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Ligninolytic Activity of *Phanerochaete chrysosporium*: Physiology of Suppression by NH_4^+ and L-Glutamate

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Abstract. Previous research showed that addition of nutrient nitrogen to ligninolytic (stationary, nitrogen-starved) cultures of the wood-decomposing basidiomycete *Phanerochaete chrysosporium* causes a suppression of lignin degradation. The present study examined early effects on nitrogen metabolism that followed addition of NH_4^+ and L-glutamate at concentrations that yield similar patterns of suppression. Both nitrogenous compounds were rapidly assimilated (> 80% in 6 h). Both caused an initial 80% or greater increase in the intracellular glutamate pool and had similar effects in increasing the specific activities of NADP- and NAD-glutamate dehydrogenases and glutamine synthetase. Differences between the effects of added NH_4^+ and glutamate showed that suppression was not correlated with intracellular pools of arginine or glutamine, nor was the maintenance of an elevated glutamate pool required to maintain the suppressed state. While a portion of the initial glutamate suppression could be attributed to an effect on central carbon metabolism through glutamate catabolism by NAD-glutamate dehydrogenase, the long term suppression by glutamate and the suppression by NH,' were more specific. Suppression by NH,' or glutamate in the presence or absence of protein synthesis (cycloheximide) followed essentially identical kinetics during 12 h. These results indicate that nitrogen additions cause a biochemical repression of enzymes associated with lignin degradation. Results are consistent with the hypothesis that nitrogen metabolism via glutamate plays a role in initiation of repression.

Key words: Wood decay – White-rot fungi – Lignin biodegradation – Fungus physiology – Repression by glutamate – Glutamate dehydrogenase – Glutamine synthetase – Intracellular amino acids

Nitrogen metabolism plays a role in regulating lignin degradation as a part of secondary metabolism in the wood-decomposing basidiomycete *Phanerochaete chrysosporium.* Ligninolytic activity and the secondary metabolite veratryl alcohol appear simultaneously 2 – 3 days after depletion of nitrogen in nitrogen-limited media (Fenn and Kirk 1981; Jeffries et al. 1981; Keyser et al. 1978). Although recent investigations have shown that carbohydrate- or sulfur limitation can also trigger lignin degradation (Jeffries et al. 1981), nitrogen must be the limiting nutrient for sustained and vigorous degradation of lignin (Jeffries et al. 1981; Keyser et al. 1978; Reid 1979). The onset of ligninolytic activity can be delayed by addition of NH_4^+ to nitrogen-starved cultures (Keyser et al. 1978). and once activity appears, it can be sharply suppressed by addition of NH_4^+ or any of several amino acids; L-glutamate was the most effective of 14 compounds tested (Fenn and Kirk 1981). Synthesis of veratryl alcohol is also suppressed markedly by added nitrogen (Fenn and Kirk 1981; Shimada et al. 1981), as is oxidation of the lignin-related phenol acetovanillone (Fenn and Kirk, 1981). Suppression by nitrogen is apparently not mediated through carbon (glucose)-catabolite repression (Fenn and Kirk 1981).

The present study examined several aspects of nitrogen metabolism during the initial phases of suppression of ligninolytic activity by added NH,' and glutamate.

Non-Standard Abbreviations: DMS = 2.2-dimethylsuccinate; TCA = trichloroacetic acid

Materials and Methods

^{14}C-Labeled Substrates

Synthetic [U-ring-^{14}C]-lignin (sp. act. 9.5×10^5 dpm/mg) was used in all experiments and was added aseptically to cultures as an aqueous suspension (Keyser et al. 1978; Kirk et al. 1975, 1978). [U-^{14}C]-glucose (sp. act. 0.081 mCi/mmol), [1,4-^{14}C]-succinic acid (sp. act. 15.1 mCi/mmol), [2-^{14}C]-uracil (sp. act. 58.0 mCi/mmol) and [U-^{14}C]-leucine (sp. act. 351 mCi/mmol) were purchased from Amersham (Arlington Heights, Ill.).

