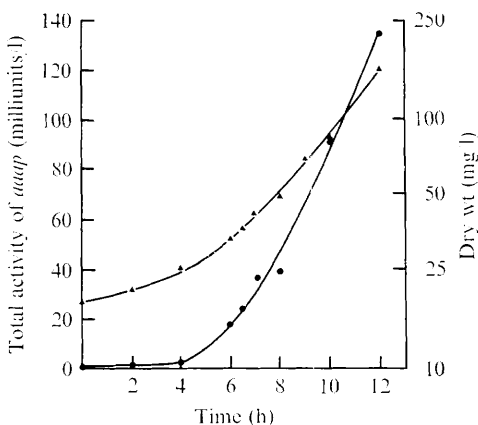


Fig. 3

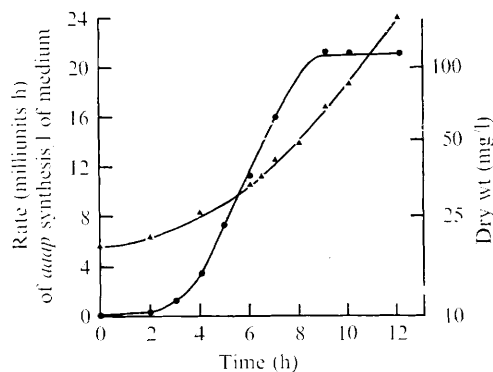


Fig. 4

Fig. 3. Increase in the total activity (milliunits/l) of acidic amino-acid permease during germination in glutamate medium. For experimental details see Fig. 2. ●, Total activity of *aaap*; ▲, dry weight.

Fig. 4. The rate of synthesis (per litre of medium) of acidic amino-acid permease (*aaap*) during germination in glutamate medium. For experimental details see Fig. 2. ●, Rate of *aaap* synthesis; ▲, dry weight.

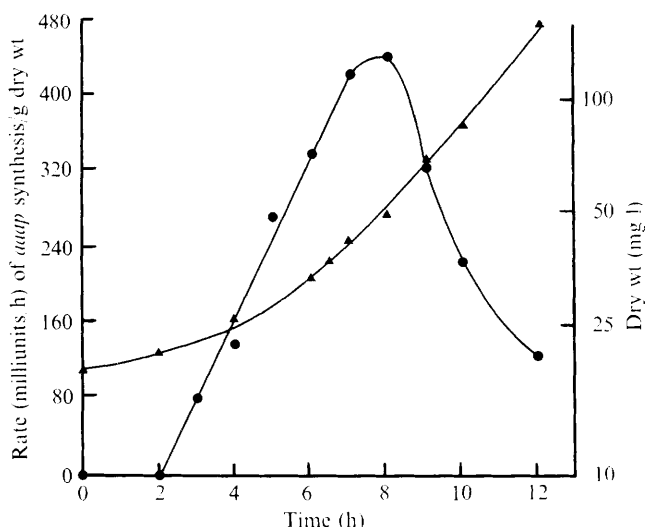


Fig. 5. The rate of synthesis (per g dry wt) of acidic amino-acid permease during germination in a glutamate medium. For experimental details see Fig. 2. ●, Rate of *aaap* synthesis; ▲, dry weight.

similar to those measured for synthesis of nitrate reductase in *Aspergillus nidulans* by Cove (1966) and may occur because hyphae grow only at the tips and there are always constant linear extension zones (Border & Trinci, 1970; Trinci, 1971) where protein synthesis is occurring.

The nature of the permease for transport of glutamate in young mycelium and in conidia germinated with ammonia as sole nitrogen source. The rates of glutamate transport by conidia germinated for 10 h with ammonia (25 mM) as sole nitrogen source, and by young mycelium germinated for 24 h with glutamate (25 mM), were low. The kinetics of transport were measured to confirm that this transport of glutamate was mediated by *aaap* rather than by a second permease with affinity for glutamate.

