Significance of the Amino Acid Pool in Nitrogen Metabolism of *Penicillium griseofulvum*

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It is now well established that the endogenous amino acid pool of yeasts and bacteria occupies a central position in the nitrogen metabolism of these organisms. The pool components are essential intermediates in the assimilation of exogenous nitrogen into macromolecular cell constituents and in the reutilization of existing cellular material for synthesis of new proteins, and are thus in a constant state of dynamic turnover. The existence of a comparable amino acid pool in moulds has been shown many times, but little is known of its significance in relation to the assimilation of exogenous nitrogen and to the synthesis and breakdown of protein. This question has a particular interest for comparative biochemistry, since filamentous fungi are in some respects intermediate between unicellular organisms and higher plants both in their morphological complexity and in their manner of growth. Steward & Bidwell (1962) have marshalled a wide range of evidence indicating that metabolically active amino acids are present generally in very small amounts in tissues of higher plants, and that the bulk of the free amino acids extractable from plants represent storage forms that are remote from anabolic sites and are produced largely by breakdown of protein rather than directly from exogenous nitrogen sources.

In the preceding papers Bent & Morton (1964*a, b*) have described the hot-water-extractable amino acid pool of the fungus *Penicillium griseofulvum* at different stages of its development and under various cultural conditions. Rapid changes in composition of the pool were induced by exhaustion of the nitrogen supply, alteration in pH of the medium, transfer to anaerobic conditions and treatment with metabolic inhibitors; pool composition was also dependent on the nature of the nitrogen source. From this susceptibility to variation it seemed probable that a large part of the pool was actively involved in the formation and inter-conversion of amino acids. The presence in conidia of a different type of amino acid pool from that in mycelium suggested that free amino acids of conidia might play a significant part in the initiation of spore germination. The present paper describes investigations into the amino acid meta-

bolism of mycelium and conidia of *P. griseofulvum* after transfer to conditions of nitrogen starvation, and also into the incorporation and redistribution of labelled nitrogen in mycelium fed with [^{15}N]-ammonium sulphate. The results indicate active participation of virtually all of the amino acid pool in the assimilation of exogenous nitrogen and in the synthesis and turnover of protein by the fungus.

METHODS

Organism and method 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- $(\text{NH}_4)_2\text{SO}_4$ -salts medium, as described by Bent & Morton (1964*a*). The N-free medium was identical with the growth medium, except that $(\text{NH}_4)_2\text{SO}_4$ was omitted. Conidia used in germination studies were obtained from agar cultures by suspension in water rather than in detergent solution (used for routine inocula), to avoid possible leaching of amino acids by detergent action.

The transfer of mycelium to fresh medium was accomplished by filtering the suspension through sintered glass (grade 3), washing the mycelial pad with 10 vol. of fresh medium for 10 sec. and resuspending the washed mycelium in fresh medium. Transfers were made quickly (under 1 min.), and care was taken that the pads did not dry during filtration. As subsequent incubations were for short periods only, there was no significant contamination. When mycelium was transferred to fresh growth medium, growth and assimilation of N continued at the same rates as before transfer, no damage to the mycelium being apparent.

Analytical methods

Measurement of dry weight and pH, extraction and analysis of mycelial constituents and analysis of the culture medium were carried out by the methods described by Bent & Morton (1964*a*).

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.

Studies with isotopic nitrogen

Preparation and treatment of cultures. Conidia were inoculated into nine flasks each containing 200 ml. of growth medium modified by decreasing the $(\text{NH}_4)_2\text{SO}_4$ concentration from 0.16 to 0.095%. The suspensions were

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incubated on the shaker, and ammonia uptake was followed by intermittent analysis of the medium in one flask. When about 12 mg. of ammonia N/flask remained, contents of the other eight flasks were mixed and redispensed into the flasks, to eliminate variation between separate suspensions. After further shaking for 5 min., 5 ml. of a solution of $(^{15}\text{NH}_4)_2\text{SO}_4$ was added to each flask. The solution was prepared by steam-distillation of ammonia from an alkaline solution of $^{15}\text{NH}_4\text{NO}_3$ into dilute H_2SO_4 . Each addition contained 2.5 mg. of ammonia N, with a ^{15}N abundance of 32.5 atoms %. Flasks were removed for analysis at intervals up to 3.5 hr. after addition of the $(^{15}\text{NH}_4)_2\text{SO}_4$; during this period the pH fell from 6.01 to 5.80. Mycelium was separated by filtration, washed with water at room temperature and immersed in boiling water to kill the cells and extract soluble components.

Measurement of isotope enrichment. Nitrogenous fractions of the medium and mycelium were converted into ammonia, which was collected in 0.05 N-HCl and used partly for analysis by the Nessler method and partly for estimation of ^{15}N enrichment. In calculating amounts of N from the Nessler coloration no correction was made for the greater mass of ^{15}N atoms; such correction was unnecessary for the present purposes and would have made interpretation difficult.

Ammonia in culture filtrates was collected by the method of Conway (1947). After removal of ammonia the samples were acidified, unlabelled $(\text{NH}_4)_2\text{SO}_4$ was added (50 μg . of ammonia N/ml. of filtrate) and ammonia was again removed by the Conway method; this 'flushing' procedure ensured that residual traces of ammonia did not interfere significantly with subsequent determination of ^{15}N abundance in extracellular organic N. The latter was converted into ammonia by the Kjeldahl method, and the ammonia released by alkaline distillation in a Markham still.