Culture Conditions and Ligninolytic Assay

Phanerochaete chrysosporium Burds. ME-446 (ATCC 35540) was maintained and cultured (10 ml cultures, 39°C, 100% O_2) as described previously (Kirk et al. 1978). The basal medium contained the mineral salts and vitamins at the concentrations previously reported (Kirk et al. 1978), plus L-asparagine (0.62 mM) and NH_4NO_3 (0.62 mM) as nitrogen sources, and b-glucose (56 mM) as growth substrate. Cultures were buffered at pH 4.2–4.3 with 0.01 M sodium 2,2-dimethylsuccinate (DMS) (Fenn and Kirk 1979). All cultures were grown without agitation and possessed maximum ligninolytic activity 5 – 6 days after inoculation.

Ligninolytic activity and the oxidations of glucose and succinate were assayed by the $^{14}CO_2$ produced following the addition of the appropriate ^{14}C-labeled compounds. Details of this assay procedure have been published (Fenn and Kirk 1981; Keyser et al. 1978). The following procedure was used in short-term assays. The labeled substrate was added in 0.5 ml of water to 5.5-day-old cultures, which were im-

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mediately flushed with 100% O_2 and incubated at 39°C. After 12 – 15 h of incubation, 0.5 ml of fresh aqueous solution of NH_4Cl, glutamate or cycloheximide were added to give the desired final concentration. The flasks were then flushed with 100% O_2, incubated at 39°C and every 2-3 h for the next 10–12 h the cultures were flushed with 100% O_2 for 15 min and the $^{14}CO_2$ collected.

Analytical

Amino acids were extracted in hot 80% v/v ethanol (Fenn and Kirk 1981). Glutamate was isolated from the extracts by the column separation procedure of Ferguson and Sims (1974) and quantitated with ninhydrin, using glutamic acid (Sigma, St. Louis, Mo.) as standard. After removal of the glutamate, glutamine in the extracts was converted to glutamate with commercial glutaminase (Sigma), and separated and quantitated as glutamate. After removal of glutamate and glutamine, the residual extract was assayed for arginine by the method of Van Pilsum et al. (1956).

Total protein was extracted from mycelia with hot 1 M NaOH (Fenn and Kirk 1981) and assayed by the method of Bradford (1976) with reagents from Bio-Rad Laboratories (Richmond, Calif.).

Ammonium in culture filtrates was assayed by the method of Robbins and Weber (1977).

Protein and RNA Synthesis

Protein and RNA syntheses were measured by the incorporation of ^{14}C-leucine (protein) or ^{14}C-uracil (RNA) into trichloroacetic acid (TCA)-insoluble cellular materials. A fresh solution of cycloheximide (to give 6.8μg/ml final culture concentration) or water (controls) was added to 6-day-old cultures, which were then flushed with 100% O_2 and incubated at 39°C. After 1, 2, 3, and 5 h ^{14}C-leucine (0.72 μCi, 2.1 nmol) or ^{14}C-uracil (0.69 μCi, 11.9 nmol) was added in 0.5 ml of water to triplicate cultures. After 15 min incubation at 39°C, 10 ml of ice-cold 20% (w/v) TCA was added to each flask and these held on ice for 30 min or more. The acid-insoluble materials were collected by vacuum filtration onto glassfiber filters (Whatman GF/A) and washed 3 times with 10 ml of cold 10% TCA followed by 3 washes with 10 ml of 95% ethanol. Filters were prepared for scintillation counting as described previously (Fenn and Kirk 1981). Cultures to which cold TCA was added simultaneously with the labeled substrate showed insignificant incorporation of the label into TCA-insoluble materials.

Enzyme Assays

Mycelium from 3-5 cultures was pooled, collected by centrifugation and washed once with the appropriate extraction buffer: 0.1 M potassium phosphate pH 7.4 for glutamate dehydrogenase assays or 0.1 M imidazole · HCl pH 6.5 for glutamine synthetase assays. The mycelial pellet, about twice its volume of acid-washed silica sand, and 1 – 2 ml of extraction buffer were ground in an ice-chilled mortar. The slurry was diluted with 2 – 3 ml of extraction buffer and centrifuged at ~ 20,000 × *g* at 4°C for 20 min. The supernatant fluids were used directly as crude enzyme preparations.