The kinetic data in Table 1 show that the V_{\max} for glutamate transport by young mycelium ($0.33 \mu\text{mol/g dry wt/min}$) was less than 10% of the V_{\max} measured in germinated conidia ($4.35 \mu\text{mol/g dry wt/min}$). The affinity of young mycelium for glutamate (apparent K_m , $190 \mu\text{M}$) was approximately equal to that of germinated conidia for glutamate (apparent K_m , $180 \mu\text{M}$).

The V_{\max} value for glutamate transport by ammonia-germinated conidia was 12% of the value measured for glutamate-germinated conidia. The apparent K_m value ($190 \mu\text{M}$) for glutamate transport by ammonia-germinated conidia was almost identical with the K_m value obtained for glutamate transport by conidia germinated with glutamate as nitrogen source.

From the similarity of the apparent K_m values it may be concluded that the glutamate permease of young mycelium and of conidia germinated on ammonia is identical with the acidic amino-acid permease (*aaap*) previously described in conidia germinated on glutamate (Robinson *et al.* 1973).

The specific activities of the acidic amino-acid permease (aaap) in conidia germinated with various nitrogen sources. The specific activities of *aaap* in conidia germinated on a variety of nitrogen sources are shown in Table 2. The growth rate of conidia depended on the nitrogen source in the germination medium. For valid comparisons with different nitrogen sources, the specific activity of *aaap* was always measured in conidia just after visible

Table 1. Apparent K_m and V_{max} values for glutamate transport in young mycelium and in conidia germinated with glutamate (25 mM), or with ammonia (25 mM)

The rate of transport of L-[U- 14 C]glutamate was measured at various concentrations of the amino acid in the assay medium. The K_m and V_{max} values were calculated from double-reciprocal plots of the kinetic data. Full experimental details are given in Methods.

Nitrogen source in germination medium	Time after inoculation (h)	Morphological state	K_m (μ M)	V_{max} (μ mol/g dry wt/min)
L-Glutamate	11	Germinated conidia	180	4.35
L-Glutamate	24	Young mycelium	190	0.33
Ammonium sulphate	11	Germinated conidia	190	0.52

Table 2. Specific activities of the amino-acid permease (*aaap*) in conidia germinated with various nitrogen sources

Full experimental details for germination of conidia and for assay of glutamate transport are given in Methods. Specific activities of *aaap* were calculated from the initial rates of uptake of L-glutamate (0.1 mM) measured in the presence of non-radioactive L-isoleucine (10 mM). The values are expressed as a percentage of the specific activity measured in conidia germinated on glutamate (1.2 units/g dry wt). Usually three germination experiments were done for each nitrogen source and the values for these experiments all fell within the percentage range indicated.

Nitrogen source	Relative transport rate (%)	Nitrogen source	Relative transport rate (%)
L-Arginine	150-170	L-Cysteic acid*	20
Acetamide	110-120	No nitrogen source*	15
L-Phenylalanine	105	Ammonia	13
L-Glutamate	100	Urea	13
L-Aspartate	100	L-Glutamate + ammonia	9
Glycine	100	Uric acid + ammonia	9
L-Alanine	70-80	L-Glutamate + urea	7
L-Lysine	70-80	L-Glutamine	4
L-Asparagine	50-60	L-Glutamate + glutamine	4
L-Proline	50-60		
Nitrate	50-60		
Uric acid	50		

* Very little growth occurred.

emergence of germ tubes (at this stage the specific activity was always the maximum reached). Germination of conidia with L-arginine as nitrogen source led to nearly twice the specific activity of *aaap* as did germination with glutamate. By contrast, germination with ammonia, urea or L-glutamine as nitrogen source gave less than 15% of the value with glutamate as nitrogen source.

The data in Table 2 suggest that the permease is regulated by repression; when ammonia, urea or L-glutamine was in the medium together with L-glutamate (or when ammonia and uric acid were present together) a low level of *aaap* resulted. If the regulatory mechanism involves repression, then the co-repressor is likely to be a common metabolite of those nitrogen sources which lead to low levels of *aaap*; it is possible that ammonia, itself, is the co-repressor. If this is the case then the high permease level in arginine-germinated conidia, for example, would be due to a lower concentration of co-repressor than is present during germination on glutamate.