Intracellular ammonia was removed from hot-water extracts by vacuum-distillation at pH 10.1 (Koch & Hanke, 1948) before the following treatments were performed. Total soluble organic N was converted into ammonia by Kjeldahl digestion. Free α -amino N was converted into ammonia by reaction with ninhydrin at pH 2.5 (Sobel *et al.* 1945) followed by vacuum-distillation at pH 10.1; samples of extracts hydrolysed with 6 N-HCl were also treated in this way, so that ^{15}N enrichment in the peptide fraction (Bent & Morton, 1964*a*) could be obtained. Total N of hot-water-insoluble mycelial residues was released as ammonia by Kjeldahl digestion, and protein α -amino N in insoluble residues was released by ninhydrin treatment after 6 N-HCl hydrolysis.

Individual free amino acids were isolated from hot-water extracts by two-dimensional paper chromatography. Eight replicate chromatograms, each containing 50 μg . of free α -amino N, were prepared for each extract. The quantitative chromatographic procedure (Bent & Morton, 1964*a*) was followed up to the stage of cutting out ninhydrin-positive areas. Amino acids were eluted from the paper with hot water (80°, for 10 min.). To remove possible traces of [^{15}N]ammonia, $(\text{NH}_4)_2\text{SO}_4$ (20 μg . of ammonia N) was added to each eluate and ammonia was removed by vacuum-distillation at pH 10.1. Eluates were then treated with ninhydrin at pH 2.5, and the α -amino N was collected as ammonia. Before ninhydrin treatment, eluates containing glutamine were treated with N- H_2SO_4 (at 100° for 2 hr.) to convert amide N into ammonia. The results for some amino

acids are incomplete, because certain chromatographic spots were not compact enough for unequivocal delimitation or were too weak for analysis.

Ammonia N obtained by these methods was converted into N_2 by treatment with alkaline hypobromite, as described by Sprinson & Rittenberg (1949), and ^{15}N abundance was determined in a mass spectrometer. The instrument was built at the Akers Research Laboratories. The incorporation of a vibrating-reed electrometer led to high sensitivity, so that reliable estimations of enrichment could be made on samples containing only 10 μg . of N. Differences between samples were less than 2% and generally less than 1% of the mean values for ^{15}N abundance.

RESULTS

Amino acid metabolism in conidia during incubation in full and nitrogen-free medium. Conidia of *P. griseofulvum* contain an amino acid pool whose composition differs from that of young growing mycelium (Bent & Morton, 1964*a*). A relatively high proportion of glutamine is present, generally together with substantial amounts of γ -aminobutyric acid; most other pool components, and especially alanine, are present in smaller amounts in conidia than in mycelium. During a 12 hr. period of germination, the pool composition changes from the conidial to the mycelial type. The behaviour at early stages of germination in full culture medium and also during incubation in the absence of an external nitrogen source was examined (Table 1), in an attempt to assess the extent to which the free amino acids of dormant conidia participate in the initiation of germination.

No morphological change was visible after shaking for 3 hr. in complete medium, but significant increases in conidial dry weight and hot-water-insoluble nitrogen occurred. The increase in insoluble nitrogen was far greater than might be accounted for by utilization of the initial soluble nitrogen, so that assimilation of exogenous nitrogen must have started during this early phase of germination. There was no change in the overall amount of free amino nitrogen, but the concentrations of glutamine and γ -aminobutyric acid decreased markedly whereas those of glutamic acid, aspartic acid and alanine increased. Over the next 5 hr. 55% of the conidia swelled to about twice their original diameter, and about 10% of the swollen spores produced germ-tube initials; large increases in dry weight, insoluble nitrogen and free amino nitrogen took place and the amino acid pool became similar in size and composition to that of young mycelium.

When conidia were incubated in nitrogen-free medium no morphological change was detectable during the first 8 hr. After a further 12 hr. the conidia were still unswollen, but about 20% of them each bore a short, very thin hypha. At this

Table 1. *Composition of conidia of Penicillium griseofulvum during incubation in full and nitrogen-free media*

Of the individual amino acids, only glutamic acid, alanine, γ -aminobutyric acid and glutamine were determined quantitatively. Detection of other amino acids is recorded as + or -. Cyst(e)ine, α -aminoadipic acid, tyrosine, asparagine and proline were not detected at any stage. Values are expressed as amounts/flask (200 ml. of suspension). Experimental details are given in the text.

Medium	Incubation period (hr.)	Dry wt. of conidia (mg.)	Insoluble N (mg.)	Free amino N (mg.)	Free amino acids (μ g. of amino N)													
					Asp	Glu	Ser	Gly	Thr	Ala	Orn	Lys + His	Arg	Aba*	Met	Phe	Glu(NH ₂)	
Full	0	9.1	0.54	0.025	-	3.7	-	-	-	-	1.8	-	-	-	5.8	-	-	7.6
	3	9.8	0.66	0.024	+	8.0	-	-	-	-	2.4	-	-	-	1.0	-	-	2.5
	8	25.4	1.87	0.114	+	40.5	+	-	-	-	20.0	+	+	+	-	-	-	5.5
N-free	0	9.2	0.55	0.024	-	3.7	-	-	-	-	1.9	-	-	-	5.4	-	-	7.8
	3	10.3	0.59	0.013	-	3.9	-	-	-	-	1.4	-	-	-	0.6	-	-	1.1
	8	11.2	0.57	0.019	+	6.0	+	+	+	+	1.7	-	+	+	-	+	+	-

* γ -Aminobutyric acid.