L-Glutamate: NADP oxidoreductase, EC.1.4.1.4 (NADP-GDH), and L-glutamate: NAD oxidoreductase, EC.1.4.1.2 (NAD-GDH), were assayed by the procedures of Kinghorn and Pateman (1973) except that 0.2 M potassium phosphate buffer pH 7.4 was used in both assays. All reagents were prepared with this buffer. The final concentrations of all reagents were those given by Kinghorn and Pateman (1973), and the buffer used in the NAD-GDH assay contained 0.1 mM ß-mercaptoethanol and 0.5 mM EDTA as described.

In the NADP-GDH assay, reaction rates were proportional to protein concentration from 0 – 40 μg protein per 3.0 ml assay volume. The specific activities reported are from assays within this range of protein. No NADP-reducing activity was detected in the crude enzyme preparations (cf. Yajima et al. 1979).

Glutamine synthetase [L-glutamate:ammonia ligase (ADP-forming) EC6.3.1.2] was assayed by the procedures of Bender et al. (1977). The final concentrations of all reagents in the assays were those given, but stock assay mixtures were twice those given so that volumes of enzyme up to 0.25 ml could be accommodated in a 0.5 ml final assay volume. Assay mixtures were adjusted to pH 6.5 and assays were run at 37°C. Preliminary experiments established that for both the γ-glutamyl transferase activity and the biosynthetic activity the optimum pH was near 6.5, that activities were proportional to enzyme concentration, and that the reactions were linear for 30 min or more. The optimum concentration of Mg^{2+} for the biosynthetic reaction was found to be near 50 mM, and the optimum Mn^{2+} concentration for the transferase activity was near 0.3 mM. These concentrations are close to those in the final assay mixtures (56 mM Mg^{2+} and 0.27 mM Mn^{2+}) by the procedures of Bender et al. (1977).

Results

Concentration Dependence of Suppression by NH_4^+ and Glutamate

Comparisons of the physiological events that accompany suppression of ligninolytic activity by added NH,' or glutamate were made using concentrations that gave a similar amount of suppression. This permitted separation of some of the general effects on nitrogen metabolism from those affecting lignin oxidation. Reproducible transient suppression by NH,' occurred at about 0.7 mM and greater concentrations, whereas less than 0.2 mM glutamate had a similar effect (Fig. 1). At 2.80 mM NH_4^+ and 0.72 mM glutamate, ligninolytic activity was suppressed strongly for more than 24 h, and returned to control or higher rates by about 48 h; these concentrations were chosen for further study. Glutamate at ≥ 1.4 mM and NH_4^+ at ≥ 5.7 mM caused long-term (> 72 h) suppression. Analyses of cultures receiving NH_4^+ to 2.8 mM or glutamate to 0.72 mM showed that assimilation of the supplemental nitrogen was rapid: after 6 h, only 18% and 14% of the NH_4^+ and glutamate remained in the culture fluids (Fig. 2). Because of this rapid uptake and the fact that suppression was evident within 6 h. further experiments were conducted with cultures 0 – 6 h after nitrogen addition.

Effects of NH_4^+ and Glutamate on Total Protein, Selected Amino Acids and Specific Activities of Enzymes

Analyses of intracellular glutamine, arginine and glutamate revealed differences in the effects of added NH_4^+ and glutamate (Fig. 3A – C). Glutamine and arginine were examined because of their pivotal roles in nitrogen uptake and storage. and glutamate because a possible regulatory role of its intracellular metabolism in lignin degradation has been