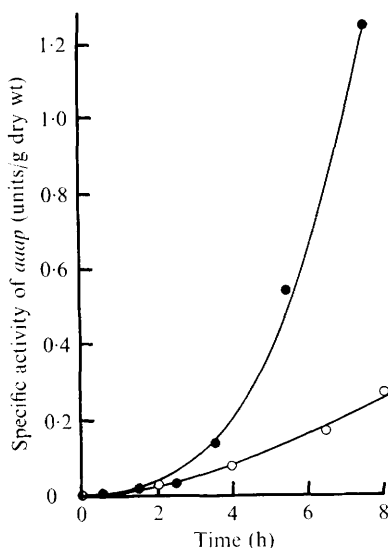


Fig. 6. Variation in the specific activity of the acidic amino-acid permease during germination with ammonia and with glutamate as sole nitrogen source. Conidia (10^7 /ml) were germinated in either 25 mM-ammonia medium, ○, or in 25 mM-glutamate medium, ●, at 30 °C. Samples were withdrawn at various times during germination, and transport of glutamate measured. Full details are given in Methods.

It has been assumed in this discussion that the differences in the measured rates of glutamate transport resulted from changes in the level of *aaap* and not from changes in the amount or activity of other permeases with affinity for glutamate. This assumption was based on the following considerations. First, at the pH value of the assay medium (6.5), glutamate is predominantly in the anionic form $^-OOC.(CH_2)_2.CH(NH_3^+)COO^-$, which by analogy with systems in other filamentous fungi (Pall, 1970*b*; Hunter & Segel, 1971) would bind tightly to an acidic amino-acid permease but not to other amino-acid permeases. Second, the initial rate of glutamate transport was measured in the presence of a hundred-fold excess of unlabelled L-isoleucine. This concentration (10 mM) of isoleucine has little effect on the transport of glutamate by *aaap* (Robinson *et al.* 1973) but it should inhibit glutamate transport by a general (or neutral) amino-acid permease.

Synthesis of the acidic amino-acid permease (aaap) during germination with ammonia as sole nitrogen source. The variation in the specific activity of *aaap* during germination with ammonia as sole nitrogen source is shown in Fig. 6; data showing the increase in specific activity of *aaap* during germination in a glutamate medium are included for comparison. The low specific activities of *aaap* observed at all stages during germination in an ammonium medium (by contrast with activities in a glutamate medium) may result from repression of synthesis of a protein component of *aaap*, and/or from inhibition of permease activity by an accumulated intracellular compound. The experiments described below were designed to investigate the extent to which inhibition of activity and of synthesis are important in the regulation of the amino-acid permease.

Development of the acidic amino-acid permease activity in ammonia-germinated conidia after transfer to glutamate medium. When ammonia-germinated conidia (10 h) were harvested, resuspended, and incubated at 30 °C in glutamate medium for 3.5 h, the specific activity of *aaap* increased seven-fold. This increase did not occur at 2 °C, or when conidia

Table 3. *Specific activities of the acidic amino-acid permease (aaap) after transfer of ammonia-germinated conidia to various media*

Conidia were germinated for 10 h in medium with ammonia as nitrogen source, harvested, and resuspended in fresh medium with either ammonia or glutamate as nitrogen source. After further incubation at 30 °C the transport of L-glutamate was measured as described in Methods.

Time after resuspension (h)	Nitrogen source in resuspension medium	Cycloheximide concn. ($\mu\text{g/ml}$)	Specific activity of aaap (units/g dry wt)
0	—	—	0.19
3.5	Ammonia (25 mM)	0	0.19
3.5	Glutamate (25 mM)	0	1.09
3.5	Glutamate (25 mM) minus glucose	0	0.36
3.5	Glutamate (25 mM)	10.0	0.24
1.75	Glutamate (25 mM)	10.0	0.24
4.75*	Glutamate (25 mM)	0.0*	0.97*

* These conidia were incubated for 1.75 h with cycloheximide, then washed, resuspended, and incubated for a further 3 h in the absence of cycloheximide.

