Effects of External Factors on the Amino Acid Pool of *Penicillium griseofulvum*

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In the preceding paper (Bent & Morton, 1964) we reported an investigation into the size and composition of the hot-water-extractable amino acid pool of *Penicillium griseofulvum* and some related fungi during submerged culture of the organisms on a glucose-ammonia medium. Characteristic differences were found between the amino acid pools of conidia and mycelium which were clearly related to distinct stages of development. In the growing mycelium, the pool composition remained almost unchanged (apart from a gradual accumulation of γ -aminobutyric acid) during a considerable period of hyphal growth involving an increase in the proportion of older non-growing parts. Extensive changes only began when the nitrogen source became exhausted and a different type of growth was initiated. It was therefore decided to investigate the effect of external factors on the amino acid pool in the growing mycelium. Effects of pH, of nitrogen source, of availability of oxygen and of certain growth inhibitors were examined, and the results are reported in the present paper.

METHODS

Organism and methods of culture

A strain of *Penicillium griseofulvum* Dierckx, Akers Culture Collection no. 375, was used.

The fungus was grown in shaken culture, on a synthetic glucose-(NH₄)₂SO₄-salts medium partially buffered with sodium succinate, as described by Bent & Morton (1964).

In some experiments (NH₄)₂SO₄ was replaced by KNO₃, casein hydrolysate (Bacto Vitamin-free Casamino Acids; Difco Laboratories Inc., Detroit, Mich., U.S.A.) or single amino acids (obtained from L. Light and Co. Ltd., Colnbrook, Bucks.). In such experiments the N source was autoclaved separately from the remainder of the medium. Glutamic acid and glutamine were sterilized by passage through sintered glass (grade 5).

Anaerobic conditions were established by replacing the cotton-wool plug of the culture flask by a rubber bung, through which passed a glass inlet tube extending almost to the bottom of the flask and a short outlet tube fitted with a one-way rubber valve. Oxygen-free nitrogen was gently

bubbled through the mycelial suspension for 3 min. The inlet tube was then sealed and the flask replaced on the shaker.

Analytical methods

Dry weight and pH measurement, extraction of free amino acids from mycelium and determination of individual amino acids were carried out by the methods described by Bent & Morton (1964). Results were corrected for dilution of mycelial suspensions in experiments where small additions of acid, alkali or inhibitor solutions were made.

Free amino nitrogen. Values for 'α-amino N' were determined as ammonia released by treatment with ninhydrin at pH 2.5 (Sobel, Hirschman & Besman, 1945). Values for 'amino N' were determined by a less-specific colorimetric ninhydrin method (Yemm & Cocking, 1955), and include the N of γ -aminobutyric acid; interference from ammonia N in mycelial extracts was slight (< 3%).

Fresh weight. A 20 ml. sample of suspension was filtered through a sintered-glass disk (grade 3). The mycelial pad was washed twice with water, placed between filter papers (six on each side), passed six times through a small mangle and weighed immediately. This procedure (MacMillan, 1956) gave a consistent measure of fresh weight, the ratio of fresh weight to dry weight in replicate samples differing by less than 3% from the mean. Tests with non-penetrating solutes (by the methods of MacMillan, 1956) indicated that adhering extracellular liquid was present in pads prepared in the above way to the extent of 0.37–0.44 ml./g. apparent fresh wt. A factor of 0.40 ml. of external solution/g. apparent fresh wt. was used to correct fresh-weight values, and also to assess the contribution of surface liquid to extracts prepared from unwashed mycelial pads.

RESULTS

Influence of pH drifts during culture. In the preceding study of the amino acid composition of *P. griseofulvum* (Bent & Morton, 1964) a gradual decline in pH of the routine glucose-ammonia medium from 6.5 to 4.6 was observed during culture, despite the inclusion of sodium succinate as a buffering agent. Effects of continually adjusting the pH during culture to maintain it between 6.0 and 6.5, or of permitting the pH to fall rapidly by omitting sodium succinate from the medium, are shown in Fig. 1.

The course of growth was similar in cultures kept at pH 6.0–6.5 and in unadjusted cultures, where despite the inclusion of succinate the pH declined to 4.6 during the phase of ammonia assimilation.

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The compositions of the amino acid pool in mycelia of these cultures, as revealed by qualitative paper chromatography, appeared to be identical except that γ -aminobutyric acid was undetectable at all stages in cultures kept above pH 6.0. In cultures where pH was not adjusted, γ -aminobutyric acid began to accumulate in the mycelium, as observed by Bent & Morton (1964). It was first detected after 21 hr. incubation, when the pH had declined to 5.83, and then gradually increased; in mycelium from the last harvest it accounted for about 15 % of the total free amino nitrogen.

The omission of succinate led to a more rapid downward drift in pH during culture, but there was no obvious effect on the growth rate or the pool size until 17 hr. At this stage (pH 4.0), however, there began a severe depletion of the amino acid pool followed by a decrease in growth rate. By 19 hr. (pH 3.36) growth had stopped completely. Subsequently pH and mycelial dry weight did not alter appreciably, but depletion of free amino acids continued; at 25 hr. the free α -amino nitrogen content was only 3.4 % of that in normal exponential-phase mycelium. It was clear from qualitative chromatograms that the depletion involved all the pool com-

ponents, including a small amount of γ -aminobutyric acid which was present before depletion started. Chromatograms prepared from samples of the culture medium showed that at least 13 amino acids, in roughly the same proportions as in the amino acid pool, accumulated in the medium at the time of pool depletion. Traces only of three amino acids (glutamic acid, alanine and γ -aminobutyric acid) were detectable in corresponding chromatograms prepared from the cultures containing succinate. Thus it appears that general leakage of free amino acids from *P. griseofulvum* was evoked when the external pH fell below 4.0. An unidentified ninhydrin-positive spot, not detected in chromatograms of mycelial extracts, was given by the succinate-free culture filtrate; this spot, which rapidly changed colour from yellow to purple and ran close to glutamine, was not given after treatment with 6N-hydrochloric acid for 3 hr. at 120°. In a separate experiment it was observed that this loss of the amino acid pool when the pH of succinate-free cultures fell below 4.0 was accompanied by a similar loss of the free nucleotide pool (determined by spectrophotometric examination of ethanolic extracts at 260 and 280 m μ). There seemed therefore to be a sharp change in the permeability relations of the mycelium at this critical external pH.