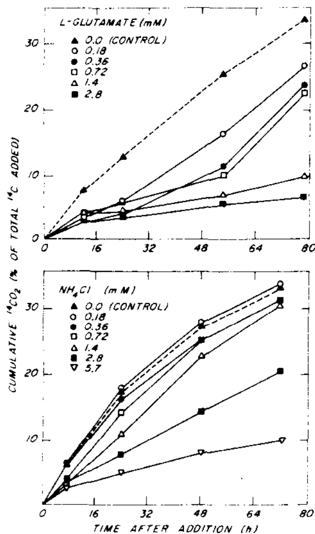

Fig. 1. Concentration dependence of suppression of lignin degradation by L-glutamate and NH_4Cl. ^{14}C-Lignin (5.2×10^4 dpm for NH_4Cl cultures, 5.6×10^4 dpm for L-glutamate cultures) was added at 5.5 days. At 6.0 days the NH_4Cl or glutamate in 0.5 ml of H_2O was added to give the indicated concentrations of added compound. Cultures were incubated under 100% O_2 at 39°C and $^{14}CO_2$ was collected at the times shown by flushing the cultures with 100% O_2. Data points are the means for 5 replicate cultures: variation was <10% of values shown. Repetition of experiment gave essentially identical results

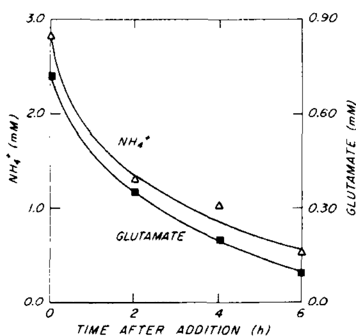

Fig. 2. Depletion of NH_4^+ and glutamate added to h-day-old ligninolytic cultures. Glutamate (0.72 mM), and NH_4Cl (2.8 mM) were added at time 0 (6 days). Cultures were flushed with 100% O_2 and incubated at 39°C

suggested (Fenn and Kirk 1981). Added NH,' and glutamate had quite different effects on the intracellular concentrations of glutamine and arginine (Fig. 3A, B). During the 6 h following addition of NH_4^+, there was a dramatic increase in glutamine (from 20 to 1200 nmol per culture) and arginine (from 6 to 210 nmol/culture). Added glutamine caused only small perturbations in the glutamine concentration in comparison with NH_4^+. Arginine was affected much less by added glutamate, although increases were observed from 6 to 35 nmol/culture over 6 h. Control cultures that received only water exhibited no variation in intracellular glutamine or arginine concentrations.

Both NH_4^+ and glutamate caused approximately an 80% increase in the intracellular pool of glutamate within 2 h (to 215 – 245 nmol/culture; Fig. 3C), at which time net protein synthesis was detected in NH_4^+ but not in glutamate cultures (Fig. 3D). The glutamate pool continued to increase in cultures receiving NH_4^+ (to 340nmol/culture by 6 h), but

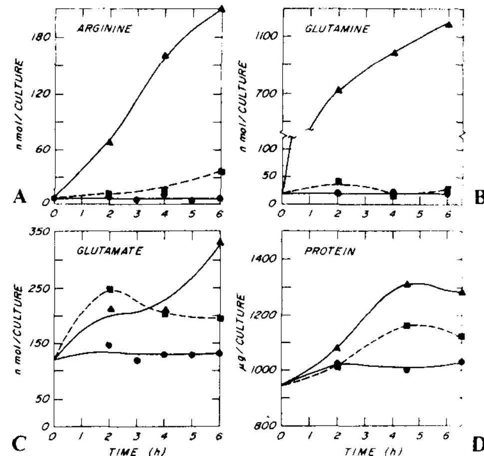

Fig. 3A–D. Changes in intracellular pools of **A** arginine, **B** glutamine, **C** glutamate, and **D** total protein after additions of NH_4Cl (2.8 mM), glutamate (0.72 mM), or water (controls). to 6-day-old ligninolytic cultures of *Phanerochaete chrysosporium.* Symbols: ▲ NH_4^+. ■ glutamate, ● control. Three replicate cultures were pooled for each analysis

slowly decreased through 6 h in cultures receiving glutamate (Fig. 3C). Further measurements of the glutamate pools after 10.5, 18, and 24 h revealed that NH_4^+, glutamate, and control cultures maintained their pools of glutamate over this period, i.e., 328–387 nmol/culture, 194–234 nmol/culture, and 139–142 nmol/culture.

