Amino Acid Composition of Fungi during Development in Submerged Culture

By K. J. BENT* and A. G. MORTON†
Imperial Chemical Industries Ltd., Pharmaceuticals Division, Akers Research Laboratories, Welwyn, Herts.

(*Received* 23 *December* 1963)

Investigations of amino acid requirements of fungal mutants and of assimilation of ^{14}C-labelled substrates by fungi have yielded extensive information about biosynthetic pathways by which carbon skeletons of amino acids are formed in these organisms. Knowledge of the amino acid composition of intact fungal mycelium and spores is less satisfactory. Reports giving qualitative or, less often, quantitative descriptions of free amino acid pools in various fungi are available (reviewed by Holden, 1962), and a few analyses of bulk-protein composition have also been made. However, very little attention has been given to the relation between amino acid composition and the physiological and morphogenetic states of the fungus, or to the influence of environment.

The aim of the present work was to provide a more comprehensive study of the free and combined amino acids of a particular fungus throughout its life cycle. For this purpose a strain of *Penicillium griseofulvum* was examined in detail, and supplementary observations were made on five other moulds for comparison. To secure relatively homogeneous samples of mycelium in a controlled environment, the fungi were grown in submerged culture in a synthetic medium. The use of surface-culture methods, which yield a heterogeneous mycelial mat in an undefinable environment, has limited the value of many previous studies in this field.

METHODS

Organisms

The main experimental material was a strain of *Penicillium griseofulvum* Dierckx, Akers Culture Collection no. 375, that has been used extensively for previous physiological work in this Laboratory. The following fungi were also used (numbers refer to strains in the culture collection of the Akers Research Laboratories): *Aspergillus niger* van Tiegh (ACC 32), *Penicillium chrysogenum* Thom. (ACC 167), *P. expansum* (Link.) Thom (ACC 57), *P. roqueforti* Thom. (ACC 509), *Trichoderma viride* Pers. ex Fries (ACC 211).

* Present address: Plant Protection Ltd., Jealott's Hill Research Station, Bracknell, Berks.

† Present address: Department of Botany and Zoology, Chelsea College of Science and Technology, London, S.W. 3.

Methods of culture

Conidia from 10- to 20-day cultures on Czapek–Dox agar slopes were suspended in a sterile 0·05% solution of an anionic detergent (Teepol; Shell Chemical Co.), centrifuged and resuspended in sterile water at $0{\cdot}8 \times 10^{-7}$–$1{\cdot}2 \times 10^{7}$ conidia/ml. (by haemocytometer count). Then 10 ml. volumes of conidial suspension were transferred to 700 ml. round flasks, each containing 200 ml. of medium. Flasks were plugged with cotton-wool and incubated at 25° on a reciprocating shaker (240 strokes/min.). The fungi grew as well-dispersed suspensions of hyphae; individual thalli were separate or in loose tangles.

For the purpose of conidia production, similarly prepared cultures of *P. griseofulvum* were grown without shaking in flat-bottomed glass culture vessels. The mycelium grew as a mat at the surface of the liquid medium, and produced abundant aerial conidiophores.

The following glucose–ammonia medium was used: glucose, 30 g.; $(NH_4)_2SO_4$, 1·6 g.; sodium succinate, 5·0 g.; KH_2PO_4, 1·0 g.; $MgSO_4,7H_2O$, 0·5 g.; trace-element mixture containing 200 μg. of Fe, 38 μg. of Cu, 225 μg. of Zn, 25 μg. of Mn, 40 μg. of Mo; deionized water to 1 l. The pH was adjusted initially to 6·6–6·7 with N-NaOH; it dropped about 0·2 pH unit during sterilization (at 15 lb./in.^{2} for 15 min.). Sodium succinate was included as a buffering agent, to mitigate the fall in pH that occurs during culture of fungi on ammonium sulphate media (Morton & MacMillan, 1954).

Preparation of samples

Mycelium. Suitable volumes of culture suspension were filtered through sintered-glass disks (grade 3). Filtrates were retained for analysis. Mycelial pads were washed twice by stirring for 10 sec. with roughly 10 pad-volumes of water at room temperature, and used for extraction as described below. Similar parallel samples of mycelium were used for dry weight.

Conidia. Conidial suspensions were obtained from agar slopes (by adding water at 2°, and gently scraping with a glass rod), from still liquid cultures (by cooling to 2°, and shaking to submerge the mycelial mat and disperse the conidia) and from sporulating submerged cultures. Hyphal material was removed by passing the suspensions through a coarse sintered-glass disk (grade 1). Suspensions were centrifuged, and the conidial sediments washed twice by mixing with cold water and recentrifuging before extraction or determination of dry weight.

Analytical methods

Dry weight. Mycelial and conidial pads were weighed after drying at 80° for 24 hr.

Glucose. Glucose content of culture medium was determined by an anthrone method (Fairbairn, 1953).

Total nitrogen. This was determined by micro-Kjeldahl analysis, with selenium dioxide as catalyst.

Ammonia. The method of Conway (1947) was used.

Amide nitrogen. This was determined as ammonia produced during 3 hr. hydrolysis of extraction residues by N-H_2SO_4 at 100°.

Free α-amino nitrogen. This was usually determined as ammonia released by reaction with ninhydrin at pH 2·5 (Sobel, Hirschman & Besman, 1945). Ammonia was collected by a vacuum-distillation method (Koch & Hanke, 1948) and measured by the Nessler technique.

Occasionally a less-specific colorimetric ninhydrin method (Yemm & Cocking, 1955) was used to determine the total free amino N of mycelial extract; the use of this method is indicated specifically in Tables.

Protein α-amino nitrogen. Samples of dried (at 80° for 24 hr.) and ground extraction residues were hydrolysed with 6 N-HCl in stoppered tubes at 120° for 3 hr. The yield of α-amino N was maximal at this time. To minimize interference from carbohydrates, at least 4 ml. of 6 N-HCl/mg. of protein was used (Dustin, Czajkowska, Moore & Bigwood, 1953). Hydrolysates were dried under vacuum and dissolved in water. Their α-amino N contents were determined by the method of Sobel *et al.* (1945).