After the addition of alkali to a succinate-free culture whose growth had been inhibited by acidity for about 20 hr., there were signs of a restoration of metabolic activity (Table 1). Production of dry matter and uptake of ammonia were resumed, intracellular accumulation of free amino acids occurred and renewed growth from many hyphal tips was detectable under the microscope. During a 3 hr. period of renewed growth the mycelial dry weight increased by about 25 % and the free α -amino nitrogen content increased to about 40 % of the normal concentration in growing mycelium. If it is assumed that the new mycelium contained a pool of normal size, then partial replenishment (to about 25 % of the normal level) of the pool in the old depleted mycelium is indicated, suggesting that the breakdown of the retention system under acid conditions is reversible. However, the assumption may not be correct; it is possible that free amino acids were more concentrated in younger parts of the mycelium.

Fig. 1. Effects of pH drifts during culture on the growth and amino acid pool of *P. griseofulvum*. Δ , Cultures on medium buffered with sodium succinate; $\bullet$, cultures on succinate-free medium; $\circ$, cultures on succinate-containing medium kept at pH 6.0-6.5 by the addition of N-NaOH at intervals. Broken lines, mycelial dry wt.; solid lines, pH and free α -amino N content of mycelium.

Table 1. *Effects of raising the pH of a culture of Penicillium griseofulvum inhibited by acid conditions*

Growth and assimilation of nitrogen in succinate-free medium had stopped at approx. 20 hr. At 40 hr. the pH was raised from 3.34 to 6.51 with N-NaOH. Other details are given in the text.				
Age of culture (hr. after inoculation)	pH of medium	Mycelial dry wt. (mg./ml.)	Ammonia N of medium (mg./ml.)	Free α -amino N of mycelium (mg./g. dry wt.)
40	3.34	0.620	0.246	0.14
43	6.51	0.782	0.227	2.51

Effects of pH and oxygen deprivation on exponential-phase mycelium. The absence of γ -aminobutyric acid from mycelium grown in medium kept above pH 6.0 suggested that the gradual accumulation of this compound during normal culture on the glucose-ammonia medium was primarily associated with the increasing acidity of the culture. It seemed possible that restriction of oxygen supply, which may well occur in older thicker mycelial suspensions, might also be involved. An experiment was performed to determine the interaction of pH and anoxia on the accumulation of γ -aminobutyric acid, and also on more general aspects of amino acid metabolism. After growth in the glucose-ammonia medium for 24 hr., during which pH declined from 6.47 to 5.54, mycelial suspensions were maintained for 1.5 hr. at various pH values in the presence and absence of air (Table 2).

During aerobic incubation at pH 6.0, 5.0 and 4.5 increase in dry weight and ammonia assimilation continued at normal rates. Concentrations of amino nitrogen in the mycelium did not alter appreciably. At pH 5.0 and especially at pH 4.5 the proportion of γ -aminobutyric acid increased and there was a slight concomitant fall in the proportions of glutamic acid and alanine; at pH 6.0, however, that of γ -aminobutyric acid diminished and those of glutamic acid and alanine changed little. Changes in the proportions of other pool components were not visible on chromatograms. At pH 4.0 growth and ammonia uptake slowed down, and a fall of about 20% in free amino nitrogen content occurred. This depletion involved most pool components except γ -aminobutyric acid, which increased.

The removal of oxygen led to almost complete inhibition of growth and ammonia uptake at all pH values. At pH 6.0 and 5.0 the free amino nitrogen content did not alter, but accumulations of γ -aminobutyric acid were greater than in air. The proportion of glutamic acid decreased, but there was little change in that of alanine or other pool constituents, except those of glutamine and asparagine which decreased markedly. Loss of these amides during anoxia was also observed in several subsequent experiments. At pH 4.5 and 4.0 the pool size was diminished by about 20 and 50% respectively; these depletions involved all the free amino acids except γ -aminobutyric acid, which accumulated strongly. Analyses of the media showed that extracellular free α -amino nitrogen remained at low concentrations (2–4 $\mu\text{g./ml.}$) in all cultures except the anaerobic culture at pH 4.0, where it rose to 9 $\mu\text{g./ml.}$ This suggests that leakage into the medium accounted for the 50% depletion of the pool observed at pH 4.0. One cannot say whether the smaller depletions that occurred under anaerobic conditions at pH 4.5 and in air at pH 4.0 were also due to leakage, since corresponding extracellular increases would have been within the limits of experimental error.

Effects of oxygen deprivation at different stages of culture. In the above experiment the removal of oxygen revealed a capacity for production of γ -aminobutyric acid in greater amounts than those actually accumulated during the normal course of culture. The question of whether this capacity for anaerobic production of γ -aminobutyric acid gradually increases during aerated culture, as does γ -aminobutyric acid accumulation itself, was

Table 2. *Growth and amino acid composition of Penicillium griseofulvum under different conditions of pH and oxygen supply*

Parallel 24 hr. cultures were treated with N-NaOH or N-HCl to give the listed pH values. The suspensions were then shaken for a further 1.5 hr. either aerobically or after flushing with N_2 . The pH of aerobic suspensions was kept within 0.1 unit of the listed values by the addition of N-NaOH or N-HCl at intervals. The pH of anaerobic suspensions did not alter by more than 0.1 unit. Other details are given in the text.