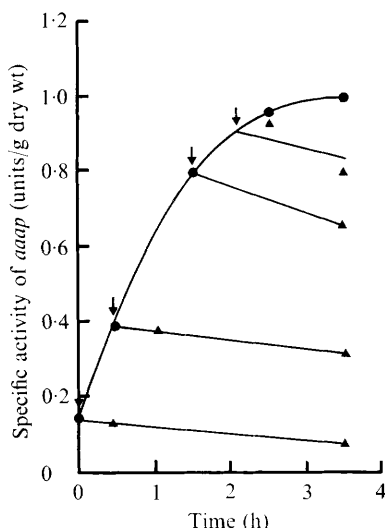


Fig. 7. The effect of cycloheximide on development of the acidic amino-acid permease activity. Conidia germinated in ammonia (25 mM) medium for 11 h at 25 °C were resuspended in glutamate (25 mM) medium at 30 °C. Cycloheximide was added at the times indicated by arrows and transport of glutamate measured as described in Methods. ●, Control, i.e. no cycloheximide; ▲, after addition of cycloheximide (10 $\mu\text{g/ml}$).

were resuspended in ammonia medium or in glutamate medium with no energy source, i.e. glucose omitted (Table 3). The data in Fig. 7 show that addition of cycloheximide (10 $\mu\text{g/ml}$) at any time during the experiment immediately prevented any further increase. This concentration of cycloheximide had no effect on the rate of transport of glutamate when measured in the usual permease assay system over a period of 20 min. The rate was 1.25 μmol L-glutamate transported/min/g dry wt in the absence of cycloheximide, and 1.20 in the presence of this inhibitor.

The incorporation of L-[U- ^{14}C]leucine into protein was measured during incubation of

ammonia-germinated conidia in glutamate medium, both with and without cycloheximide (10 $\mu\text{g/ml}$). The incorporation of radioactivity in the protein fraction was 6858 d.p.m./mg dry wt of germinated conidia in the absence of cycloheximide, and 391 d.p.m./mg dry wt in the presence of cycloheximide. This experiment showed that cycloheximide inhibited protein synthesis in *Aspergillus nidulans* by at least 95 %. The inhibitory effect of this protein synthesis inhibitor was reversible; germinated conidia which had been incubated in a medium containing cycloheximide (10 $\mu\text{g/ml}$) and glutamate (25 mM) for 105 min synthesized *aaap* when resuspended in fresh glutamate medium without cycloheximide (Table 3).

These data suggest that the resuspension of ammonia-germinated conidia in glutamate medium results in a *de novo* synthesis of the permease system; the cycloheximide prevented this by inhibiting the necessary protein synthesis. An alternative explanation is that the inhibition of protein synthesis by cycloheximide prevented the utilization of, and perhaps resulted in the accumulation of, an intracellular inhibitor such as ammonia (or derivative of ammonia such as glutamine). The concentration of inhibitor decreased on suspension in glutamate medium (in the absence of cycloheximide) and de-inhibition of the permease resulted. The slow appearance of *aaap* activity on transfer from ammonia medium to glutamate medium perhaps favours the first of these explanations. The following experiments were designed to give further information on the repression and inhibition of the acidic amino-acid permease by ammonia (or metabolite of ammonia).

Variation in the specific activity of the acidic amino-acid permease in a methylamine-resistant mutant of Aspergillus nidulans. In *Aspergillus nidulans*, ammonia represses a number of proteins including nitrate reductase, nitrite reductase, xanthine dehydrogenase, uric-acid oxidase and allantoinase (Arst & Cove, 1969) and an extracellular protease (Cohen, 1972). A mutant strain (*mea^R-8*), resistant to the toxic effects of methylamine, is derepressed with respect to those enzymes usually repressed by ammonia (Arst & Cove, 1969). It has been suggested above that ammonia may also repress synthesis of *aaap*. If the *mea^R-8* locus codes for a protein involved in the regulation of all ammonia-repressible systems, and if the permease is indeed repressed by ammonia, then the effect of ammonia on the permease of the mutant should differ from that observed in the parent strain.