Peptide nitrogen. The term 'peptide nitrogen' is used in this paper to denote α-amino N released by acid hydrolysis of mycelial extracts. Equal volumes of conc. HCl were added to samples of extracts. Hydrolysis and analysis were then carried out as for protein α-amino N.

Separation of amino acids. Two-dimensional chromatograms of extracts and hydrolysates were prepared on Whatman no. 1 paper, with butan-1-ol–acetic acid–water (50:11:25, by vol.) and phenol–water (10:3, w/v, containing 0·01% of 8-hydroxyquinoline) as successive solvent systems. Amino acids were detected by spraying with a ninhydrin solution [0·2% in 95% (v/v) ethanol]. Spots were identified by co-chromatography with authentic markers and by specific tests given by Block, Durrum & Zweig (1958). The 2-methylpropan-2-ol solvent systems of Boissonas (1950) were used to separate phenylalanine from leucine + isoleucine and methionine from valine in protein hydrolysates. Histidine and lysine in protein hydrolysates were separated by one-dimensional development for 5 days with the butan-1-ol–acetic acid–water solvent.

Quantitative determination of amino acids. Two-dimensional chromatograms of samples containing 50 μg. of α-amino N were prepared with the butan-1-ol–acetic acid–water and phenol–water solvent systems described above. After drying in a cool air-stream for 16 hr., residual phenol was removed by washing with ether. Papers were sprayed on both sides with 0·2% KOH in ethanol, heated at 60° for 5 min. to remove ammonia, dipped in ninhydrin solution (0·1% in acetone), dried and again heated at 60° for 5 min. The lightly coloured spots were cut out, weighed, cut into pieces and placed in test tubes. Amino N contents were determined by the method of Yemm & Cocking (1955). Proline was measured at 440 mμ. Three blank areas of each chromatogram were used to determine background extinctions. From chromatograms containing amino acids at four known amounts (2, 4, 6 and 8 μg.) a standard curve was prepared for each spot. To check reproducibility, control chromatograms containing 4 μg. of each amino acid were run with each batch of experimental chromatograms. Cysteine and cystine were determined jointly after oxidation to cysteic acid; extracts and insoluble residues were treated with performic acid, according to the method of Schram, Moore & Bigwood (1954), before direct chromatography or hydrolysis with 6 N-HCl. A correction for the low yield (90%) of cysteic acid was applied. To avoid concomitant degradation of free serine and threonine separate samples of extracts were used for this procedure.

Except for proline, replication between triplicate standard samples was ±7% and recoveries were 92–104% of the expected values. With proline replication was ±10% and recovery 87–108%. Replication of experimental samples depended on the relative concentration of the amino acid. The mean value for each amino acid is presented as a percentage of total amino N of the sample, and individual values differed from this mean value by amounts representing up to 2·5% of the total amino N.

RESULTS

Fractionation of mycelium

It was necessary at the outset of the present study to find a suitable means of separating free amino acids from protein of the mould, the simplest approach being treatment of fresh material with an agent that would destroy permeability barriers of the cell, dissolve free amino acids and precipitate protein. Several extracting agents have been used in previous studies on fungi, but comparative data on efficiency and specificity of extraction are not available. The most widely used solvents have been aqueous ethanol, aqueous trichloroacetic acid and boiling water. The fractionation of the mycelium of *P. griseofulvum* by these three agents was investigated.

Mycelial samples from a 21 hr. culture were immersed in roughly 10 times their volume of extractant. The mixtures were stirred briefly at the beginning and end of each extraction period. After extraction the mixtures were brought to room temperature, insoluble residues were filtered off and rinsed twice with extractant at room temperature, and extracts and washings were combined. Since trichloroacetic acid and ethanol interfere with some analytical methods, trichloroacetic acid was removed by three successive extractions with equal volumes of ether, and ethanol by drying under vacuum at 40°. Extracts were then diluted to standard volume with water and stored at −10° until required. After storage, precipitates were found in hot-trichloroacetic acid and hot-ethanol extracts; these were removed by centrifugation.

To test for completeness of extraction, the residues were re-extracted by the same procedures.

Each system was tested in duplicate, and average values are shown in Table 1. Duplicate values differed by less than 4%, except those of peptide nitrogen which differed by up to 12%.

Table 1. *Comparison of compositions of extracts prepared from* Penicillium griseofulvum *by different procedures*

Experimental details are given in the text. Results are expressed as mg./g. dry wt. of mycelium. The values in parentheses refer to second extractions.

Extraction procedure	Total solid matter	Total N	Free α-amino N	Peptide α-amino N
Hot water (100°, 10 min.)	155	9·58 (0·74)	5·43 (0·11)	0·62 (0·20)
Hot 5% (w/v) trichloroacetic acid (100°, 1 hr.)	375	26·20 (3·15)	5·78 (0·32)	3·38 (0·88)
Cold 5% (w/v) trichloroacetic acid (4°, 24 hr.)	97	9·70 (1·10)	5·55 (0·07)	0·52 (0·16)
Boiling 65% (v/v) ethanol (10 min.)	164	9·60 (1·30)	5·38 (0·14)	0·59 (0·16)
Cold 65% (v/v) ethanol (4°, 1 hr.)	124	9·25 (0·35)	5·20 (0·06)	0·58 (0·09)

Four of the five procedures extracted almost identical amounts of total nitrogen, free α-amino nitrogen and peptide nitrogen. Hot-trichloroacetic acid extracts, however, were richer in total solids, total nitrogen and peptide nitrogen, although the free α-amino nitrogen content was similar to that in the other extracts. Obviously considerable protein breakdown took place during hot-trichloroacetic acid extraction.

The free amino acid composition as revealed by qualitative paper chromatography was similar in all extracts, except that glutamine was absent and the asparagine spot weaker in hot-trichloroacetic acid extracts. After acid hydrolysis four of the extracts again showed a common amino acid pattern, but hot-trichloroacetic acid extracts had a different composition with higher proportions of threonine, lysine + histidine, arginine, methionine + valine, phenylalanine + leucine + isoleucine and proline.