Conditions during 1.5 hr. incubation period		Mycelial dry wt. (mg./ml.)	Ammonia N of medium (mg./ml.)	Total free amino N of mycelium (mg./g. dry wt.)	Free amino acids of mycelium (mg. of amino N/g. dry wt.)		
Gas phase	pH of medium				Glu	Ala	Aba*
Initial culture		1.60	0.166	5.78	2.35	1.15	0.20
Air	6.0	2.31	0.106	5.76	2.33	1.17	0.11
Air	5.0	2.36	0.102	5.86	2.25	1.11	0.36
Air	4.5	2.28	0.105	5.59	2.09	1.04	0.52
Air	4.0	2.10	0.112	4.46	1.83	0.81	0.35
N_2	6.0	1.66	0.161	5.81	2.02	1.27	0.45
N_2	5.0	1.63	0.160	5.61	1.19	1.08	0.96
N_2	4.5	1.68	0.157	4.63	1.01	0.92	1.06
N_2	4.0	1.63	0.162	2.82	0.85	0.63	0.73

* γ -Aminobutyric acid.

Table 3. *Effects of oxygen removal at three stages of culture on the amino acid pool of Penicillium griseofulvum*

Cultures were grown on the glucose-ammonia medium. In half of the cultures the pH was kept above 6.0 by the addition of *N*-NaOH at intervals. After 20, 24 and 27 hr. of growth suspensions were adjusted to pH 5.0 and shaken for a further 1.5 hr., either aerobically or after flushing with N_2 , at pH 4.9–5.1 (*N*-NaOH was added at intervals to aerobic cultures to maintain pH in this range). Other details are given in the text.

Age of culture (hr.)	pH of culture	Treatment for 1.5 hr. at pH 5.0	Mycelial dry wt. (mg./ml.)	Total free amino N of mycelium (mg./g. dry wt.)	Free amino acids of mycelium (mg. of amino N/g dry wt.)		
					Glu	Ala	Aba*
20	6.01	Initial	0.66	5.90	2.45	1.28	0.00
		Air	0.87	5.82	2.40	1.26	0.11
		N_2	0.69	7.02	2.36	2.27	0.05
24	5.60	Initial	1.68	5.74	2.39	1.15	0.16
		Air	2.39	5.70	2.13	1.10	0.39
		N_2	1.73	5.53	1.31	1.14	1.04
27	4.84	Initial	3.10	5.71	2.17	1.03	0.50
		Air	3.97	5.61	1.80	0.95	0.62
		N_2	3.16	4.79	0.15	0.80	1.68
20	6.0–6.5	Initial	0.64	5.84	2.38	1.19	0.00
		Air	0.91	5.83	2.42	1.16	0.05
		N_2	0.68	6.88	2.30	2.07	0.00
24	6.0–6.5	Initial	1.64	5.69	2.50	1.26	0.00
		Air	2.29	5.64	2.22	1.20	0.21
		N_2	1.66	5.71	1.74	1.26	0.56
27	6.0–6.5	Initial	3.02	5.70	2.24	1.13	0.00
		Air	3.94	5.66	2.15	1.02	0.29
		N_2	3.15	5.25	1.12	0.85	0.92

* γ -Aminobutyric acid.

investigated by removing oxygen at three successive stages (20, 24 and 27 hr.) of culture of *P. griseofulvum* on the glucose-ammonia medium. Behaviour during subsequent anaerobic incubation for 1.5 hr. at pH 5.0 was examined (Table 3). Similar treatments were also given to cultures whose pH was artificially maintained above 6.0 before the removal of oxygen.

Analyses of 'initial' samples confirmed the previous results showing that γ -aminobutyric acid accumulated gradually when the external pH drifted below 6.0, but not when cultures were kept above 6.0. However, aerobic incubation for 1.5 hr. at pH 5.0 evoked some accumulation of γ -aminobutyric acid even in mycelium grown at pH 6.0–6.5, and, moreover, accumulations induced in this way increased with culture age. This suggests that enzyme systems involved in γ -aminobutyric acid production did develop to some extent during growth at pH 6.0–6.5, although accumulation occurred only when the pH was lowered.

Anaerobic incubation at 24 and 27 hr. led to enhanced γ -aminobutyric acid accumulation in mycelium grown at pH 6.0–6.5, and to stronger accumulation in mycelium from uncontrolled cultures. This effect was most marked in the 27 hr. uncontrolled culture, where almost complete depletion of free glutamic acid and correspondingly high accumulation of γ -aminobutyric acid occurred. On

the other hand, at 20 hr. anaerobic incubation inhibited the initial accumulation of γ -aminobutyric acid in both types of culture. Evidently oxygen was necessary for the formation of the γ -aminobutyric acid-producing system, although apparent activity of the system was favoured by anaerobiosis.

Other changes in the response to oxygen deprivation were observed as the cultures aged. At 24 hr., as noted above (Table 2), the removal of oxygen for 1.5 hr. did not affect the overall pool size. At 27 hr., however, pool depletion was induced, especially in the culture without pH control; it seems likely that leakage of amino acids from the mycelium was responsible for the depletion, but measurement of corresponding slight rises in extracellular amino nitrogen was not attempted. Conversely, in young (20 hr.) mycelium oxygen deficiency led to an augmented free amino nitrogen content, largely attributable to the accumulation of alanine.