When growth (increase in total protein, Fig. 3D, Table 1) was considered over this time period it was calculated that following NH_4^+ addition, the intracellular concentration of glutamate increased from 135 nmol/mg protein (controls) to ~ 270 nmol/mg protein after 6 h and then remained nearly constant (~ 260 nmol/mg protein) through 18 h. After addition of glutamate the pool increased to 240 nmol/mg protein in 2 h and then decreased by 6 h to 177 nmol/mg protein and to ~ 150 nmol/mg protein, or near control levels. by 18 h.

Additions of NH_4^+ and glutamate resulted in concentration-dependent increases in net protein. Both nitrogen sources yielded approximately the same net increase in protein per equivalent of nitrogen added after 18 h (Table 1).

Changes in the specific activities of both NADP- and NAD-glutamate dehydrogenases, and of glutamine synthetase, occurred as a result of nitrogen additions (Table 2). The specific activity of NADP-glutamate dehydrogenase, which serves a synthetic role (Kinghorn and Pateman 1973), more than doubled by 3 h after NH_4^+ addition and doubled after addition of glutamate. NAD-glutamate dehydrogenase, which probably functions in glutamate catabolism (Kinghorn and Pateman 1973), was not detected in control cultures, but low specific activities which increased with time were found following both glutamate and NH_4^+ additions. The transferase activity of glutamine synthetase was increased by both NH_4^+ and glutamate additions, but a significant increase in the synthetic activity was detected only 6 h after glutamate addition.

Suppression by NH_4^+, Glutamate and Cycloheximide

The time course of suppression of ligninolytic activity by NH,' and glutamate in relation to the capacity of the fungus to synthesize protein was followed. Initial experiments estab-

Table 1. Effects of added NH_4Cl and glutamate on protein content of ligninolytic cultures of *Phanerochaete chrysosporium*

Nitrogen source	Final concentration of added compound (mM)	Increase in total protein (% of controls)
NH_4Cl[a]	0.18	7
	0.36	20
	0.72	36
	1.42	60
	2.80	72
L-Glutamate[a]	0.18	9
	0.36	15
	0.72	30
	1.42	63

[a] Nitrogen sources were added in 1.0 ml of water to 6-day-old cultures. Protein was extracted and assayed after 18 h. Each analysis was performed on triplicate cultures. Values shown are the means; standard deviation was less than 10 %

Table 2. Effects of added NH_4^+ and glutamate on the specific activities of glutamine synthetase and NADP- and NAD-glutamate dehydrogenases (GDH) in ligninolytic cultures of *Phanerochaete chrysosporium*

Time after nitrogen source added	Specific activities, nmol/min · mg protein[a,b]			
	Glutamine synthetase		NADP-GDH	NAD-GDH
	Transferase activity	Synthetic activity		
3 h				
None	130	100	320	ND[c]
NH:	250	80	740	< 10
Glutamate	260	90	590	10
6 h				
None	150	60	440	ND[c]
NH_4^+	360	90	820	20
Glutamate	220	170	660	20

[a] Enzyme preparations were prepared 3 and 6 h after NH_4Cl (2.8 mM), glutamate (0.72 mM) or water (controls) were added to 6-day-old ligninolytic cultures. For details see Materials and Methods

[b] Glutamine synthetase activities were assayed by the formation of glutamyl hydroxamate; NADP- and NAD-GDH activities were assayed by oxidations of NADPH and NADH respectively. For details see Materials and Methods. For each analysis, 3 – 5 replicate cultures were pooled. Entire experiment was repeated twice with same relative values being obtained

[c] ND = no activity detected

lished that cycloheximide at 6.8 µg/ml inhibited protein synthesis by 98 % within 1 h. RNA synthesis was also inhibited after 3 h at this concentration (Fig. 4). Results showed that suppression of ligninolytic activity by added NH_4^+ glutamate or cycloheximide exhibited the same kinetics (Fig. 5). No augmentation of suppression was seen when cycloheximide was added together with NH_4^+, but a slight initial additive effect was seen when glutamate and cycloheximide were both administered (Fig. 5).