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stage (20 hr.), ammonium sulphate was added to the suspension; within only 5 hr. over 90 % of the conidia were swollen, 45 % bearing normal germ-tubes, and subsequently 95 % of the conidia gave rise to mycelia. The thin protrusions produced during nitrogen starvation did not develop further.

Despite the absence of morphological change, several signs of metabolic activity were observed during the 8 hr. period of nitrogen starvation (Table 1). Over the first 3 hr. slight increases in dry weight and in insoluble nitrogen were detectable, together with striking falls in the concentrations of glutamine and γ -aminobutyric acid, indicating that synthesis of insoluble nitrogenous material at the expense of these two components occurred. Little change was detected in the concentrations of glutamic acid or alanine, the other major pool components. In the next 5 hr. dry weight continued to rise but the insoluble nitrogen content did not increase and possibly declined slightly. The pool was partially replenished and several previously undetectable amino acids appeared, suggesting that protein degradation had become predominant over synthesis.

Amino acid metabolism of mycelium during nitrogen starvation. Exhaustion of the nitrogen source in shaken cultures of *P. griseofulvum* and other fungi is followed by a decrease in the size and a change in the composition of the amino acid pool; the likelihood that these changes reflect participation of the pool in a continuation of protein synthesis has been discussed (Bent & Morton, 1964a). Behaviour during nitrogen starvation was studied further by transferring young growing mycelium to fresh medium from which the nitrogen source was omitted. This technique gives a precise starting point for nitrogen starvation, after which samples can be taken at short intervals. Also, the ratio of mycelium to medium can be raised during transfer, so that extracellular accumulation of metabolites becomes easier to detect.

Results of an experiment where exponential-phase mycelium was transferred to nitrogen-free medium maintained at three pH values (6.0, 3.6 and 3.0) are shown in Table 2. The two lower pH values are about 0.3 pH unit above and below the pH at which growth and nitrogen assimilation become completely inhibited during culture on unbuffered (succinate-free) medium (Bent & Morton, 1964b). Treatment at these pH values was originally included with the separate aim of studying leakage of amino acids from the mycelium under acidic conditions, for which purpose the experimental procedure was particularly suitable, but the results are germane to the present problem and are therefore presented here.

Behaviour differed markedly according to pH. At pH 6.0 mycelial dry weight increased by 26 %

and hyphal length by roughly 20 % during the 6 hr. period of nitrogen starvation. Over the first hour there was a rapid depletion of pool α -amino nitrogen, to about 40 % of the initial amount. Since this depletion was accompanied by an increase of similar magnitude in insoluble nitrogen, continuation of net synthesis of macromolecular nitrogenous material at the expense of the initial amino acid pool was indicated. Subsequently, however, a steady decline in the amount of insoluble nitrogen occurred, together with a partial replenishment of the depleted amino acid pool, a marked increase in the peptide fraction (hot-water-extractable combined α -amino nitrogen) and an appearance of combined α -amino nitrogen in the culture medium. Clearly proteolytic activity had become predominant, leading to a net breakdown of protein to peptides and amino acids.

Qualitative chromatograms revealed that the initial depletion of the amino acid pool involved all its components (described by Bent & Morton, 1964*a*), but especially glutamine, arginine and γ -aminobutyric acid, all of which became undetectable within 15 min. of transfer. The ensuing replenishment also involved all the pool components, but increases were proportionately higher in serine, glycine, threonine, lysine + histidine,

methionine + valine + tryptophan and phenylalanine + leucine + isoleucine. Since these amino acids are present in higher proportions in mycelial protein than in the original amino acid pool (Bent & Morton, 1964*a*), this shift in pool composition is a further sign of replenishment by protein degradation. The initial peptide fraction consisted largely of GSH; during nitrogen starvation the amount of GSH changed little, and chromatograms of hydrolysed extracts showed that the increase in the peptide fraction was attributable to compounds containing a wide range of amino acid residues. A similar accumulation of peptidic material in the mycelium was observed in nitrogen-exhausted cultures by Bent & Morton (1964*a*).

At pH 3.6, the dry weight increased by 11 % during the 6 hr. starvation period, but increase in hyphal length was barely significant. Pool depletion again ensued directly after transfer, but was more severe than at pH 6.0 and continued throughout the incubation period. Falls in the concentrations of all pool components occurred, the loss of glutamine being particularly marked. Net synthesis of insoluble nitrogenous material occurred during the first 2 hr., at a slower rate than at pH 6.0; thereafter this fraction underwent little change and there were no signs of protein degradation to

Table 2. *Composition of mycelium and medium during incubation of Penicillium griseofulvum in nitrogen-free medium at three pH values*

Mycelium from 21 hr. cultures on glucose-ammonia medium was placed in N-free medium at the listed pH values; the pH was kept within 0.1 unit of these values by the addition of N-NaOH or N-HCl at intervals. Except where stated, values are expressed as mg./flask (200 ml. of suspension). Other details are given in the text.