Effects of growth-inhibitory substances. The effects of adding 2,4-dinitrophenol, sodium azide and potassium cyanide to 24 hr. cultures of *P. griseofulvum* are shown in Table 4. Each compound completely inhibited growth and ammonia uptake, and induced diminution of the amino acid pool by 35–45% in 1.5 hr. Qualitative chromatograms indicated that all the free α -amino acids decreased, but decrease of γ -aminobutyric acid only occurred in the

presence of cyanide. Treatment with dinitrophenol and azide led to enhanced accumulation of this compound and also to more severe depletion of glutamic acid than was induced by cyanide (Table 4).

In inhibited cultures, the free α -amino nitrogen content of the medium rose by amounts corresponding roughly to the concomitant falls in the amino acid pool. Qualitative chromatography showed that amino acids appeared in the medium. The pattern of the released amino acids was in each case broadly similar to that of the initial amino acid pool, except that in the presence of dinitrophenol or azide the proportion of γ -aminobutyric acid was higher in the medium.

The results of a separate experiment with five other inhibitors are also given in Table 4. Diminution of the amino acid pool was induced by hydroxylamine, and, more markedly, by arsenite or malonic acid. Hydroxylamine-induced depletion involved all the pool constituents except γ -aminobutyric acid and glutamine, which increased in amount. Malonic acid led to depletion of all components except γ -aminobutyric acid, which again accumulated. Treatment with arsenite, however, evoked an accumulation of alanine, the remaining free amino acids being strongly depleted. Analyses for extracellular α -amino nitrogen and qualitative chromatograms prepared from samples of media revealed release from the mycelium of free amino acids during incubation with hydroxylamine or

arsenite, but not with malonic acid. In the presence of hydroxylamine or arsenite the whole range of pool constituents were released; the larger amount of amino nitrogen released with arsenite (which was greater than the amount by which the pool had diminished) was accounted for by a heavy accumulation of alanine in the medium.

Treatment with thiosemicarbazide or *p*-fluorophenylalanine strongly inhibited growth but had generally little effect on the size and composition of the amino acid pool. However, no γ -aminobutyric acid was detectable in thiosemicarbazide-treated mycelium, and the glutamine content increased (roughly twofold) in *p*-fluorophenylalanine-treated material.

Amino acid pool during growth on different nitrogen sources. In the experiments described above ammonium sulphate was provided as the sole source of nitrogen for growth. A comparison of intracellular concentrations of free amino acids between mycelia growing on ammonia, nitrate, casein hydrolysate and a range of single amino acids as sole nitrogen sources is shown in Table 5.

The size and composition of the amino acid pool was similar in cultures growing on ammonia and on nitrate; the presence of γ -aminobutyric acid in ammonia-grown mycelium reflects the low pH in the medium at the time of harvest. With casein hydrolysate all the pool constituents except ornithine, α -aminoadipic acid, glutamine and asparagine were present in increased amounts, but

Table 4. *Effects of growth inhibitors on the amino acid pool of Penicillium griseofulvum*

Samples (10 ml.) of solutions of inhibitors at pH 5.0 were added to 24 hr. cultures, to give the concentrations shown. Cultures were then incubated for a further 1.5 hr. at pH 4.9–5.1 (maintained by the addition of *N*-NaOH at intervals). The results of two separate experiments are shown. Other details are given in the text.

Addition	Mycelial dry wt. (mg./ml.)	Ammonia N of medium (mg./ml.)	Free α -amino N of medium (mg./ml.)	Total free amino N of mycelium (mg./g. dry wt.)	Free amino acids of mycelium (mg. of amino N/g. dry wt.)		
					Glu	Ala	Aba*
Initial	1.54	0.171	0.002	5.74	2.49	1.05	0.16
Water	2.18	0.114	0.002	5.69	2.26	0.94	0.28
2,4-Dinitrophenol (2 mM)	1.49	0.174	0.007	3.04	0.95	0.55	0.73
Sodium azide (0.02 M)	1.51	0.170	0.004	3.72	1.07	0.74	0.82
Potassium cyanide (0.01 M)	1.48	0.169	0.005	3.66	1.63	0.68	0.12
Initial	1.47	0.154	0.003	5.43	2.16	0.94	0.07
Water	2.19	0.077	0.002	4.97	2.10	0.85	0.14
<i>p</i> -Fluorophenylalanine (0.2 M)	1.56	0.126	—	5.24	2.03	0.88	0.16
Hydroxylamine (0.2 M)	1.49	0.147	0.005	4.15	1.81	0.80	0.36
Thiosemicarbazide (0.02 M)	1.65	0.123	0.003	5.20	1.97	0.84	0.00
Sodium arsenite (0.01 M)	1.28	0.133	0.010	2.46	0.60	1.33	0.00
Malonic acid (0.05 M)	1.72	0.130	0.003	2.87	1.28	0.61	0.29

* γ -Aminobutyric acid.

Cultures were grown on glucose-succinate medium (Bent & Morton, 1964) with nitrogen sources at initial concentrations of 0.34 mg. of N/ml. Exponential-phase mycelia were harvested when growth had reached 0.7-1.6 mg. dry wt./ml. (0.23 mg./ml. with hydroxyproline). Final pH values were between 5.85 and 7.00, except with ammonium sulphate (pH 5.48 and 5.54 in two separate cultures). The incubation time varied between 22 and 34 hr. Amino acid concentrations are expressed as mg. of amino N/g. dry wt. (means of triplicate determinations; individual values were within 0.15 mg./g. dry wt. of the mean); the occurrence of an amino acid at a concentration below 0.2 mg./g. dry wt. is recorded as +, and its absence as -. Other details are given in the text.