The results in Table 4 show that the specific activity of *aaap* in *mea^R-8* was more than twice that measured in the parent strain after germination in ammonia or glutamine media. No significant difference in the specific activities of *aaap* in the two strains was measured after germination of conidia in a glutamate medium. This evidence gives support to the hypothesis that ammonia represses synthesis of *aaap*; it should be noted that in the derepressed mutant strain the activity of *aaap* in conidia germinated on ammonia was still not equal to that in glutamate-germinated conidia (Table 4). It is probable, therefore, that ammonia inhibited the acidic amino-acid permease as well as repressing its synthesis.

Effects of ammonia on the activity of the acidic amino-acid permease (aaap). In the previous sections some evidence has been presented indicating that *aaap* was repressed, and also perhaps inhibited from within the organism, by ammonia or by a metabolite of ammonia. By measurement of the changes in the specific activity of *aaap* occurring after addition of ammonia to a suspension of conidia germinating in a glutamate medium, it was possible to determine whether ammonia repressed or inhibited the permease. The results in Fig. 8 show that 30 min after the addition of ammonia to germinating conidia the specific activity of *aaap* was 50 % of that in conidia with no added ammonia. After 3 h the specific activity was less than 10 % of the control value. The total activity of permease/litre of culture fell to 50 % of the control shortly after addition of ammonia, and subsequently to 30 % of the control value. This low level of activity was maintained over a subsequent fivefold

Table 4. The specific activities of the acidic amino-acid permease (*aaap*) in a methylamine-resistant mutant (*bi-1*, *mea^R-8*) and the parent strain (*bi-1*) of *Aspergillus nidulans* germinated on various nitrogen sources

Conidia were germinated and glutamate transport assayed as described in Methods.

Strain	Nitrogen source	Specific activity of <i>aaap</i> (units/g dry wt)
Parent strain (<i>bi-1</i>)	Glutamate	1.38 (100%)
	Glutamine	0.16 (12%)
	Ammonia	0.13 (10%)
<i>(bi-1, mea^R-8)</i>	Glutamate	1.35 (100%)
	Glutamine	0.38 (28%)
	Ammonia	0.30 (22%)

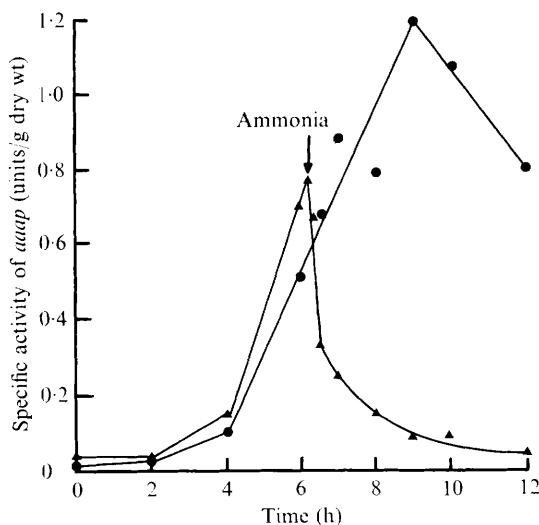


Fig. 8. The effect of ammonia on the specific activity of the acidic amino-acid permease (*aaap*) in *Aspergillus nidulans* (wild-type). Conidia (3.5×10^6 /ml) were germinated in glutamate medium (25 mM) at 30 °C. ▲, Ammonia (final concn. 25 mM) added to culture after 6 h (↓); ●, no ammonia added. Samples were withdrawn periodically and the transport of glutamate measured.

increase in mass (Fig. 9). The immediate 50% reduction in the measured activity of *aaap* occurred without any significant increase in the mass of the germinated conidia. Such a rapid decrease in activity of *aaap* could not be accounted for by cessation of synthesis of *aaap* unless an active component of the permease was rapidly inactivated. The data of Fig. 7 indicate that the permease was degraded at a very low rate after protein synthesis had been inhibited with cycloheximide. Thus the rapid decline in total activity of *aaap* after ammonia was added to the growth medium was more likely to have resulted from inhibition of the activity of preformed permease. The low level of permease observed during the subsequent five-fold increase in mass (Fig. 9) showed that little net synthesis of permease took place after the initial inhibition by ammonia; this confirms the conclusion (above) that ammonia also repressed synthesis of the permease.