Subsequent extraction removed further nitrogenous material (4–14% of that in primary extracts), but the amounts of free α-amino nitrogen in second extracts were low. Chromatographic examination showed that these low amounts were due to a mixture of amino acids in roughly the same proportions as in primary extracts, i.e. no particular amino acids were extracted more slowly than the others.

Thus hot water, cold 5% (w/v) trichloroacetic acid, cold 65% (v/v) ethanol or boiling 65% (v/v) ethanol all appeared to be suitable agents for extracting the amino acid pool from *P. griseofulvum*. Smaller amounts of combined amino acids were also consistently extracted by these solvents. The hot-water procedure was used for all further experiments; subsequent references to 'soluble' and 'insoluble' fractions refer to extractability by hot water.

A decrease in the period of hot-water extraction from 10 to 5 min. led to lower extraction of free α-amino nitrogen; extending the period to 30 min. had no effect. Disruption of mycelium and conidia by various means before extraction with hot water did not increase the amount of free amino acids extracted. It was found that the procedure of

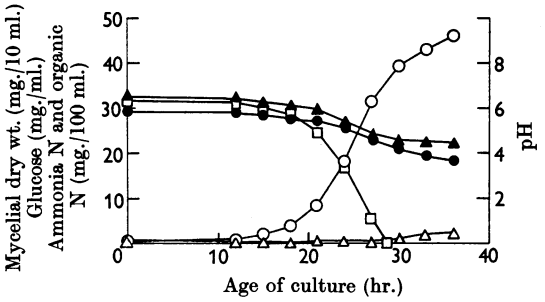

Fig. 1. Growth of mycelium and changes in composition of the medium during shaken culture of *P. griseofulvum*. ○, Mycelial dry wt.; ▲, pH; ●, glucose; □, ammonia N; △, extracellular organic N.

washing with cold water led to a small loss (4·8%) of free α-amino nitrogen; the washings contained the whole range of pool components, no particular amino acids being leached out preferentially. Prolonged washing led to more serious losses.

Amino acid composition of Penicillium griseofulvum *during growth in shaken culture*

Vegetative growth. A suspension of conidia from 15-day agar cultures was used to inoculate flasks of glucose–ammonia medium. Analyses were made of the conidia used for inoculation and of the mycelium at 3 hr. intervals during growth from 12 to 36 hr. after inoculation. In this medium and in these experimental conditions the fungus grows vegetatively but never sporulates even after the nitrogen in the medium is exhausted.

Results are shown in Fig. 1 and in Tables 2–4. In comparison with growing mycelium, conidia had a low total nitrogen content (5·8% of dry wt.). Proportions of soluble nitrogen and free α-amino nitrogen were also relatively low in conidia (respectively 8·5% and 3·6% of total mycelial nitrogen). During germination, which was complete at 12 hr., total nitrogen content increased to 8·7% and remained at a high level (8·7–9·0%) during the ensuing phase of exponential growth. Proportions of soluble nitrogen and free α-amino nitrogen rose

during germination to 11·7 and 7·1 % of total nitrogen, and then remained fairly steady at slightly lower levels. The proportion of free α-amino nitrogen declined somewhat towards the end of the rapid growth phase. Small amounts of peptide nitrogen, equivalent to 10–20 % of the free α-amino nitrogen, were present in conidia and exponential-phase mycelium.

Ammonia nitrogen in the medium was exhausted at about 28·5 hr. During the subsequent 7·5 hr. the fungus continued to grow in length and to increase in dry weight at the expense of the residual glucose in the medium. A 20–25 % increase in length of individual mycelia was observed under the microscope, while mycelial dry weight increased by about 30 %. There was a concomitant fall in mycelial nitrogen content from 8·7 to 6·6 % of dry wt. (Table 2). This fall was mostly a reflexion of the accumulation of non-nitrogenous cellular material (found in separate experiments to be mainly carbohydrate), since the absolute amounts of mycelial nitrogen declined relatively slowly (Table 3). A small release of nitrogenous organic material into the medium started at about the time of ammonia exhaustion (Fig. 1 and Table 3). In the final (36 hr.) culture medium 10–15 % of this material was free α-amino nitrogen and 50–55 % was combined α-amino nitrogen which was released by treatment with 6 N-hydrochloric acid. The absolute amounts of free α-amino nitrogen in the mycelium fell to about half in the first 4 hr. after ammonia exhaustion and then remained steady at the new level (Table 3). However, the amount of intracellular peptide nitrogen increased more than twofold after ammonia exhaustion.

The composition of the amino acid pool at different stages of growth is shown in Table 4. In conidia the major components were glutamic acid, γ-aminobutyric acid and glutamine, the last-named accounting for 37 % of the pool amino nitrogen. Apart from alanine, other amino acids were at low concentrations or were not detected. A striking change in pool composition occurred during germination. At 12 hr. after inoculation, when the spores were swollen and bore short germ-tubes, the chief components were glutamic acid (39 %) and alanine (18 %). Other amino acids, including α-aminoadipic acid, were detected, but apart from aspartic acid (7 %) and glutamine (7 %) none of these accounted for more than 3 % of the pool; γ-aminobutyric acid was absent. A similar pattern of free amino acids persisted throughout the period of rapid growth, the only substantial change being a reappearance of γ-aminobutyric acid at about 21 hr. This amino acid then gradually increased in amount and at 27 hr. accounted for

Table 2. *Relative amounts of nitrogenous fractions in the mycelium of* Penicillium griseofulvum *during growth in shaken culture*

Experimental details are given in the text.

Age of culture (hr. after inoculation)	Total N (% of dry wt.)	Soluble N (% of total N)	Free α-amino N (% of total N)	Peptide α-amino N (% of total N)
0 (conidia)	5·83	8·50	3·62	0·70
12	8·70	11·65	7·08	0·81
15	9·05	10·49	5·85	0·79
18	8·84	10·71	6·02	0·70
21	8·93	10·42	5·72	0·72
24	8·85	10·30	5·51	0·67
27	8·71	10·66	5·23	0·81
30	8·20	7·92	2·93	0·97
33	7·24	7·95	2·20	1·51
36	6·59	9·71	2·27	2·07

Table 3. *Amounts of nitrogen per flask at late stages of shaken culture of* Penicillium griseofulvum

Experimental details are given in the text. All values are expressed as mg./flask (200 ml. of suspension).