pH	Incubation period (min.)	Mycelium							Medium		
		Dry wt.	Length* (μ)	Insoluble N†	Insoluble N‡	Soluble N	Free α -amino N	Peptide α -amino N	Total N	Free α -amino N	Combined α -amino N
6.0	2	728	223	55.8	55.8	8.21	4.42	0.71	1.0	0.36	0.40
	15	—	—	57.2	57.1	6.72	3.27	0.60	1.2	0.46	0.46
	30	—	—	57.6	57.7	6.00	2.60	0.66	1.3	0.46	0.56
	60	—	—	58.0	57.8	6.03	1.91	1.07	1.2	0.28	0.68
	120	—	—	55.3	54.0	9.60	1.88	3.39	1.4	0.18	1.04
	180	—	—	52.0	51.4	12.02	2.32	4.76	1.6	0.12	1.12
	360	920	293	47.8	47.8	15.09	2.60	5.80	2.1	0.20	1.52
3.6	2	728	223	54.4	54.4	8.01	3.87	0.73	1.4	0.66	0.46
	15	—	—	54.8	55.7	5.78	2.78	0.58	2.3	1.24	0.60
	30	—	—	55.2	55.5	5.08	1.86	0.46	3.2	1.78	0.72
	60	—	—	56.7	56.7	3.22	1.05	0.45	3.9	2.04	0.90
	120	—	—	57.8	58.2	2.17	0.76	0.40	3.4	1.10	1.26
	180	—	—	58.0	58.5	2.40	0.40	0.34	2.9	0.52	1.28
	360	807	244	58.2	58.5	2.91	0.19	0.31	2.4	0.30	1.20
3.0	2	728	233	54.8	54.8	7.84	3.76	0.66	1.5	1.00	0.50
	15	—	—	54.5	54.7	5.20	2.05	0.48	4.2	2.54	0.70
	30	—	—	54.4	55.3	3.91	1.41	0.41	4.9	3.04	0.72
	60	—	—	54.5	55.9	1.83	0.63	0.27	6.4	3.90	0.82
	120	—	—	54.0	55.8	1.46	0.42	0.28	6.8	4.02	0.88
	180	—	—	54.4	55.9	1.22	0.26	0.30	7.0	4.36	0.80
	360	736	232	54.1	55.3	1.35	0.18	0.33	7.5	4.64	0.84

* Mean of 20 measurements with an eye-piece micrometer; significant difference 18μ (P 0.01).

† Determined directly.

‡ Values calculated from difference between changes in soluble N and total medium N.

intra- or extra-cellular amino acids or peptides. The decrease in free α -amino nitrogen was initially faster than the concomitant increase in insoluble nitrogen. This discrepancy was accounted for by leakage of amino acids (and other nitrogenous material) out of the mycelium. Leakage of endogenous amino acids was encountered when the pH of unbuffered cultures fell below 4.0 (Bent & Morton, 1964b). The accumulation of extracellular amino acids, which were present in proportions similar to those of the original amino acid pool, was maximal at about 1 hr. after transfer, but then declined to a negligible level. Evidently amino acids released into the medium were ultimately all incorporated into insoluble cellular material.

At pH 3.0, rapid leakage of almost all the amino acid pool again occurred, but there was no sign of reabsorption of the released amino acids. Dry weight, hyphal length and insoluble mycelial nitrogen did not alter appreciably during the 6 hr. starvation period. It appears that both anabolic and catabolic processes were completely inhibited at this degree of acidity.

It is clear from these results that the free amino acid pool was readily available for the maintenance of continued synthesis of insoluble nitrogenous material directly after the withdrawal of the exogenous nitrogen supply. The difference between behaviour at pH 6.0, where depletion of the pool was only partial and net formation of insoluble nitrogen was transient, and at pH 3.6, where an almost complete utilization of the amino acid pool and a corresponding permanent increase in insoluble nitrogen occurred, is attributable to an inhibition of intracellular degradation of protein to amino acid and peptides during incubation at the lower pH. The presence of an intracellular proteinase system in *P. griseofulvum* has been demonstrated by Morton, Dickerson & England (1960); proteinase content, which increased during nitrogen starvation, was determined as the increase in free α -amino nitrogen during incubation of previously

frozen mycelium for 4 hr. in phosphate buffer, pH 7.0, at 25°. With this technique it was found that incubation of mycelium for 3 hr. in a nitrogen-free medium at pH 6.0 led to a sevenfold increase in proteinase content, whereas at pH 3.6 the increase was only twofold. Proteinase activity of frozen- and thawed preparations was 5–7 times as strong at pH 6.0 as at pH 3.6. A similar response to pH was also observed in intact mycelium, net rates of autolytic protein degradation during incubation (for 6 hr. at 25°) in buffer being 3–4 times as great at pH 6.0 as that at pH 3.6. Thus lowering the pH of nitrogen-starved suspensions to 3.6 appeared to inhibit both the synthesis and the activity of the proteolytic system, whereas overall protein synthesis was relatively unimpaired.

Assimilation and redistribution of isotopic nitrogen in mycelium. [^{15}N]Ammonium sulphate was added to 24 hr. cultures of *P. griseofulvum* growing on glucose-ammonia medium. Isotopic enrichment in the free amino acid, peptide and hot-water-insoluble fractions of the mycelium and in the culture medium was measured at intervals. Observations were continued after exhaustion of ammonia, so that subsequent redistribution of mycelial nitrogen could be followed. The analytical results are shown in Table 3, and ^{15}N enrichments in Figs. 1 and 2. The degree of labelling is expressed in the form:

$$\frac{\text{atoms } \% \text{ excess of } ^{15}\text{N} \text{ of fraction}}{\text{atoms } \% \text{ excess of } ^{15}\text{N} \text{ of N source}} \times 100$$

Values expressed in this way indicate the percentage of nitrogen in each fraction which was derived from exogenous ammonia after the addition of [^{15}N]ammonium sulphate. Isotopic enrichment of the nitrogen source was taken as the mean of the five successive values obtained; these did not vary significantly from each other.