Nitrogen source	Total free amino N		Cys + CysSH	Asp	Glu	Aad*	Ser	Gly	Thr	Ala	Orn
	(mg./g. dry wt.)	amino N (% of insoluble N)									
Ammonium sulphate	5.57	6.71	+	0.29	2.22	+	+	+	+	1.10	+
Ammonium sulphate	5.65	6.91	+	0.26	2.37	+	+	+	+	0.98	+
Potassium nitrate	4.73	6.42	+	0.25	1.88	+	+	+	+	0.90	+
Casein hydrolysate	6.30	7.58	+	0.34	2.45	+	0.32	+	+	1.21	+
L-Aspartic acid	7.02	8.12	+	0.74	2.32	+	+	+	+	1.80	+
L-Glutamic acid	5.85	8.41	+	0.29	2.79	+	+	+	+	1.37	+
Glycine	6.73	8.40	+	0.31	2.33	+	0.66	0.89	+	1.26	+
L-Alanine	8.48	11.09	+	0.19	2.04	+	+	+	+	3.75	+
L-Ornithine	7.66	9.49	+	0.44	2.32	+	+	+	+	1.42	1.87
L-Lysine	5.26	8.10	+	+	1.24	-	+	+	+	0.98	-
L-Arginine	7.58	9.07	+	0.24	2.63	+	+	+	+	1.07	0.30
γ -Aminobutyric acid	6.83	9.09	+	0.34	2.45	+	+	+	+	2.35	+
L-Methionine	4.45	6.38	+	0.21	1.91	+	+	+	+	0.92	+
L-Leucine	6.01	8.05	+	0.21	2.29	+	+	+	+	0.96	+
L-Glutamine	5.66	7.30	+	0.21	2.14	+	+	+	+	0.74	+
L-Asparagine	7.82	9.71	+	0.70	2.42	+	+	+	+	1.71	+
L-Proline	7.26	8.70	+	0.27	3.02	+	+	+	+	1.40	+
L-Hydroxyproline	4.20	6.54	+	0.22	1.65	+	0.28	+	+	0.76	+
L-Citrulline	5.03	6.98	+	+	1.80	+	+	+	+	0.70	+
Lys + His		Arg	Aba*	Tyr	Met + Val + Try	Phe + Leu + Ileu	Glu(NH ₂)	Asp(NH ₂)	Pro	Hypro	Cit*
Ammonium sulphate	+	+	+	-	+	+	0.38	+	+	+	+
Ammonium sulphate	+	+	+	-	+	+	0.40	+	-	-	-
Potassium nitrate	+	+	+	-	+	+	0.35	+	-	-	-
Casein hydrolysate	0.32	0.27	-	+	+	+	+	+	-	-	-
L-Aspartic acid	+	+	-	-	+	+	0.35	+	-	-	-
L-Glutamic acid	+	+	-	-	+	+	0.21	+	-	-	-
Glycine	+	+	-	-	+	+	0.28	+	-	-	-
L-Alanine	+	+	-	-	+	+	+	+	-	-	-
L-Ornithine	+	+	-	-	+	+	0.27	+	+	-	-
L-Lysine	1.79	+	-	-	+	+	+	+	+	-	-
L-Arginine	+	1.61	-	-	+	+	0.48	+	-	-	-
γ -Aminobutyric acid	+	+	0.72	-	+	-	+	+	-	-	-
L-Methionine	+	+	-	-	0.56	-	+	-	-	-	-
L-Leucine	+	+	-	-	+	1.03	0.24	+	-	-	-
L-Glutamine	0.34	0.25	-	-	+	+	1.10	+	-	-	-
L-Asparagine	+	+	-	-	+	+	0.72	0.84	-	-	-
L-Proline	+	+	-	-	+	+	0.56	+	0.89	-	-
L-Hydroxyproline	+	+	-	-	-	-	+	+	-	-	-
L-Citrulline	+	0.39	-	-	+	+	0.35	+	-	1.45	0.66

* Aad, α -Aminoadipic acid; Aba, γ -aminobutyric acid; Cit, citrulline.

the overall pattern, with glutamic acid and alanine as main components, was retained.

The total free amino nitrogen content of the mycelium tended to be larger during growth on single amino acids than during growth on inorganic nitrogen. This trend is more obvious when the amino nitrogen content is expressed as a proportion of hot-water-insoluble mycelial nitrogen rather than dry weight, since some of the amino acid-grown mycelia (particularly those which developed more slowly) contained relatively low proportions of insoluble nitrogen.

Utilization of an amino acid was always associated with intracellular accumulation of the same amino acid to a higher concentration than in mycelium grown on ammonia or nitrate. Increases above the 'endogenous' concentrations (taken as means of amounts present in nitrate- and ammonia-grown material) varied from 0.47 mg./g. dry wt. (aspartic acid) up to 2.75 mg./g. dry wt. (alanine). Moreover, the ratio of internal concentration (mg./g. fresh wt.) to external concentration (mg./ml. of medium) for each amino acid except methionine (0.85) was greater than unity, varying from 1.14 (glycine) to 8.76 (arginine), indicating that the nitrogen sources were taken into the mycelium in an intact form against an apparent concentration gradient. Stress is not laid on comparative degrees of accumulation of each amino acid, since at the time of sampling relevant factors such as mycelial age, external concentration of the amino acid and pH varied widely between cultures growing on different nitrogen sources. Suffice it to say that there was no obvious correlation between the observed extents of accumulation and the chemical properties of the amino acid or the rate of growth of the fungus.