Age of culture (hr. after inoculation)	Mycelium					Medium		Total N (mycelium + medium)
	Total N	Insoluble N	Soluble N	Free α-amino N	Peptide α-amino N	Organic N	Ammonia N*	
27	54·8	49·0	5·82	2·86	0·44	0·5	10·9	66·2
30	64·9	59·8	5·14	1·90	0·63	1·5	0	66·4
33	63·4	58·0	5·02	1·40	0·95	2·2	0	65·6
36	62·6	56·6	6·08	1·42	1·29	3·2	0	65·8

* Exhausted at approx. 28·5 hr.

12·5 % of the free amino nitrogen, the proportions of glutamic acid, alanine and aspartic acid being depressed.

On exhaustion of the inorganic nitrogen supply rapid depletions of free glutamine, ornithine and arginine occurred, together with more gradual falls in the proportions of glutamic acid and alanine. Minor pool components (such as lysine + histidine, threonine, methionine + valine tryptophan and phenylalanine + leucine + isoleucine) increased in proportion, so that by 36 hr. a more even pattern of free amino acids was apparent.

The bulk-protein composition, determined as amino acids released from hot-water-insoluble material by 6N-hydrochloric acid hydrolysis, was stable throughout culture (Table 4). The distribution of constituent amino acids was more uniform than in the pool. Ornithine, γ-aminobutyric acid, α-aminoadipic acid and hydroxyproline were not detected in the hydrolysates. Protein α-amino nitrogen represented 45–48 % of the total insoluble nitrogen at all stages of culture. Since α-amino nitrogen accounted for only 69·5 % of the protein nitrogen, it follows that 65–69 % of the hot-water-insoluble nitrogen was attributable to protein. It can be deduced from the data presented that protein accounted for 21 % of dry weight in conidia, rose to 29–32 % in exponential-phase mycelium and declined to 23 % in older nitrogen-starved mycelium.

The presence of combined α-amino nitrogen in hot-water extracts of the fungus at all stages of culture has been mentioned above. Hydrolysis of extracts with 6N-hydrochloric acid before chromatography led to disappearance of the two amide spots and of a spot near the origin, and to intensification of all other spots except those of ornithine, γ-aminobutyric acid and α-aminoadipic acid. Increases in the proportions of glutamic acid, cysteic acid and glycine were particularly strong. Material eluted from the spot near the origin was identified as GSH by separation of the hydrolysis products and by co-chromatography with an authentic sample. The amount of GSH nitrogen was sufficient to account for 50–65 % of the peptide nitrogen of spores and exponential-phase mycelium, and for 32 % of the larger-peptide fraction of 36 hr. (nitrogen-starved) material. The presence of GSH in mould mycelia is well-documented (e.g. De Flines, 1955; Fuerst & Wagner, 1957; Arnstein, Artman, Morris & Toms, 1960).

Table 4. *Composition of free amino acid pool and of bulk protein of* Penicillium griseofulvum *during growth in shaken culture*

Analyses of the amino acid pool at ten stages of culture are shown. Protein composition is the average of analyses at five stages of culture (0, 12, 21, 27 and 36 hr.); extreme values obtained are given in parentheses. The results express amino N of individual amino acids as percentages of total amino N (total α-amino N + N of γ-aminobutyric acid and proline) of the sample. —, Amino acid not detected. Experimental details are given in the text.

	Amino acid pool										
Age of culture (hr. after inoculation)	0 (conidia)	12	15	18	21	24	27	30	33	36	Protein
Cyst(e)ine	—	1·6	0·9	1·7	1·4	0·7	1·3	1·3	1·7	2·0	1·1 (0·7–1·2)
Aspartic acid	3·1	7·1	6·4	6·8	5·0	5·3	4·4	5·0	5·1	3·2	9·9* (9·6–10·1)
Glutamic acid	23·9	39·3	45·0	43·7	39·1	39·2	37·1	36·4	32·2	22·7	13·5* (12·9–13·8)
α-Aminoadipic acid	—	2·0	0·8	0·9	1·2	0·9	1·5	1·0	1·5	1·2	—
Serine	1·9	2·1	2·0	1·6	2·5	2·2	2·9	3·1	3·3	5·0	6·3 (5·8–6·4)
Glycine	—	1·7	1·5	2·2	1·5	1·6	1·9	2·4	2·8	4·1	6·9 (6·4–7·2)
Threonine	—	—	1·4	1·6	1·1	1·8	1·0	1·0	2·5	4·3	5·5 (5·2–5·6)
Alanine	7·2	18·2	17·3	15·2	17·5	15·7	12·6	13·0	13·0	12·4	10·2 (9·8–10·5)
Ornithine	—	2·2	2·5	2·5	1·4	1·9	2·1	1·0	1·0	1·5	—
Lysine Histidine	—	1·4	1·8	1·8	1·5	1·9	1·3	2·3	3·8	4·9	4·9 (4·6–5·1) 2·7 (2·4–2·9)
Arginine	0·8	2·8	2·6	2·9	3·0	2·3	2·0	1·3	1·6	1·2	4·9 (4·7–5·3)
γ-Aminobutyric acid	19·3	—	—	—	1·3	5·1	12·5	12·9	11·1	13·8	—
Tyrosine	—	—	—	0·9	—	—	1·1	—	1·2	1·6	3·0 (2·7–3·1)
Methionine Valine Tryptophan	—	1·4	1·0	1·1	2·0	1·8	1·2	1·9	4·0	4·4	1·6 (1·5–1·8) 6·4 (6·0–6·6) —
Phenylalanine Leucine + isoleucine	—	1·3	1·7	1·9	1·6	2·0	1·6	1·9	2·9	5·1	3·2 (3·0–3·4) 9·7 (9·2–9·9)
Glutamine	36·8	7·2	5·1	6·3	5·9	6·6	5·6	1·8	1·5	1·5	—
Asparagine	—	1·3	1·6	1·7	1·3	2·2	1·7	—	—	—	—
Proline	—	—	—	—	1·6	—	—	—	—	1·4	4·9 (4·0–5·6)
Total	93·0	89·6	91·6	92·8	88·9	91·2	91·8	86·5	89·2	90·3	94·8

* These values include amino N from asparagine and glutamine. Amide N of protein was equivalent to 9·4 % (9·2–9·6 %) of the total protein amino N.