Uptake of labelled ammonia proceeded in a roughly linear manner until it was exhausted (1.5–2 hr. after addition of [^{15}N]ammonium sulphate).

Table 3. *Composition of mycelium and medium during and after the assimilation of [^{15}N]ammonia by Penicillium griseofulvum*

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

Period after addition of ($^{15}\text{NH}_4$) $_2\text{SO}_4$ (min.)	Mycelium						Medium	
	Insoluble N	Soluble organic N	Free α -amino N	Peptide α -amino N	Glutamic acid N	Glutamine amino N	Ammonia N	Organic N
3.25	24.10	4.04	1.71	0.30	0.74	0.086	10.80	—
10	24.66	4.19	1.72	0.34	0.74	0.094	10.00	—
30	27.03	4.56	1.78	0.34	0.75	0.098	8.10	—
60	30.48	5.00	1.87	0.43	0.79	0.101	4.15	—
90	32.56	5.27	1.88	0.54	0.78	0.095	1.40	0.44
120	34.40	4.72	1.46	0.72	0.64	0.056	0.0	0.63
150	32.61	5.45	1.23	1.05	0.52	0.036	0.0	0.74
210	30.47	7.54	1.18	2.00	0.52	0.028	0.0	1.50

There was a rapid accumulation of ^{15}N in the hot-water-soluble fraction of the mycelium, particularly in the free α -amino nitrogen fraction (Fig. 1) which became 86 % labelled before the external ammonia was used up. The rates of labelling of total nitrogen and of free α -amino nitrogen were fastest initially and tended to fall off with time, whereas

Fig. 1. Labelling of nitrogenous components in a culture of *P. griseofulvum* after the addition of $(^{15}\text{NH}_4)_2\text{SO}_4$. Labelling in medium: $\blacktriangle$, ammonia N; $\blacksquare$, organic N. Labelling in mycelium: $\square$, hot-water-insoluble N; $\triangle$, total soluble N; $\bullet$, peptide α -amino N; $\circ$, free α -amino N. Experimental details are given in the text.

Fig. 2. Labelling of free amino acids of mycelium in a culture of *P. griseofulvum* after the addition of $(^{15}\text{NH}_4)_2\text{SO}_4$. (a) $\circ$, Glutamic acid; $\bullet$, serine; $\triangle$, aspartic acid; (b) $\circ$, glutamine (amide N); $\bullet$, glutamine (amino N); $\triangle$, alanine; $\blacktriangle$, arginine. Experimental details are given in the text.

the rate of labelling of the insoluble fraction gradually increased during the first hour. Labelling of free α -amino nitrogen resided mainly in glutamic acid (Fig. 2); after 1 hr., 88 % of the glutamic acid nitrogen was derived from the labelled nitrogen source. The amino nitrogen of glutamine was also labelled rapidly, but amino groups in other pool components were enriched at lower rates and initial lags were shown in most cases. The amide nitrogen of glutamine was labelled more strongly than the amino group, and showed the highest ^{15}N abundance of all mycelial components examined. Peptide α -amino nitrogen was enriched at a low rate, ^{15}N abundance in this fraction being little higher than that in the much larger insoluble fraction.

The assimilation of exogenous nitrogen can be represented by the following model:

Assuming complete and instantaneous mixing of labelled and unlabelled molecules of the primary product, then at any instant the rate of ^{15}N assimilation is given by the equation:

$$d(bB)/dt = aw_1 - bw_2 + cw_3$$

where w_1 , w_2 and w_3 are rates of transfer of nitrogen ($^{14}\text{N} + ^{15}\text{N}$) to and from the pool of primary product. Therefore:

$$Bdb/dt + bdB/dt = aw_1 - bw_2 + cw_3$$

But:

$$dB/dt = w_1 - w_2 + w_3$$

Hence:

$$db/dt = [(a-b)w_1 + (c-b)w_3]/B$$

Therefore:

$$w_1 = B(db/dt)/(a-b) - w_3(c-b)/(a-b) \quad (1)$$

If w_3 is neglected, then:

$$w_1 = B(db/dt)/(a-b) \quad (2)$$

Values for a , b and B were measured experimentally, and estimates of db/dt were made by constructing tangents to the curves of isotopic enrichment. Values for w_1 were then calculated with respect to ^{15}N incorporation into total soluble nitrogen, total free α -amino nitrogen, glutamic acid nitrogen and amide nitrogen of glutamine (Table 4). Values for zero time can be regarded as the truest estimates of incorporation rates, since at zero time $b = 0$ and $c = 0$, and hence derivation of w_1 from eqn. (2) is independent of the assumptions that perfect mixing occurred and that formation of 'primary product' from endogenous sources was negligible. The validity of these assumptions and possible explanations of the apparent decline of

incorporation rates with time are discussed below. The initial values themselves must be regarded as minimal estimates for several reasons. First, initial rates of labelling (db/dt) were derived from the slopes between 0 and 3.25 min., whereas the true initial tangents would be greater than this. Secondly, some inhibition of ^{15}N uptake must undoubtedly have occurred during the process of filtration and washing (which was started about 20 sec. before extraction). Thirdly, unlabelled ammonia will have been present within the mycelium at zero time. Separate tests indicated an intracellular concentration of 15–20 μg . of ammonia nitrogen/100 ml. of suspension; thus there may have been an appreciable initial dilution of incoming labelled ammonia, which was utilized at an average rate of 54 μg . of nitrogen/100 ml./min.