Effect of NH_4^+, Glutamate and Cycloheximide on Glucose and Succinate Oxidation

Ammonia, glutamate, and cycloheximide had quite different effects on oxidation of glucose and succinate to CO_2 (Fig. 6).

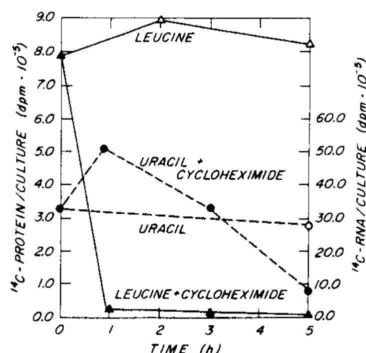

Fig.4. Inhibition of protein and RNA synthesis by cycloheximide (6.8 µg/ml) in ligninolytic cultures of *P. chrysosporium.* Synthesis was measured by the incorporation of radioactivity into TCA-insoluble materials after a 15 min pulse of ^{14}C-leucine (protein) or ^{14}C-uracil (RNA) administered at the times indicated

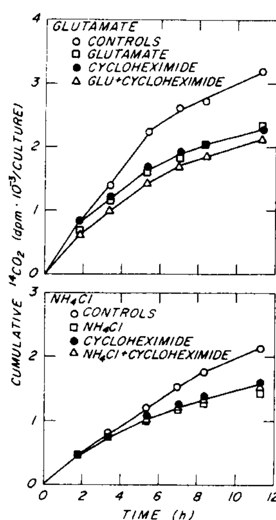

Fig.5. Kinetics of the suppression of ^{14}C-lignin oxidation to $^{14}CO_2$ by added L-glutamate (0.72 mM), NH_4Cl (2.8 mM), or cycloheximide (6.8 µg/ml). Details were as described in Fig. 1. In this experiment, glutamate cultures initially contained 7.6×10^4 dpm of ^{14}C-lignin and NH_4Cl cultures contained 4.9×10^4 dpm of ^{14}C-lignin. Values shown are means of 6 replicates. Standard deviations for each data point were < 10% of values shown

As anticipated, oxidation of glucose and succinate was markedly stimulated by NH_4^+ addition, and rates continued to increase over controls for > 10 h. In contrast, glutamate caused a slight and transient decrease in oxidation rates lasting about 6 – 7 h. Addition of cycloheximide resulted in a severe and progressive shutdown of the oxidation of both succinate and glucose.

Discussion

Nature of Suppression by NH_4^+ and Glutamate

These results demonstrate that the suppressive effect of NH_4^+ on lignin degradation is not via suppression of the rates of one or more common reactions of central carbon catabolism – i.e., those shared by glucose and succinate oxidation. This conclusion is supported by the fact that NH_4^+ stimulated glucose and succinate oxidations but suppressed lignin oxidation. The stimulatory effect of NH_4^+ on central carbon

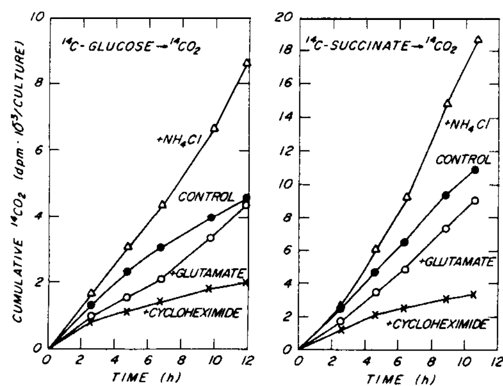

Fig.6. Effects of added glutamate (0.72 mM) and NH_4Cl (2.8 mM) on oxidation of ^{14}C-glucose and ^{14}C-succinate to $^{14}CO_2$. Details were as described in Fig. 1. Cycloheximide was added at 6.8 μg/ml. ^{14}C-Glucose was added to give 2×10^5 dpm/culture; ^{14}C-succinate was initially 4.9×10^5 dpm/culture. Data points are the means of 5 replicate cultures: standard deviations were < 10% of values shown

oxidative pathways was presumably due to an increase in activities of enzymes catalyzing the rate-limiting steps. Failure of NH_4^+ to cause even transient stimulation of lignin oxidation suggests two possibilities: (1) the suppressive effect was at some step before lignin carbon entered the central pathways, and (2) there was no significant pool of lignin degradation products past the NH_4^+-affected step(s).