Qualitative chromatograms were also prepared from acid hydrolysates of extracts in which free amino acids had previously been destroyed by reaction with ninhydrin at pH 2·5 (a procedure recommended by Markovitz & Steinberg, 1957). These confirmed that, in addition to GSH components, the peptide fraction included a wide range of other amino acid residues. The latter were distributed in roughly the same proportions as in protein, and markedly increased in quantity during the nitrogen-deficient phase of culture.

Analysis of peptide α-amino nitrogen in dialysed extracts showed that in conidia and exponential-phase mycelium 70–85 % of the 'non-GSH' peptide material was diffusible through cellophan, whereas in 36 hr. material only 41 % was diffusible. Thus the increase in mycelial peptide nitrogen observed at later stages of culture was due to accumulation in the soluble fraction of material that had broadly the same amino acid composition as protein and of which only 25–30 % was diffusible through cellophan. A separate test showed that this hot-water-extractable peptidic material that accumulated after exhaustion of ammonia could not be extracted with cold 5 % (w/v) trichloroacetic acid and was only partially (15–20 %) extracted with 65 % (v/v) ethanol, whereas all three treatments extract similar amounts of peptide nitrogen from exponential-phase mycelium (Table 1).

Sporogenous growth. Like many other moulds, *P. griseofulvum* does not form conidia in submerged culture on a normal glucose–ammonia medium. However, abundant conidiation can be induced if pure glucose is replaced by a crude commercial glucose (Morton, England & Towler, 1958), an effect shown to be due to the presence of traces of Ca^{2+} ions and anhydroglucose (Armstrong, England, Morton & Webb, 1963). Since metabolic changes associated with morphogenetic changes of mycelium from the vegetative to the sporogenous state are at present unknown, a comparison was made between the amino acid compositions of mycelia growing on normal glucose–ammonia medium and on crude-glucose–ammonia medium (reagent-grade glucose replaced by crude glucose). In the former medium the mycelium remained vegetative. In crude-glucose–ammonia medium signs of incipient sporulation were visible at 21 hr., the hyphae showing increased branching and septation. Formation of conidiophores ensued, and at 27 hr. 60 % of the hyphal branches ended in conidiophores bearing chains of conidia.

Sporogenous mycelium had a consistently lower overall nitrogen content, but proportions of soluble nitrogen, free α-amino nitrogen and peptide nitrogen were not significantly different from those in vegetative mycelium (Table 5). No differences between patterns of free, peptide or protein amino acids in vegetative and sporogenous mycelia of the same age were detectable in qualitative paper chromatograms. Quantitative estimations of free and protein amino acids of each 27 hr. mycelium were not significantly different from one another, or from corresponding data for vegetative mycelium presented above (Table 4).

Amino acid pool in various samples of conidia of Penicillium griseofulvum

Results shown in Table 4 indicated that conidia of *P. griseofulvum* had a different pattern of free amino acids from that in mycelium. Further analyses of conidia were made, to determine the range of variation in their amino acid pools with respect to conditions of conidiation and to length of the dormant period.

Four samples of conidia, with different histories, were obtained: (i) from 15-day cultures grown on Czapek–Dox agar slopes; (ii) from 15-day surface cultures grown on liquid crude-glucose–ammonia medium; (iii) a sample similar to (ii) which was dried in a vacuum desiccator and stored at 2° for 3 years, when the conidia were 93 % viable; and (iv) from 45 hr. shaken cultures in crude-glucose–ammonia medium. In the last case parent mycelium also was analysed; it included adhering spores, but the weight of these was less than 1 % of that of mycelium.

It has been noted above that conidia freshly

Table 5. *Nitrogenous compositions of vegetative and sporogenous mycelia of* Penicillium griseofulvum

Experimental details are given in the text.

Medium	Age of culture (hr. after inoculation)	No. of sporogenous branches (%)	Mycelial dry wt. (mg./ml.)	Total mycelial N (mg./g. dry wt.)	Soluble N (% of total N)	Free α-amino N (% of total N)	Peptide α-amino N (% of total N)
Normal medium	21	0	0·81	89·3	11·7	6·2	0·88
	24	0	1·65	87·4	11·3	6·1	0·80
	27	0	3·05	86·4	11·6	5·9	0·83
Sporulation medium	21	0	0·73	77·3	11·3	6·0	0·87
	24	15	1·42	73·4	11·4	6·3	0·85
	27	60	2·56	70·3	11·7	5·8	0·90

harvested from mycelium on agar slopes show differences in composition from vegetative mycelium in shaken culture (Tables 2 and 4). Conidia had a lower content of free amino nitrogen (in relation to insoluble nitrogen), a lower proportion of free alanine and of many minor components in the amino acid pool, and a higher content of free glutamine.

The results in Table 6 show that these characteristics occurred consistently in conidia of *P. griseofulvum*, regardless of the culture conditions under which they were produced and of the length of the dormant period. The same distinctions were also apparent between conidia and parent mycelium harvested at the same time from submerged cultures. In view of the heterogeneity of mycelium grown in surface culture, analysis of parent mycelia from such cultures was not undertaken.

Certain significant variations in the size and composition of the amino acid pool between conidial samples with different histories are, however, evident from Table 6, particularly with respect to the proportions of glutamic acid and γ-aminobutyric acid which tended to vary inversely. The factors responsible for this variation in conidial composition were not investigated.