The inhibitory effect of ammonia was time-dependent; ten minutes after addition of ammonia, *aaap* activity was not inhibited, but 20 min later the activity was reduced by 50% (Fig. 8). Furthermore, another experiment showed that when germinated conidia were

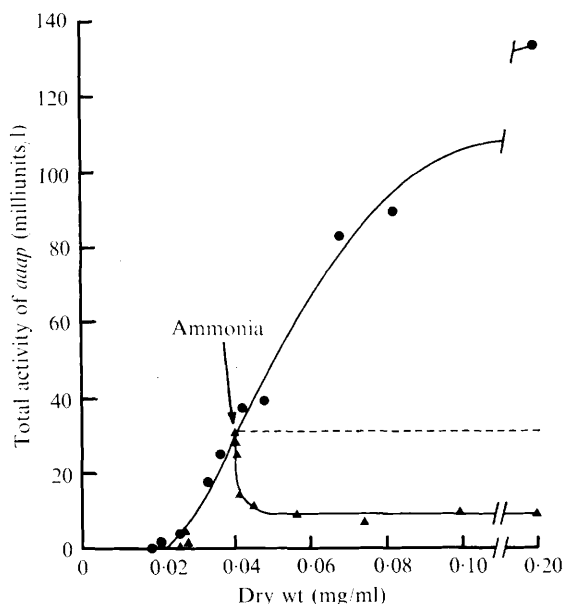


Fig. 9. The effect of ammonia on total activity (per litre of medium) of the acidic amino-acid permease (*aaap*) in *Aspergillus nidulans*. For experimental details see Fig. 8. ▲, Ammonia added to culture after 6 h; ●, no ammonia added. The dashed line represents the value expected if repression is the sole regulatory mechanism.

pre-incubated in assay medium with ammonia for 30 min before assay of uptake of glutamate, the permease was inhibited by 40%. The observation that ammonia did not inhibit the permease when added to the assay medium at the same time as glutamate (Robinson *et al.* 1973) suggests that ammonia (or a metabolite of ammonia) inhibited only when it had accumulated within the germinated conidia or when it had been metabolized to an inhibitory product.

Nature of the intracellular effector. The results described above suggested that both the synthesis and the activity of *aaap* were controlled by the intracellular concentration of an effector substance, which may have been ammonia or a metabolite of ammonia. The nature of the intracellular effector was investigated by measuring both the specific activity of *aaap* and the intracellular concentrations of amino acids and ammonia in conidia which had been germinated with various nitrogen sources. The intracellular pool of amino acids and ammonia was extracted with trichloroacetic acid (5%) at 2 °C to preserve glutamine and other heat-labile compounds. The acidic conditions of the extraction minimized loss of ammonia.

No correlation was found between the pool size of the substrates of the permease (glutamate and aspartate) and the specific activity of *aaap* (Table 5). A high intracellular concentration of ammonia was always associated with a low specific activity of *aaap* and vice versa. This observation supports the previous conclusion that the intracellular effector which regulated the activity and synthesis of *aaap* was ammonia or a metabolite of ammonia. Some of the ammonia detected during analysis could have arisen from the breakdown of glutamine. Sufficient data are not available to give an estimate of this conversion and therefore correlation between levels of *aaap* and pool size of ammonia must be treated with caution.