The slight, transient suppression of glucose and succinate oxidations by glutamate is considered to be an anaplerotic effect of the entrance into the TCA cycle of α-ketoglutarate derived from the deamination of added glutamate or by its transamination. This also can explain why a slight initial, but non-progressive, additive suppression of lignin oxidation was seen when cycloheximide and glutamate were added simultaneously. [That glutamate plus cycloheximide had this additive effect also with lignin provides evidence that the carbon of lignin actually enters central carbon catabolic pathways; incorporation of lignin carbon (side chain-*b* - and γ-) into veratryl alcohol (Kirk and Fenn 1981) also indicates this.] Although the short-term (6 h) effect of glutamate on lignin oxidation and the greater effect of glutamate than NH_4^+ could be explained, in part, by an effect on central carbon catabolism, the effect over a 12-h period clearly could not. Over the longer term, other mechanisms must be responsible for the more suppressive effect of glutamate.

The combined evidence from the present study and earlier investigations indicates that the effect of adding certain nitrogenous compounds is one of biochemical *repression* of a key enzyme or enzymes involved in lignin degradation. When the effect of nitrogen was first discovered (Keyser et al. 1978; Kirk et al. 1978), it was recognized that it might be competition by nitrogen metabolism for cofactors needed for lignin oxidation or even direct inhibition of the ligninolytic system, although repression seemed more probable. The following results favor repression and discredit competition and inhibition: (a) Repression but not inhibition or competition would be expected to take several hours for maximum development, as observed here; (b) the kinetics of repression by the protein synthesis inhibitor cycloheximide and suppression by NH_4^+ and glutamate were identical; and (c) ligninolytic activity can be triggered by carbon- or sulfur-limitation in the presence of nitrogen (Jeffries et al. 1981) at concentrations which would be suppressive in nitrogen-starved cultures if the mechanism were competition or inhibition. In accord with repression is the fact that the suppressive effect of nitrogen is a general one affecting veratryl alcohol synthesis (Fenn and Kirk 1981; Shimada et al. 1981) and oxidation to $^{14}CO_2$ of methoxyl- and side chain- as well as ring-labeled lignin (Kirk et al. 1978).

The possibility must also be considered that added nitrogen simply causes lignin carbon to be diverted to biosynthesis. However, this seems most unlikely, since glucose and succinate oxidations to CO_2 were stimulated by added NH_4^+ Carbon from these substrates would be expected to be diverted to biosynthesis as efficiently as carbon from lignin.

Evidence presented here is consistent with nitrogen repression being at the level of either protein or RNA synthesis or both. Hynes (1974) has reached a similar conclusion that added nitrogen (glutamate and glutamine) can initiate repression of enzyme synthesis in *Aspergillus nidulans.*

Physiology of Nitrogen Repression

Evidence indicates that the repression by glutamate and NH_4^+ may ultimately occur by the same mechanism: The kinetics during the initial onset (11 h) of repression by both are the same, and both nitrogen sources affect veratryl alcohol synthesis as well as ligninolytic activity (Fenn and Kirk 1981). Comparison of the physiological effects of added NH_4^+ and glutamate therefore helps distinguish effects that are general responses to added nitrogen from those that may be more closely related to the development of repression. Thus, the large changes in glutamine and arginine pools in NH_4^+-repressed cultures were not found in cultures similarly repressed by glutamate, so that the high levels of these amino acids would appear not to be essential for, or indicative of, the initiation or maintenance of repression. On the other hand, both nitrogen sources increased the glutamate pool prior to the onset and during the first hours of repression, which leaves open the suggestion (Fenn and Kirk 1981) that glutamate metabolism may play a more direct role in initiating nitrogen-based repression. However, the fact that the glutamate pool per unit protein returns to levels found in fully ligninolytic cultures by 18 h after glutamate addition, with no relief of repression shows that maintenance of an elevated glutamate pool is not essential to continued repression.