Studies with other fungi

For comparison with the data from *P. griseofulvum*, limited observations were made on conidia and mycelia of five other moulds (Tables 7 and 8). The results were generally similar to those obtained with *P. griseofulvum*. Thus conidia of all the species had a lower total nitrogen content (per unit of dry wt.) than growing mycelium, and their proportions of soluble nitrogen and free α-amino nitrogen were also lower. As in *P. griseofulvum*, amino acid pools of conidia contained high proportions of glutamine; apart from glutamic acid and γ-aminobutyric acid, the remaining amino acids, especially alanine, tended to be at lower relative proportions than in mycelium.

In growing mycelia the contents of total nitrogen, total soluble nitrogen and free α-amino nitrogen resembled those of *P. griseofulvum*, except that the contents of soluble nitrogen in *A. niger* and *T. viride* were considerably higher. Patterns of free amino acids were also similar, glutamic acid again being generally the major component and alanine the second. γ-Aminobutyric acid occurred in most samples of conidia and mycelia. Two samples of *P. expansum* were obtained during the phase of rapid growth; as in *P. griseofulvum* γ-aminobutyric acid disappeared during early growth and reappeared at a later stage.

The second mycelial samples of *A. niger* and *T. viride* were taken after exhaustion of the nitrogen source. Analyses of these samples indicated falls in overall nitrogen content and in the pro-

Table 6. *Amino acid pool of various samples of conidia and of a sporogenous mycelium of* Penicillium griseofulvum

Experimental details are given in the text. Only glutamic acid, alanine, γ-aminobutyric acid and glutamine were determined quantitatively; their amino N concentrations are expressed as percentages of total free amino N which was determined colorimetrically (Yemm & Cocking, 1955). The detection of the remaining amino acids on paper chromatograms is recorded as + or −.

Origin of sample	Free amino N (mg./g. dry wt.)	Free amino N (% of insol. N)	CyS + CySH	Asp	Glu	Aad*	Ser	Gly	Thr	Ala	Orn	Lys + His	Arg	Aba*	Tyr	Met + Val + Try	Phe + Leu + Ileu	Glu(NH_2)	Asp(NH_2)	Pro
15-day agar culture	2·45	4·7	−	+	17·4	−	+	−	−	6·5	−	−	−	25·3	−	−	−	36·1	−	−
15-day still culture	1·88	3·6	−	+	26·2	−	+	+	−	8·8	−	−	+	11·0	−	−	−	27·6	−	−
15-day still culture (stored 3 years)	1·98	4·4	−	+	27·2	−	+	+	−	6·1	−	−	−	7·3	−	−	−	37·6	+	−
45 hr. shaken culture (conidia)	1·56	4·0	−	+	33·7	−	+	+	−	8·4	−	−	−	4·9	−	−	−	25·5	−	−
45 hr. shaken culture (sporogenous mycelium)	2·95	6·1	−	+	37·3	+	+	+	+	4·0	−	+	+	10·1	+	+	+	4·0	−	+

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

Table 7. *Nitrogenous composition of conidia and mycelia of various fungi*

Experimental details are given in the text.

Material		Age of culture (hr. after inoculation)	Mycelial dry wt. (mg./ml.)	pH of medium	Ammonia N of medium (μg./ml.)	Total mycelial N (mg./g. dry wt.)	Soluble N (% of total N)	Free α-amino N (% of total N)
P. chrysogenum	Conidia	—	—	—	—	56·7	9·0	3·3
	Mycelium	27	2·6	3·78	140	80·4	12·5	7·0
P. expansum	Conidia	—	—	—	—	36·2	7·2	2·5
	Mycelium	23	1·0	5·84	270	75·8	13·1	7·4
	Mycelium	28	2·6	4·93	160	70·1	12·5	7·0
P. roqueforti	Conidia	—	—	—	—	35·9	8·2	3·4
	Mycelium	50	1·0	5·50	230	91·1	11·4	7·1
A. niger	Conidia	—	—	—	—	46·0	9·0	3·0
	Mycelium	24	2·2	3·72	150	89·9	17·6	7·7
	Mycelium	31	4·0	3·34	0	66·9	16·1	4·6
T. viride	Conidia	—	—	—	—	52·0	12·1	4·1
	Mycelium	26	1·4	5·40	200	89·3	18·3	6·7
	Mycelium	38	3·3	4·76	0	52·6	13·9	2·1

portion of free amino nitrogen, rises in total soluble nitrogen, and changes in pool composition which were similar to those observed in *P. griseofulvum* after ammonia exhaustion.

DISCUSSION

The amino acid composition of bulk protein is known to be largely independent of age and environment in micro-organisms (Stokes & Gunness, 1946; Lugg, 1949; Sueoka, 1961) and in higher plants (Pleshkov & Fowden, 1959). Thus the uniformity of protein composition observed in *P. griseofulvum* throughout the growth cycle is not unusual. Dormant tissues of plants, such as seeds and bulbs, generally have different overall protein compositions from vegetative tissues (Steward & Thompson, 1954); no such distinction could be detected between conidia and mycelium of *P. griseofulvum*. The protein analysis given here is broadly similar to previous analyses of protein hydrolysates of various moulds (e.g. Stokes & Gunness, 1946; Mansford & Raper, 1956; Ross, 1959; Gotz & Pascher, 1962) and of yeast protein (e.g. Lindan & Work, 1951).

The size of the amino acid pool in growing mycelium of *P. griseofulvum* and the other fungi studied (4–5 % of dry wt.) is of the same order as in unicellular yeasts and Gram-positive bacteria (Holden, 1962). This would suggest that free amino acids are located throughout the mycelium, rather than being restricted to the hyphal tips where growth in length occurs. The overall similarity in pool composition of the six moulds examined recalls the generally negative outcome of attempts to establish free amino acid patterns as taxonomic criteria for fungi (e.g. Venkata Ram, 1956; Close, 1960; Chattaway, Toothill & Barlow, 1961). Fluctuations in pool size and composition with age of fungal cultures have been described by several investigators (see references cited by Holden, 1962; also Rao & Ventkataraman, 1952; Meyers & Knight, 1961; Chattaway, Toothill & Barlow, 1962). However, the relationship of such changes to stages of development and to environmental conditions have not been made clear. In the present study we observed changes that occurred during the successive phases of spore germination, exponential growth and nitrogen starvation.