Table 5. *The intracellular concentrations of amino acids and ammonia, and relative specific activities of the acidic amino-acid permease, in conidia germinated on various nitrogen sources*

Compound in extracts	Nitrogen sources*										
	Arginine	Acetamide	Phenylalanine	Glutamate	Alanine	Nitrate	Glutamate	Urea	Ammonia	Glutamine	
Ammonia	3.41	6.10	2.92	5.68	4.95	6.55	10.22	11.53	13.14	11.07	
Aspartate	1.65	1.48	0.81	1.65	1.19	2.08	2.02	1.70	2.19	1.79	
Glutamate	27.66	42.49	18.86	19.62	25.02	64.44	38.84	53.85	54.14	26.96	
Asparagine	0.58	—	—	0.54	1.52	1.20	1.65	1.73	1.55	1.75	
Glutamine	17.74	26.93	16.46	16.41	17.76	17.25	34.92	32.86	27.49	20.19	
Lysine	2.22	—	—	1.05	0.74	2.59	2.52	1.13	1.98	2.23	
Histidine	0.44	—	—	0.38	—	0.73	0.57	0.77	0.64	0.87	
Arginine	13.27	—	—	1.14	0.79	7.18	3.83	5.85	6.94	5.60	
Threonine	1.76	0.86	1.52	2.08	1.95	2.52	2.94	2.29	3.17	3.95	
Serine	1.51	1.42	1.07	2.01	1.32	2.72	2.93	2.81	3.04	5.17	
Glycine	0.70	0.53	0.24	0.56	0.57	1.45	2.30	1.40	1.90	1.82	
Alanine	1.95	5.04	0.30	0.93	3.22	3.87	6.59	6.80	2.46	2.76	
Cysteine	0.92	—	—	0.92	1.13	0.73	1.24	0.85	1.16	0.75	
Valine	0.21	1.80	0.32	2.63	2.97	3.07	3.89	3.31	3.55	1.37	
Methionine	0.46	—	—	0.81	—	0.70	0.64	0.74	0.77	0.70	
Isoleucine	1.36	1.25	0.24	1.31	1.43	1.59	1.90	1.54	1.96	1.41	
Leucine	—	—	—	—	—	—	0.28	—	—	0.25	
Phenylalanine	—	—	0.58	—	—	—	—	—	—	—	
Relative sp. act. of <i>aatp</i> (%)	172	120	106	100	78	54	47	19	14	10	

* The concentration of all nitrogen sources was 25 mM except where specified otherwise (†).

† The concentration was 250 mM.

—, Not detected.

Table 6. *Initial rates of uptake of [¹⁴C]amino acids after germination of conidia in a glutamate medium*

Conidia were germinated at 30 °C in a glutamate (25 mM) medium. Samples were taken at various times after inoculation and the rates of transport of the four [¹⁴C]amino acids determined. Full experimental details are given in Methods. Rates of transport are expressed as μmol substrate transported/g dry wt/min.

Amino acid	Concn. in assay medium (μM)	Time after inoculation (h)				
		0	9.2	11.5	15.0	19.0
L-Glutamate	100	0.01	1.29	1.23	1.18	0.37
Glycine	100	0.01	0.85	0.81	1.38	0.33
L-Lysine	100	0.02	1.26	1.23	1.30	0.61
L-Methionine	100	0.03	0.95	0.96	1.41	0.76

Table 7. *Initial rates of uptake of ¹⁴C amino acids in conidia germinated on various nitrogen sources*

The initial rate of uptake of an amino acid was measured during the first 3 min of assay as described in Methods. The growth rate of conidia depended on the nitrogen source in the germination medium. For valid comparisons with different nitrogen sources the specific activity of *aaap* was measured in conidia just after visible emergence of germ-tubes (at this stage the specific activity was always the maximum reached.)

Radioactive amino acid (0.1 mM) in assay medium	Rate of amino-acid transport ($\mu\text{mol}/\text{min}/\text{g}$ dry wt)			
	Nitrogen source (25 mM) of germination medium:			
	Glutamate	Arginine	Glutamine	Ammonia
L-Glutamate	1.20	1.63	0.16	0.16
L-Aspartate	0.63	0.91	0.08	0.08
L-Methionine	1.22	1.10	0.62	0.55
Glycine	0.86	0.84	0.19	0.19
L-Lysine	1.07	0.79	0.50	0.50
L-Phenylalanine	1.33	0.92	0.33	0.37
L-Arginine	2.38	1.55	0.72	0.79