Other findings suggestive of a role of glutamate metabolism in repression are that glutamate figures prominently in the changes in the amino acid pool that occur between primary and secondary metabolism (Fenn and Kirk 1981), that it is the most effective of the examined nitrogenous compounds in suppressing ligninolytic activity (Fenn and Kirk 1981), and that it can also repress lignin degradation in carbon-limited ligninolytic cultures containing excess nitrogen (Jeffries et al. 1981). Other strongly suppressive amino acids include glutamine and histidine (Fenn and Kirk 1981), which can be catabolized via glutamate.

Assays for enzymes important in nitrogen metabolism and cellular regulation revealed changes in their activities that may be associated with repression. Increased NADP-glutamate dehydrogenase activity after NH_4^+ addition showed that in *Phanerochaete chrysosporium,* as in other fungi (Hynes 1974; Kinghorn and Pateman 1973; Pateman 1969), NH_4^+ is important in regulating the activity of this enzyme. Increased intracellular release of NH_4^+ from glutamate by NAD-

glutamate dehydrogenase may explain why added glutamate also increased NADP-glutamate dehydrogenase activity. The important role of increased NADP-glutamate dehydrogenase in ammonium repression in fungi (Hynes 1974; Kinghorn and Pateman 1973; Pateman 1969) suggests the need for detailed studies of this enzyme in relation to regulation of secondary metabolism.

Glutamine synthetase showed increased specific activities (transferase assay) which were consistent with the elevated glutamate pools following addition of both NH_4^+ and glutamate (Ferguson and Sims 1974; Pateman 1969). However, the 45-fold increase (on a protein basis) in the intracellular glutamine pool after NH_4^+ addition would have been expected to cause a severe decrease, not an increase, in glutamine synthetase activity. In other fungi glutamine has been shown to be a strong repressor of this activity (Ferguson and Sims 1974; Pateman 1969). This observation suggests that there may be a major difference in the mode of regulation of glutamine synthetase in *P. chrysosporium* compared to other fungi and possibly in any role this enzyme may play in nitrogen-based repression.

Results here and from earlier work (Fenn and Kirk 1981) indicate that repression is not tied closely to net increase in cellular protein. which is an indication of renewed primary growth. Added histidine (2.8 mM) strongly suppressed ligninolytic activity but caused no net increase in protein during the 18 h assay period (Fenn and Kirk 1981). Similarly, in the present study added glutamate (0.18 mM) strongly repressed ligninolytic activity but caused less than 10% net increase in protein. Conversely, added NH_4^+ (0.36–0.72 mM) resulted in a 20 – 36% net increase in protein but caused no significant repression.

These observations indicate that this ligninolytic fungus can be maintained in a mixed primary/secondary state in which growth and ligninolytic activity (secondary metabolism) occur concurrently. Grootwassink and Gaucher (1980) have shown recently that the concentration of nitrogen in cultures of *Penicillium urticae* determines whether primary and secondary metabolism are temporally separated or whether they overlap (they measured growth and enzymes of patulin biosynthesis). Results here show that the same situation obtains in the effects of NH_4^+ concentration on growth and lignin metabolism. Aharonowitz and Demain (1980) have recently reached the general conclusion that the separation of primary from secondary metabolism ["trophophase" from "idiophase" (Bu'Lock 1975)] is a function of nutrition. and that the two phases can be made to occur simultaneously by manipulation of nutrition or by mutation. This concept has important practical implications for lignin biodegradation, as it has for other secondary metabolic processes.

It must be kept in mind that the assay for ligninolytic activity used here and in past work (^{14}C-lignin → $^{14}CO_2$) does not disclose which degradative step or steps are secondary metabolic and repressible by nitrogenous compounds. This will require detailed understanding of the chemistry and biochemistry of lignin degradation and the development of assays for the intermediate steps. Current research seeks this information.

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