Comparable detailed analyses of conidia and mycelium of moulds have not been available previously. Meyers & Knight (1961) reported that conidia and mycelium of *P. roqueforti* showed the same qualitative patterns of free amino acids. However, Shepherd (1957) observed that germination of *Aspergillus nidulans* conidia was associated with changes in pool composition, and also with increases in acid-soluble and protein nitrogen. Our evidence indicates that amino acid pools in conidia of *P. griseofulvum* and related moulds are smaller than in mycelium and tend to have relatively high proportions of glutamine and low proportions of alanine and of most minor components. Since such differences were detected between conidia and parent mycelium taken from a submerged culture, it seems likely that conidial composition is not merely a reflexion of overall mycelial composition during sporulation, but is a specific feature of the conidia themselves.

The only appreciable change in pool composition of *P. griseofulvum* during the exponential growth phase was the appearance and gradual accumulation of γ-aminobutyric acid. This compound has often been detected in fungi (Holden, 1962). Similar accumulation of γ-aminobutyric acid has been observed in advanced cultures of yeast (A. P. Sims, quoted by Yemm & Folkes, 1958) and of

Table 8. *Amino acid pools of conidia and mycelia of various fungi*

Experimental details are given in the text. The results express amino N of each amino acid as percentages of total free amino N. The values are based on semi-quantitative estimation only by visual comparison of experimental chromatograms with standard chromatograms.

Material	Age of culture (hr. after inoculation)	CyS + CySH	Asp	Glu	Aad*	Ser	Gly	Thr	Ala	Orn	Lys + His	Arg	Aba*	Tyr	Met + Val + Try	Phe + Leu + Ileu	Glu-(NH_2)	Asp-(NH_2)	Pro
P. chrysogenum Conidia	—	2	2	30	0	2	0	0	10	0	0	0	10	0	0	0	20	0	0
Mycelium	27	2	5	35	2	5	2	0	20	0	2	0	0	0	2	2	15	0	0
P. expansum Conidia	—	0	0	45	0	0	0	0	5	0	0	0	15	0	0	0	25	0	0
Mycelium	23	0	5	40	2	2	2	0	25	2	0	10	0	0	0	0	15	0	0
Mycelium	28	0	5	30	2	2	2	0	20	2	0	10	15	0	2	0	15	0	0
P. roqueforti Conidia	—	0	2	30	0	0	0	0	10	0	0	2	5	0	0	0	25	0	0
Mycelium	50	2	2	30	2	2	2	0	25	2	0	5	10	0	0	0	5	0	0
A. niger Conidia	—	0	2	40	0	2	0	0	5	0	0	0	10	0	0	0	15	0	0
Mycelium	24	2	5	30	2	2	2	0	25	0	0	2	20	0	2	2	10	0	0
Mycelium	31	0	5	10	0	5	5	2	15	0	2	0	15	0	2	5	2	0	0
T. viride Conidia	—	2	2	35	0	2	2	2	5	0	0	2	5	0	2	2	15	0	0
Mycelium	26	2	5	35	2	5	2	2	20	2	2	0	25	0	2	2	15	0	0
Mycelium	38	0	5	20	0	5	2	2	15	2	2	0	10	2	2	5	5	0	0

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

Lactobacillus arabinosus (Holden, 1962). Evidence that the formation of γ-aminobutyric acid is closely associated with a fall in pH of the culture medium is presented by Bent & Morton (1964).

There is now much evidence (for reviews see Mandelstam, 1960; Halvorson, 1962) that, when yeasts and bacteria are starved of nitrogen, synthesis of new protein can continue at the expense of the amino acid pool and of pre-existing protein, the size of the pool during nitrogen starvation being determined by relative rates of protein breakdown and resynthesis. Very little work on this subject has been done with fungi, although it is known that mycelium can continue to grow and even sporulate without an exogenous nitrogen supply (Morton, 1961) and that intracellular proteinase systems are present (Morton, Dickerson & England, 1960). Meyers & Knight (1961) reported that the pool size of *P. roqueforti* increased after transfer to nitrogen-free medium, and suggested that internal replenishment by breakdown of labile protein was responsible. Under our conditions nitrogen starvation led to initial pool depletion in *P. griseofulvum*, but the subsequent halting of depletion and the change in proportions of amino acids, which became more evenly distributed as they are in bulk protein, suggest the onset of protein turnover.

Peptide pools have not been studied in moulds, although a number of individual peptides have been detected. We have used the term 'peptide nitrogen' for convenience to denote α-amino nitrogen released by acid hydrolysis of hot-water extracts. For conidia and exponential-phase mycelium, where the fraction (mostly GSH) was 87–93% diffusible through cellophan, this usage seems justifiable; it may not be so for the additional soluble combined α-amino nitrogen produced during nitrogen starvation, which was largely non-diffusible and not extractable with trichloroacetic acid. The metabolic significance of these hot-water-soluble proteins or large peptides that accumulated during nitrogen starvation is unknown; possibly they are involved in the mobilization of existing protein nitrogen for sustaining further hyphal growth.

Morton & Broadbent (1955) reported that surface cultures of *P. griseofulvum* produce extracellular nitrogenous material, largely of a peptidic nature, the amount present at any stage of growth corresponding to some 20% of the inorganic nitrogen previously assimilated. The present results show that in shaken culture such material only accumulates after exhaustion of the nitrogen supply, and then in relatively small amounts. Possibly production of extracellular nitrogenous material in surface cultures is associated with the physiological heterogeneity of the mycelial mat. It is notable that the blue–green alga *Nostoc* similarly produces much less extracellular nitrogenous material in

shaken culture than in still culture (Henriksson, 1957).