Although accumulation of the supplied amino acid itself was the most general response observed, significant changes in other pool constituents were specifically induced in some instances: feeding with aspartic acid led to an increase in alanine; glycine to increased serine; ornithine to increased proline; lysine to decreased glutamic acid, aspartic acid and α -aminoadipic acid; arginine to increased glutamic acid and ornithine; γ -aminobutyric acid to increased alanine; glutamine to increased lysine or histidine; asparagine to increased glutamine, alanine and aspartic acid; proline to increased glutamic acid; citrulline to increased arginine and decreased aspartic acid; hydroxyproline to increased serine. Many of these effects, such as augmentation of serine by exogenous glycine, proline by ornithine, ornithine by arginine and aspartic acid by asparagine, can be readily accounted for by direct interconversion through well-established metabolic pathways. Others, such as the depression of α -aminoadipic acid by lysine and augmentation

of glutamic acid by proline and arginine, may reflect feedback inhibition or repression of biosynthetic enzymes. Glutamine appears to be a particularly labile constituent of the amino acid pool; marked differences in amounts of this compound occurred with different nitrogen sources (Table 5), and were also observed between cultures at different stages in the growth cycle (Bent & Morton, 1964).

The bulk-protein composition, measured as amino acids released by acid hydrolysis of hot-water-insoluble residues, was examined in material grown on ammonia, alanine and glycine. No effects of varying the nitrogen source were detected, and the results were not significantly different from analyses of ammonia-grown mycelium presented by Bent & Morton (1964).

In the work described above, mycelium was washed twice with distilled water at room temperature before extraction of the amino acid pool with water at 100°; preliminary tests showed that this washing procedure led to only slight (< 5%) loss of intracellular free amino nitrogen, and that the loss involved the whole range of pool components. Cowie & McClure (1959) found that exogenous threonine accumulated in cells of *Candida utilis* differed from endogenous amino acids in being readily extractable by cold water. In view of this evidence, the question of the removal of amino acids during washing was re-examined with mycelium of *P. griseofulvum* grown on ammonia, alanine and arginine as sole nitrogen sources. Hot-water-extractable free amino nitrogen was measured in unwashed material and in material given two or ten successive washes with cold water. Further samples were treated similarly with fresh nitrogen-free culture medium, diluted with water to give a glucose concentration (2.6%, w/v) roughly iso-osmotic with that of the cultures at time of harvest (2.3–2.6%, w/v).

Two successive washes with water led to slight leaching of amino nitrogen from ammonia-grown mycelium, as observed above, and ten washes caused extensive loss (Table 6). These effects were more marked when pools were augmented with exogenous alanine or arginine. However, treatment with iso-osmotic medium removed much smaller quantities of amino nitrogen, and in this case amounts removed from ammonia-, alanine- and arginine-grown mycelia were similar.

Visual examination of chromatograms prepared from extracts and washings showed that all treatments removed the whole range of endogenous pool components, and that the extent of this process did not vary appreciably between mycelia grown on the different nitrogen sources. However, alanine or arginine which accumulated in the presence of exogenous alanine or arginine was removed in proportionately greater amounts by washing with

Table 6. *Effects of different washing procedures on the free amino nitrogen content of Penicillium griseofulvum grown on ammonia, alanine or arginine as nitrogen source*

The cultures were grown as described in Table 5. Mycelial pads were blotted to ensure uniform amounts of adhering medium (as for fresh wt. determination, see the Methods section), washed repeatedly with approx. 10 vol. of water or iso-osmotic N-free medium at room temperature for 10 sec. and then extracted with water at 100°. Values for free amino N are corrected where appropriate for amino N present in adhering culture medium. Other details are given in the text.

Nitrogen source ...	Free amino N content (mg./g. dry wt.)					
	(NH ₄) ₂ SO ₄		L-Alanine		L-Arginine	
	Mycelium	Washings	Mycelium	Washings	Mycelium	Washings
Unwashed	5.96	—	9.46	—	8.45	—
Water (twice)	5.55	0.36	7.96	1.25	7.20	1.34
Water (ten times)	3.29	2.71	4.38	5.35	4.65	3.38
Medium (twice)	5.76	0.09	9.44	0.06	8.15	0.28
Medium (ten times)	3.98	1.69	7.91	1.91	6.98	1.53

water, although even after ten washes the relatively high intracellular concentrations of these compounds were still apparent. When quantitative determinations of alanine and arginine were made in unwashed and twice-washed material, these indicated that 26 and 34 % respectively of accumulated alanine and arginine were removed by two washes with water, whereas only 4.2 and 6.7 % of the remaining (endogenous) amino acid contents were removed. On the other hand two washes with iso-osmotic medium removed less than 5 % of the accumulated amino acids and less than 3 % of the endogenous pools.

DISCUSSION

In the absence of suitable precautions (such as the inclusion of sodium succinate as a buffering agent), a decline of pH to growth-inhibitory values occurs frequently in fungal cultures with an ammonium salt as nitrogen source (Morton & MacMillan, 1954). Points of interference with the growth process under such circumstances are unknown, but the present observation that in *P. griseofulvum* cessation of growth is preceded by the onset of almost complete leakage of free amino acids and nucleotides from the mycelium suggests that growth may become inhibited by intracellular deficiency of one or more essential metabolites. In view of the simultaneous loss of the whole range of free amino acids, and also of nucleotidic material, it seems likely that some alteration in an overall permeability barrier is induced when the external pH falls below 4.0. Ross (1959) found that exposure of *Verticillium albo-atrum* to growth-inhibitory concentrations of the polyene antibiotic fungichromin led to complete loss of the amino acid pool, and there is now a good deal of evidence that polyene antifungal agents act primarily by damaging the cytoplasmic membrane (Marini, Arnow & Lampen, 1961; Kinsky, 1962). However, the possibility that the liberation of endogenous pool constituents depend on disruptive effects on specific cytoplasmic

binding sites, as suggested for yeast cells by Cowie & McClure (1959), cannot be excluded. Since mycelium remained viable for some hours after the growth-inhibiting pH was reached, it is clear that the inhibitory effect is reversible and that decrease of the endogenous amino acid pool to less than 5 % of the normal overall concentration does not preclude subsequent growth.