In Table 4 the initial rates of ^{15}N incorporation are also expressed as percentages of the mean overall rate of nitrogen uptake. At least 39.8 % of the nitrogen entering the mycelium was incorporated as α -amino nitrogen in glutamic acid. Thus synthesis of glutamic acid could account for at least 85 % of the total synthesis of free and combined α -amino groups (which comprised about 47 % of the total mycelial nitrogen). The ratio [(proportion of incoming nitrogen incorporated into a fraction): (amount of the fraction as a proportion of total mycelial nitrogen)] is equivalent to the ratio [(gross rate of synthesis): (net rate of synthesis)] and is thus a measure of nitrogen turnover in the fraction; from the present data, ratios of 11.2, 15.0 and 19.6 were indicated for total free α -amino nitrogen, glutamic acid nitrogen and glutamine amide nitrogen respectively.

Exhaustion of the external nitrogen supply was followed by changes in mycelial composition (Table 3) similar to those found in nitrogen-limited cultures (Bent & Morton, 1964*a*) and in mycelium transferred to nitrogen-free medium at pH 6.0 (Table 2). Continued increase in dry weight was accompanied by a net decline in insoluble nitrogen, partial depletion of the amino acid pool, increase in

the peptide fraction and accumulation of extracellular organic nitrogen. Changes also occurred in the distribution of ^{15}N . The ^{15}N enrichment of hot-water-insoluble material continued, at a diminishing rate, but labelling of the soluble fractions and of all the individual amino acids declined markedly. Hence the operation of a cyclic system of nitrogen exchange between the soluble and insoluble fractions is indicated. The system was unbalanced, since a net loss of insoluble nitrogen and a net increase in soluble nitrogen (particularly peptide nitrogen) and in extracellular nitrogen occurred. At the end of the experiment ^{15}N enrichments of all fractions seemed to be approaching the same final level (Figs. 1 and 2). This suggests that breakdown and synthesis of insoluble nitrogen were of general occurrence in the mycelium. If, on the other hand, protein synthesis were confined to hyphal tips (where growth in length occurs) and degradation were occurring in older, relatively unlabelled, regions, then ultimately almost all the labelled nitrogen would have resided in the insoluble fraction and the soluble fraction would have tended to become free of label.

Assuming that dilution of ^{15}N enrichment in the soluble fraction was due to solubilization of nitrogen labelled at the overall level observed in the insoluble fraction, it may be calculated from an equation analogous to eqn. (2) that at 2.5 hr. after the addition of [^{15}N]ammonium sulphate (0.5–1 hr. after ammonia exhaustion) nitrogen was entering the soluble fraction at 2.95 mg./100 ml. of suspension/hr. This is equivalent to a loss of 17.5 % of the insoluble nitrogen/hr. Since at this time insoluble nitrogen decreased at a net rate of only 7.4 %/hr., simultaneous synthesis at approx. 10.1 %/hr. is indicated (this value may be compared with a net rate of synthesis of 23.8 %/hr. in the period before ammonia exhaustion). Similar calculations indicate replenishment of pool α -amino nitrogen from insoluble (protein) α -amino nitrogen, labelling of which was measured at 2.5 hr. only, at a rate equivalent to 6.4 % of the protein α -amino nitrogen/

Table 4. *Rates of incorporation of nitrogen from [^{15}N]ammonium sulphate into nitrogenous fractions of Penicillium griseofulvum*

Rates are expressed as μg . of N/200 ml. of suspension/min. Initial rates are also expressed (in parentheses) as percentages of the overall rate of uptake of ammonia N. Experimental details and methods of calculation are given in the text.

Time after addition of (NH_4) $_2\text{SO}_4$ (min.)	Rate of incorporation into			
	Soluble organic N	Total free α -amino N	Free glutamic acid	Free glutamine (amide N)
0	50.5 (93.5 %)	36.2 (67.1 %)	21.5 (39.8 %)	3.2 (5.9 %)
3.25	49.5	35.8	19.8	2.8
10	42.6	28.3	14.8	2.4
30	35.6	25.7	13.7	1.9
60	27.3	24.7	13.6	—

hr., and removal of pool α -amino nitrogen at a gross rate equivalent to 7.7% of the protein α -amino nitrogen/hr. Since the ratio of protein nitrogen to total insoluble nitrogen remains stable throughout culture (Bent & Morton, 1964a), the net rate of loss of insoluble nitrogen (7.4%/hr.) may be taken as the net rate of protein breakdown. Therefore, if removal and replenishment of pool α -amino nitrogen represent protein synthesis and breakdown respectively, then protein synthesis at 7.7%/hr. and simultaneous protein degradation at 15.1% (7.4% + 7.7%)/hr. are indicated for the nitrogen-starved mycelium.

Labelling of the increasing peptide fraction was consistent with its formation by breakdown of protein; however, the change in ^{15}N enrichment was too small to permit any attempt to estimate whether reincorporation of peptide nitrogen into insoluble material was occurring. The enrichment data for extracellular organic nitrogen indicates that this material stemmed from insoluble mycelial components rather than from the amino acid pool, which was much more highly labelled. However, the first (1.5 hr.) value of extracellular ^{15}N enrichment was significantly higher than later ones, and higher than the labelling of the insoluble fraction at that time, so that the initial output of organic nitrogen, which occurred before exhaustion of ammonia, may have been derived partly from the soluble fraction.