No composition differences between vegetative and sporogenous mycelia of *P. griseofulvum* were revealed by the present study, apart from the lower overall nitrogen content of the latter. This does not imply that induction of sporulation, which leads to changes in the morphological character of the entire mycelium, does not also involve changes in amino acid metabolism. It can only be concluded that there are no gross changes in composition, such as could be revealed by bulk analyses of free and combined amino acids of whole mycelium.

SUMMARY

1. Determination of free amino acids and of bulk-protein composition in *Penicillium griseofulvum* were made at different stages of shaken culture on a glucose–ammonia medium. Changes in size and composition of the amino acid pool occurred during conidial germination, the exponential growth phase and the subsequent phase of nitrogen starvation. The bulk-protein composition was stable throughout culture.

2. A hot-water-extractable fraction yielding amino acids on hydrolysis ('peptide' fraction), of which glutathione was a major component, was present in mycelium and conidia. The non-glutathione part of this fraction increased during nitrogen starvation. Combined amino acids also accumulated in the culture medium after exhaustion of the inorganic nitrogen supply.

3. The composition of the amino acid pool of conidia, particularly the glutamic acid and γ-aminobutyric acid contents, varied between samples with different histories. The pool was smaller than in mycelium and contained consistently a higher proportion of glutamine and a lower proportion of other components, especially alanine.

4. No differences in amino acid composition were detected between vegetative and sporogenous cultures of *P. griseofulvum*.

5. Five other moulds had amino acid pools that were generally similar to those of *P. griseofulvum* at comparable stages of development.

REFERENCES

Armstrong, J. J., England, D. J. E., Morton, A. G. & Webb, J. A. (1963). *Nature, Lond.*, **197**, 723.

Arnstein, H. R. V., Artman, M., Morris, D. & Toms, E. J. (1960). *Biochem. J.* **76**, 353.

Bent, K. J. & Morton, A. G. (1964). *Biochem. J.* **92**, 270.

Block, R. J., Durrum, E. L. & Zweig, G. (1958). *A Manual of Paper Chromatography and Paper Electrophoresis*, 2nd ed., p. 128. New York: Academic Press Inc.

Boissonas, R. A. (1950). *Helv. chim. acta*, **33**, fasc. 6, 1966.

Chattaway, F. W., Toothill, C. & Barlow, A. J. E. (1961). *Nature, Lond.*, **190**, 87.

Chattaway, F. W., Toothill, C. & Barlow, A. J. E. (1962). *J. gen. Microbiol.* **28**, 721.

Close, R. (1960). *Nature, Lond.*, **185**, 609.

Conway, E. J. (1947). *Microdiffusion Analysis and Volumetric Error*, p. 95. London: Crosby and Lockwood.

De Flines, J. (1955). *J. Amer. chem. Soc.* **77**, 1676.

Dustin, J. P., Czajkowska, C., Moore, S. & Bigwood, E. J. (1953). *Analyt. chim. acta*, **9**, 256.

Fairbairn, N. J. (1953). *Chem. & Ind.* p. 86.

Fuerst, R. & Wagner, R. P. (1957). *Arch. Biochem. Biophys.* **70**, 311.

Gotz, H. & Pascher, G. (1962). *Dermatologica*, **124**, 31.

Halvorson, H. O. (1962). In *Amino Acid Pools*, p. 646. Ed. by Holden, J. T. Amsterdam: Elsevier Publishing Co.

Henriksson, E. (1957). *Physiol. Plant.* **10**, 943.

Holden, J. T. (1962). In *Amino Acid Pools*, p. 73. Ed. by Holden, J. T. Amsterdam: Elsevier Publishing Co.

Koch, F. C. & Hanke, M. E. (1948). *Practical Methods in Biochemistry*, 5th ed., p. 383. Baltimore: Williams and Wilkins Co.

Lindan, O. & Work, E. (1951). *Biochem. J.* **48**, 337.

Lugg, J. W. H. (1949). *Advanc. Protein Chem.* **5**, 229.

Mandelstam, J. (1960). *Bact. Rev.* **24**, 289.

Mansford, K. & Raper, R. (1956). *Ann. Bot., Lond., N.S.*, **20**, 287.

Markovitz, A. & Steinberg, D. (1957). *J. biol. Chem.* **228**, 285.

Meyers, E. & Knight, S. G. (1961). *Mycologia*, **53**, 115.

Morton, A. G. (1961). *Proc. Roy. Soc.* B, **153**, 548.

Morton, A. G. & Broadbent, D. (1955). *J. gen. Microbiol.* **12**, 248.

Morton, A. G., Dickerson, A. G. F. & England, D. J. F. (1960). *J. exp. Bot.* **11**, 116.

Morton, A. G., England, D. J. F. & Towler, D. A. (1958). *Trans. Brit. mycol. Soc.* **41**, 49.

Morton, A. G. & MacMillan, A. (1954). *J. exp. Bot.* **5**, 232.

Pleshkov, B. P. & Fowden, L. (1959). *Nature, Lond.*, **183**, 1445.

Rao, P. L. N. & Ventkataraman, R. (1952). *Experientia*, **8**, 350.

Ross, J. P. (1959). *Phytopathology*, **49**, 422.

Schram, E., Moore, S. & Bigwood, E. J. (1954). *Biochem. J.* **57**, 33.

Shepherd, C. J. (1957). *J. gen. Microbiol.* **16**, i.

Sobel, A. E., Hirschman, A. & Besman, L. (1945). *J. biol. Chem.* **161**, 99.

Steward, F. C. & Thompson, J. F. (1954). In *The Proteins*, vol. 2, part A, p. 513. Ed. by Neurath, H. & Bailey, K. New York: Academic Press Inc.

Stokes, J. L. & Gunness, M. (1946). *J. Bact.* **52**, 195.

Sueoka, N. (1961). *Cold Spr. Harb. Symp. quant. Biol.* **26**, 35.

Venkata Ram, C. S. (1956). *Proc. nat. Inst. Sci. India*, B, **22**, 227.

Yemm, E. W. & Cocking, E. C. (1955). *Analyst*, **80**, 209.

Yemm, E. W. & Folkes, B. F. (1958). *Annu. Rev. Plant. Physiol.* **9**, 245.