Regulation of various amino-acid permeases in germinated conidia. Conidia transported glycine, L-lysine and L-methionine at low rates (0.01, 0.02 and 0.03 $\mu\text{mol}/\text{g}$ dry wt/min, respectively, Table 6). During germination in a glutamate medium, the initial rates of uptake of the three amino acids increased at least fifty-fold. The initial rates of uptake of these, and of three further amino acids (L-arginine, L-aspartate and L-phenylalanine) were measured in conidia germinated with various nitrogen sources (Table 7). Conidia germinated with glutamine or ammonia transported each amino acid at a lower rate than conidia germinated with glutamate or arginine as nitrogen source. The initial rates of uptake of glutamate and aspartate, which are both substrates of *aaap*, were the lowest following germination with ammonia or glutamine. With the exception of glutamate and aspartate, all of the amino acids were transported at slightly lower rates by conidia germinated with arginine as nitrogen source compared with conidia germinated with glutamate. The results in Table 7 show that the relative initial rates of uptake of glutamate and aspartate were similar under the various germination conditions. This supports the conclusion that these two acidic amino acids were transported by one permease (Robinson *et al.* 1973).

Data given in Table 7 show that the permeases for L-arginine, glycine, L-lysine, L-methio-

nine and L-phenylalanine were not regulated by the same system as the acidic amino-acid permease. If the same regulatory system were operating, then the relative rates of transport of each amino acid (compared with the rate for glutamate) would be constant under the various germination conditions.

DISCUSSION

The activities of amino acid permeases of *Aspergillus nidulans* are very low in conidia but increase markedly during germination. It is possible that derepression of synthesis of proteins of the *aaap*, and of other permeases, may be a direct consequence of the onset of germination in this organism. Derepression of a variety of structural genes occurs during development and differentiation of other organisms. For example, during differentiation of the slime mould *Dictyostelium discoideum* from amoeba to spore, simultaneous derepression of several enzymes is required for each morphological change (Sussman & Sussman, 1969).

There are probably further mechanisms for the regulation of amino-acid transport in *Aspergillus nidulans*. Germination of conidia in a medium containing ammonia or glutamine as nitrogen source resulted in a relatively low specific activity of *aaap* without affecting the growth rate. These nitrogen sources do not therefore interfere with the general derepression of the synthesis of proteins required for germination, but must lead to some repression of the synthesis and/or inhibition of the acidic amino-acid permease by a more specific mechanism.

The results presented in this paper suggest that intracellular ammonia may regulate the transport of acidic amino acids, by both repressing the synthesis of the permease and by inhibiting the preformed permease.

Germination of conidia in a medium with a nitrogen source such as arginine, producing a low intracellular concentration of ammonia, led to high activity of the permease during all the stages of germination. Germination with a nitrogen source such as ammonia or glutamine, producing a high intracellular concentration of ammonia, resulted in inhibition and also some repression of the permease. Sufficient information is not available to determine whether or not ammonia was the sole regulatory metabolite during the earlier stages of germination.

The activities of the permeases for transport of glycine, L-methionine, L-lysine, L-phenylalanine and L-arginine were also lower after germination in ammonia or glutamine medium than after germination in glutamate or arginine medium. It is possible that permeases for these amino acids were also regulated by intracellular ammonia (or metabolite of ammonia).

The regulation of amino-acid permeases by ammonia can be rationalized as follows. During growth in the presence of ammonia the low permease activities are likely to provide sufficient amino acids for protein synthesis but not sufficient for any one amino acid to be a major nitrogen source. In ammonia-deficient media an increased activity of the permeases is required so that amino acids are able to supply all the nitrogen for growth.

The effects of ammonia on the transport of glutamate in *Aspergillus nidulans* probably explains the diminished incorporation of [¹⁴C]glutamate (from the growth medium) into the organism during the first 18 h of germination and mycelial growth, when ammonia is included as nitrogen source together with glutamate (C. Anthony & R. J. Cook, unpublished). Similar mechanisms to those described in this paper may also be involved in *Aspergillus clavatus* when this organism uses ammonia in preference to other nitrogen sources provided in the medium (Robinson, 1973).

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