DISCUSSION

It can be concluded from the study of ^{15}N incorporation that virtually the whole of the amino acid pool of *P. griseofulvum* participated in the assimilation of inorganic nitrogen, and that a large proportion (at least 67%) of the assimilated nitrogen passed through the pool. Evidently the amino acid pool of fungal mycelium, like that of unicellular micro-organisms, occupies a central position in the metabolism of inorganic nitrogen to protein, and cannot be regarded as a group of storage or breakdown products. Since the pool constituted about 5% of the mycelial weight it follows that anabolic activity was not confined to the 'growing' tips but extended for some distance along the hyphae. Such behaviour has also been inferred by Zalokar (1959), partly on the basis of a radioautographic examination of hyphae after the incorporation of [^{14}C]proline into protein of *Neurospora crassa*.

The high rate of ^{15}N incorporation into glutamic acid suggests that the amination of α -oxoglutaric acid was the principal mechanism for the biosynthesis of α -amino groups from inorganic nitrogen by the fungus, the formation of other amino acids being accomplished largely by secondary transamination reactions. Similar conclusions were reached by A. P. Sims (quoted by Folkes,

1959) from a kinetic study of [^{15}N]ammonia assimilation by the yeast *Torulopsis utilis*. A specific requirement for glutamate-dehydrogenase activity in the utilization of ammonia as nitrogen source was revealed by work with 'amination-deficient' mutants of *N. crassa* (Fincham, 1954). The possibility that amino acids other than glutamic acid also participate in primary amination reactions is not excluded by our data, although the presence of lags in most of the incorporation curves makes it improbable. Several authors have found that the amination of pyruvic acid to alanine is a major pathway of nitrogen assimilation in certain bacilli. Also, the NADP-linked glutamate dehydrogenase of *N. crassa* catalyses the amination of pyruvic acid and other oxo acids to a limited extent (Bürk & Pateman, 1962; Barratt & Strickland, 1963). However, if the initial labelling observed in alanine did represent direct incorporation of ^{15}N , it could account for only 3.3% of the total ^{15}N assimilation. Initial ^{15}N enrichments of other amino acids correspond to still lower values.

The labelling of glutamine was consistent with its formation by the direct amination of glutamic acid. The rapid turnover of the amide group of glutamine showed it to be a highly active intermediate in the assimilation of ammonia. The accumulation of glutamine is a prominent feature when ammonia is fed to nitrogen-starved mycelium of *Scopulariopsis brevicaulis* (MacMillan, 1956) and *Neocosmospora vasinfecta* (Budd & Harley, 1962). The severe depletions of glutamine observed after exhaustion of the nitrogen source, after transfer of mycelium to nitrogen-free medium and during conidial germination presumably reflected the known demand for amide nitrogen in the biosynthesis of nucleotides, amino sugars, indoles, histidine and possibly (as established in the fungus *Agaricus bispora*; Levenberg, 1962) carbamoyl phosphate.

Almost complete utilization of the amino acid pool as a nitrogen reserve was demonstrable in mycelium transferred to nitrogen-free medium at pH 3.6; visible growth was not detected in the present experiment, but the development of aberrant globose structures on the hyphae, involving protoplasmic synthesis, can occur during nitrogen starvation at this pH, particularly at high glucose concentration (Bent & Morton, 1963). It is clear that the growth in hyphal length (which can be accompanied under certain circumstances by profuse sporulation; Morton, 1961) observed during nitrogen starvation at higher pH depends on a rapid turnover of protein involving the utilization and replenishment of the amino acid pool. Redistribution of the released α -amino groups, by transamination or deamination-amination processes, no doubt occurs; formation of amino acids from [^{14}C]glucose in the absence of exogenous nitrogen has

been shown in the mould *Zygorynchus moelleri* (Moses, 1959).

The occurrence of intracellular protein turnover in nitrogen-starved cells of yeast and bacteria at rates of 2–7 %/hr. is well established (for reviews see Halvorson, 1962; Mandelstam, 1963). It is not certain whether the more rapid turnover encountered in the present work is an entirely intracellular process. However, several lines of evidence suggesting that protein synthesis and breakdown are not restricted to separate regions of the mycelium have been mentioned above; also, the development in surface cultures of aerial hyphae producing apical conidiophores and the occurrence of cytoplasmic streaming in hyphae signify a potentiality for extensive intrahyphal movement of metabolites in mould mycelium. Meyers & Knight (1961) reported that the amino acid pool of *Penicillium roqueforti* increased during incubation in nitrogen-free medium, suggesting that under their conditions replenishment of the pool proceeded faster than its utilization; the addition of a mixture of purines and pyrimidines led to pool depletion, probably by changing the balance between protein synthesis and degradation. No significant effect on the amino acid pool was found when a similar mixture of bases was added to suspensions of *P. griseofulvum* growing in full or nitrogen-free medium (K. J. Bent, unpublished work).