The promotion of pool leakage by treatment with several metabolic inhibitors points to an energy requirement for the retention process. Similar findings have been reported for bacteria (Mandelstam, 1958). However, efflux was not generally induced by the removal of oxygen, although growth was strongly inhibited (Tables 2 and 3); presumably anaerobic respiration, a well-established feature of oxygen-starved mould mycelium (Cochrane, 1958), provided sufficient energy for amino acid retention. The induction of pool depletion by malonic acid deserves special comment, since it was not accompanied by concomitant release of amino acids into the medium. A limited increase in mycelial dry weight did occur in the presence of malonic acid, and pool depletion here possibly may reflect greater inhibition of amino acid synthesis than of protein synthesis.

Quantitative variation in the concentrations of free amino acids between fungal mycelia growing on different nitrogen sources has not been investigated previously. However, the capacity for active intracellular accumulation of exogenous amino acids, a phenomenon intensively studied in unicellular micro-organisms, has been demonstrated in a number of moulds under various conditions (Mathieson & Catcheside, 1955; Zalokar, 1961; Jones, 1963). The observation that pools of arginine and alanine concentrated from the medium are washed out of the mycelium of *P. griseofulvum* by cold water much more readily than endogenous pool constituents suggests that the concept of an exogenous 'expandable' or 'concentrating' pool and a separate 'internal' or 'conversion' pool (dis-

tinguishable by the greater sensitivity of the former to osmotic shock), developed by Cowie & McClure (1959) from a study of threonine accumulation by *Candida utilis*, may in some respects be applicable to *P. griseofulvum*. Thus the quoted accumulations (Table 5), which refer to water-washed material, may reflect even higher concentrations *in vivo*; the need for careful selection of washing procedures in further work in this field is clearly indicated. Cowie & McClure (1959) have postulated that interconversion of amino acids does not occur in the 'expandable' pool, but the examples observed in the present work of large increases in pool constituents other than the provided amino acid suggest that in *P. griseofulvum* enzyme systems for interconversion are readily accessible to some exogenous amino acids. Kinetic evidence indicating the presence of separate 'internal' and 'expandable' pools of proline in *Neurospora crassa* has been reported by Zalokar (1961).

γ -Aminobutyric acid will serve as nitrogen source for the growth of *P. griseofulvum* (Table 4) and other moulds (Emmerling, 1902; Roberts, Ayengar & Posner, 1953; Morton & Broadbent, 1955). It is also a direct precursor for the biosynthesis of the antibiotic variotin by *Paecilomyces varioti* (Tanaka, Sashikata & Umezawa, 1962). The endogenous formation of γ -aminobutyric acid has often been detected in fungi (Holden, 1962), but little is known of factors governing its accumulation in the mycelium. Simonart & Chow (1953) found that *Aspergillus oryzae* only formed γ -aminobutyric acid when the external pH was below 4.0. In *Microsporium canis* γ -aminobutyric acid accumulated during growth on several ammonium salts, where pH declined during culture, but only rarely during growth on amino acid media (Chattaway, Toothill & Barlow, 1962). This connexion between γ -aminobutyric acid accumulation and low external pH is amply confirmed by the present results.

Indirect evidence from work with mammalian nervous tissue (reviewed by Roberts, 1960), higher plants (Fowden, 1959; Dixon & Fowden, 1961) and yeasts (Pietruszko & Fowden, 1961) suggests that γ -aminobutyric acid is an intermediate in a series of reactions which may bypass part of the tricarboxylic acid cycle (Scheme 1).

Scheme 1.

Preparations showing glutamate-decarboxylase activity (Gitterman & Knight, 1952; Natarajan, 1962; Tanaka *et al.* 1962) and γ -aminobutyrate-transaminase activity (Roberts *et al.* 1953) have been obtained from several fungi. Since optimum pH seems invariably to be lower for the decarboxylase (generally pH 4–5) than for the transaminase (pH 8–9), the association between γ -aminobutyric acid accumulation and low external pH in *P. griseofulvum* is readily explainable in terms of changing activities of these enzymes. The corresponding losses of glutamic acid observed under anaerobic conditions (Table 5) suggest that γ -aminobutyric acid originates from the glutamic acid pool, and that almost the whole of the latter is susceptible to decarboxylation. An inverse relationship between amounts of glutamic acid and γ -aminobutyric acid was also observed in conidia of *P. griseofulvum* (Bent & Morton, 1964). In the fungus *Fusarium vasinfectum* (Natarajan, 1962), as in bacteria (Gale, 1946), glutamate decarboxylase is synthesized best under acidic conditions and forms gradually during culture. Conversely, synthesis of γ -aminobutyrate transaminase in *Aspergillus fumigatus* was stronger during growth at pH 8.5 than at 5.6 (Roberts *et al.* 1953). Thus the evidence (Table 4) that a system responsible for γ -aminobutyric acid accumulation gradually develops during culture, especially when the pH declines, is again consistent with the formation and metabolism of γ -aminobutyric acid by decarboxylation and transamination reactions respectively. The enhancement of γ -aminobutyric acid accumulation by transfer to anaerobic conditions (an effect observed in barley leaves by Naylor & Tolbert, 1956) and by treatment with azide or malonate supports the view that it is further metabolized by an oxidative pathway. Inhibition of its accumulation by cyanide does not detract from this view, since glutamate decarboxylases of bacteria and plants are cyanide-sensitive (Gale, 1946; Okunuki, 1937). The opposing effects of the carbonyl-trapping agents hydroxylamine and thiosemicarbazide on the concentration of γ -aminobutyric acid are in keeping with similar observations by Baxter & Roberts (1962) on brain tissue.