Estimated rates of ^{15}N incorporation into soluble mycelial fractions gradually declined with time (Table 4), although ^{15}N uptake proceeded at a steady rate. Except for the initial values, rates were calculated on the assumption that there was no contribution to these fractions from insoluble intracellular sources. If protein turnover occurred during growth on exogenous nitrogen, i.e. if w_3 in eqn. (1) was not in fact negligible, then a decline in the calculated incorporation rates would be apparent. There is at present no evidence on the turnover of insoluble nitrogen during growth of fungi on an external nitrogen supply. In yeast and bacteria the rates of protein turnover are generally much lower in rapidly growing cells than in nitrogen-starved cells (Halvorson, 1962; Mandelstam, 1963). Nevertheless, degradation of mycelial protein may have occurred to some extent before exhaustion of the nitrogen source, particularly since rapid breakdown (about 17.5 % of the insoluble nitrogen/hr.) was detectable within 1 hr. of nitrogen exhaustion. Slower degradation, at say 5 %/hr., would account largely for the falls in the estimated rates of incorporation.

It was also assumed that all molecules of a particular 'nitrogen acceptor' in the mycelium were equally liable to combine with exogenous nitrogen. If, however, a small and more accessible portion of a pool became fully labelled in a short time, the

remainder being labelled more slowly, then overall rates of labelling would decrease when the 'active' portion became saturated with ^{15}N . Similarly, a gradual fall in metabolic activity from the elongating tips to the older parts of the mycelium could account for the apparent declines in incorporation rates.

The significance of the specialized amino acid pool of dormant conidia is difficult to assess from the present data. It can hardly be regarded as a reserve of nitrogen since an external source was required for the earliest visible stage of spore germination. However, the initial depletions in glutamic acid and γ -aminobutyric acid, observed during incubation in both full and nitrogen-free media, may be an integral part of the process of the breaking of dormancy, particularly since prior incubation in nitrogen-free medium led to faster germination on transfer to full medium. Results rather similar to our own were reported by Shepherd (1957) for conidia of *Aspergillus nidulans*; early synthesis of protein was accompanied by the depletion of several pool components, particularly γ -aminobutyric acid and asparagine. Yanagita (1957), on the other hand, recorded an initial transient accumulation of free amino acids during the germination of conidia of *Aspergillus niger*, and postulated that in conidial germination the synthesis of amino acids from exogenous nitrogen precedes the onset of protein synthesis. Such behaviour was not shown, under the present conditions, by conidia of *P. griseofulvum*.

SUMMARY

1. Conidia of *Penicillium griseofulvum* were incubated in full and nitrogen-free media, and changes in the amino acid pool were followed. Utilization of free amino acids present in dormant conidia, especially glutamine and γ -aminobutyric acid, occurred at an early stage in both media, although an external nitrogen source was required before visible signs of normal germination could be detected.

2. Net utilization of over 90 % of the initial amino acid pool in the synthesis of hot-water-insoluble nitrogenous components of *P. griseofulvum* was demonstrable when the mycelium was transferred to nitrogen-free medium at pH 3.6. At pH 6.0 this process became masked by the onset of net protein degradation to amino acids and peptides. At pH 3.0 both synthetic and degradative systems were inhibited.

3. A study of incorporation of ^{15}N -labelled ammonia revealed that almost all of the free amino acid fraction of the mycelium was involved in the assimilation of inorganic nitrogen, and that at least 67 % of the assimilated nitrogen passed through

this fraction. Incorporation into glutamic acid was sufficient to account for the synthesis of at least 85% of the total mycelial α -amino nitrogen content. The redistribution of label observed after the exhaustion of exogenous nitrogen confirmed previous non-isotopic evidence that a rapid turnover of insoluble nitrogen, involving breakdown to the amino acid level, occurred during nitrogen starvation. Extracellular organic nitrogen released after exhaustion of ammonia arose from the breakdown of insoluble mycelial material.

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Studies on the Nature of Polysomes

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Polysomes are aggregates of ribosomes held together by RNA. These structures have been obtained from rat liver (Wettstein, Staehelin & Noll, 1963; Noll, Staehelin & Wettstein, 1963; Jackson, Munro & Korner, 1963), reticulocytes (Marks, Burka & Schlessinger, 1962; Warner, Knopf & Rich, 1963; Gierer, 1963; Williamson & Mathias, 1963), HeLa cells (Penman, Scherrer, Becker & Darnell, 1963), ascites-tumour cells (White, Pedersen & Hultin, 1963), rat heart (Earl & Korner, 1963) and from bacteria (Spyrides & Lipmann, 1962; Barondes & Nirenberg, 1962; Staehelin, Brinton, Wettstein & Noll, 1963*a*). Further, electron-micrographs have been published which indicate that aggregates of ribosomes can be attached to a thin strand which might be messenger RNA (Warner, Rich & Hall, 1962; Williamson, Mathias, Huxley & Page, 1963).

Polysomes are considered to be the functional

units of protein biosynthesis by Wettstein *et al.* (1963) and Noll *et al.* (1963). They are called 'ergosomes' by these workers to indicate that these aggregates are the working particles and that the aggregate structure is necessary for protein synthesis *in vivo* and for amino acid incorporation *in vitro*. This viewpoint is supported by other workers (Goodman & Rich, 1963) and models of the protein-biosynthetic mechanism have been propounded by them.

In this paper we first consider details of conditions necessary for optimum amino acid incorporation into protein with rat-liver polysomes, ribosomes and a total ribonucleoprotein particle preparation. We produce evidence that the strand to which ribosomes are attached in polysomes isolated from rat liver has some of the properties of messenger RNA. We question some details of the models of the protein-synthetic mechanism suggested by Wettstein