Thus our results indicate that the metabolic system outlined in Scheme 1 may well operate in the mycelium of *P. griseofulvum*, and also that the activity of such a system varies with the age of the mycelium and with the pH of the medium.

The accumulation of endogenous alanine is a prominent feature in young mycelium deprived of oxygen, and in arsenite-treated mycelium; possibly alanine formation under these circumstances originates from an increased availability of pyruvate. Similar accumulation of alanine under anaerobic conditions has been observed in houseflies (Price,

1963) and in yeast (Pietruszko & Fowden, 1961). No explanation can be offered at present for the absence of alanine accumulation on the removal of oxygen at later stages of culture.

SUMMARY

1. The amino acid pool of *Penicillium griseofulvum* changed in size and composition in response to downward drifts in pH which occurred during culture on media containing ammonium sulphate as nitrogen source. Intracellular accumulation of endogenous γ -aminobutyric acid occurred only when the pH fell below 5.8. An almost complete efflux of amino acids from the mycelium started when the pH fell below 4.0.

2. Oxygen starvation generally enhanced the accumulation of endogenous γ -aminobutyric acid, to a degree which depended on pH during anoxia and on the age and pH of the culture at the time of the removal of oxygen; concomitant depletions of glutamic acid occurred. Removal of oxygen from young cultures, however, inhibited γ -aminobutyric acid formation and led to an increase in the concentration of alanine.

3. Leakage of amino acids into the medium and alterations in pool composition were observed after the addition of certain growth-inhibitory reagents.

4. When compared with mycelium grown on an inorganic nitrogen supply, mycelia grown on single amino acids as sole nitrogen sources contained increased amounts of the supplied amino acids; transport against apparent concentration gradients was indicated. Changes in amounts of other pool constituents were induced by some amino acids. Amino acids derived from the medium were more readily washed out of mycelium by water (but not by iso-osmotic medium) than were endogenous amino acids.

REFERENCES

Baxter, C. F. & Roberts, E. (1962). In *Amino Acid Pools*, p. 499. Ed. by Holden, J. T. Amsterdam: Elsevier Publishing Co.

- Bent, K. J. & Morton, A. G. (1964). *Biochem. J.* **92**, 260.
 Chattaway, F. W., Toothill, C. & Barlow, A. J. E. (1962). *J. gen. Microbiol.* **28**, 721.
 Cochran, V. W. (1958). *Physiology of Fungi*, p. 212. New York: John Wiley and Sons Inc.
 Cowie, D. B. & McClure, F. T. (1959). *Biochim. biophys. Acta*, **31**, 236.
 Dixon, R. O. D. & Fowden, L. (1961). *Ann. Bot., Lond., N.S.*, **25**, 513.
 Emmerling, O. (1902). *Ber. dtsh. chem. Ges.* **35**, 2289.
 Fowden, L. (1959). *Symp. Soc. exp. Biol.* **13**, 283.
 Gale, E. F. (1946). *Advanc. Enzymol.* **6**, 1.
 Gitterman, C. O. & Knight, S. G. (1952). *J. Bact.* **64**, 223.
 Holden, J. T. (1962). In *Amino Acid Pools*, p. 83. Ed. by Holden, J. T. Amsterdam: Elsevier Publishing Co.
 Jones, O. T. G. (1963). *J. exp. Bot.* **14**, 399.
 Kinsky, S. C. (1962). *Proc. nat. Acad. Sci., Wash.*, **48**, 1049.
 MacMillan, A. (1956). *J. exp. Bot.* **7**, 113.
 Mandelstam, J. (1958). *Biochem. J.* **69**, 103.
 Marini, F., Arnow, P. & Lampen, J. O. (1961). *J. gen. Microbiol.* **24**, 51.
 Mathieson, M. J. & Catcheside, D. G. (1955). *J. gen. Microbiol.* **13**, 72.
 Morton, A. G. & Broadbent, D. (1955). *J. gen. Microbiol.* **12**, 248.
 Morton, A. G. & MacMillan, A. (1954). *J. exp. Bot.* **5**, 232.
 Natarajan, S. (1962). *J. Indian bot. Soc.* **41**, 305.
 Naylor, A. W. & Tolbert, N. E. (1956). *Physiol. Plant.* **9**, 220.
 Okunuki, K. (1937). *Bot. Mag., Tokyo*, **51**, 270.
 Pietruszko, R. & Fowden, L. (1961). *Ann. bot., Lond., N.S.*, **25**, 491.
 Price, G. M. (1963). *Biochem. J.* **86**, 372.
 Roberts, E. (1960). In *Inhibition in the Nervous System and γ -Aminobutyric Acid*, p. 144. Ed. by Roberts, E. New York: Pergamon Press.
 Roberts, E., Ayengar, P. & Posner, I. (1953). *J. biol. Chem.* **203**, 195.
 Ross, J. P. (1959). *Phytopathology*, **49**, 422.
 Simonart, P. & Chow, K. Y. (1953). *Leeuwenhoek ned. Tijdschr.* **19**, 121.
 Sobel, A. E., Hirschman, A. & Besman, L. (1945). *J. biol. Chem.* **161**, 99.
 Tanaka, N., Sashikata, K. & Umezawa, H. (1962). *J. Antibiot. A*, **15**, 228.
 Yemm, E. W. & Cocking, E. C. (1955). *Analyst*, **80**, 209.
 Zalokar, M. (1961). *Biochim. biophys. Acta*, **46**, 423.