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Choline- and acetylcholine-induced changes in the morphology of *Fusarium graminearum*: evidence for the involvement of the choline transport system and acetylcholinesterase

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The response of *Fusarium graminearum* to choline, acetylcholine and a number of related analogues was investigated and their ability to induce a morphological response quantified. A number of mutants resistant to the alkylating agent nitrogen mustard (*nim* strains) were generated and found to have lost the ability to transport choline. These mutants were found to be insensitive to choline and acetylcholine but not to betaine, ethanolamine and other analogues. In addition, the non-competitive inhibitor hemicholinium-3 was also found to reduce the morphological effect of choline, proving that transport of choline into the hypha is essential for the morphological response. Acetylcholinesterase inhibitors blocked the morphological response to acetylcholine but had no effect on the response to choline, suggesting the presence of a membrane- or wall-bound acetylcholinesterase that hydrolyses acetylcholine to choline which subsequently induces the morphological response. Studies on the *in vivo* chitin synthase activity revealed that addition of choline caused a transient 75% increase in chitin synthase activity within 30 s, the rate rapidly returning to that observed before the addition of choline. No such effect was observed with the *nim* mutants.

Keywords: *Fusarium graminearum*, choline, hyphal growth, nitrogen mustard, chitin synthase

INTRODUCTION

Choline and the related analogue betaine, were isolated from wheat anthers and identified as virulence enhancers for *Fusarium graminearum* (Strange & Smith, 1971; Strange *et al.*, 1974). Later they were found to cause an increase in the spread of the mycelium due to stimulation of the hyphal extension rate, although the overall growth rate remained unchanged (Wiebe *et al.*, 1989). A number of other related compounds, including phosphorylcholine, ethanolamine and acetylcholine, have also been shown to stimulate hyphal extension in *F. graminearum* to differing degrees (Strange & Smith, 1978; Wiebe *et al.*, 1989).

Recently, we have characterized a constitutive choline (Robson *et al.*, 1992) and a constitutive betaine (Robson *et al.*, 1994) transport system in *F. graminearum*, which may be involved in mediating the morphological effects of these two compounds. Previously however, it has not been possible to distinguish whether the morphological effect of choline on *F. graminearum* acted externally or required transport into the hypha. In *Saccharomyces cerevisiae*, resistance of mutants to the alkylating agent nitrogen mustard has been found to be due to the loss of a single recessive gene encoding choline permease (Li *et al.*, 1991), resulting in the inability of these mutants to transport choline.

In this paper, we quantify the stimulatory effects of choline and related analogues on the colony radial growth rate of *F. graminearum* A3/5. In addition, we have isolated a number of nitrogen-mustard-resistant mutants which lack the ability to transport choline, and have investigated the morphological effects of choline and related analogues on these mutants.

Abbreviations: 1,5(4-ADAP), 1,5-bis(4-allyldimethyl-ammoniumphenyl)-pentan-3-one dibromide; GlcNAc, *N*-acetylglucosamine.

METHODS

Organism, media and chemicals. *F. graminearum* strain A3/5 was obtained from Mr T. W. Naylor, (Marlow Foods,

Billingham, UK) and maintained in 20% (v/v) glycerol at −80 °C.

All cultures were grown on modified Vogel's medium (Vogel, 1956) in which 10 g D-glucose l^{-1} replaced sucrose as the carbon source. For liquid cultures, *F. graminearum* A3/5 was grown in 50 ml Vogel's medium in 250 ml flasks on a rotary shaker (200 r.p.m.) and inoculated with 1 ml of a 1×10^6 spore ml^{-1} suspension derived from a 7–10 d-old culture. For agar medium, 1·5% (w/v) Tayo agar (Lucas Meyer) was added. All cultures were incubated at 25 °C.

All additions to the medium were prepared as filter-sterilized (0·22 μm pore size, Whatman) stock solutions in distilled water (DMSO for hemicholinium-3) and stored in aliquots at −20 °C until required. Stock solutions were diluted × 100 to give the final concentration required in the medium and added either to molten agar at 55 °C, or to liquid medium at room temperature.

[^{3}H]*N*-Acetylglucosamine (GlcNAc) was obtained from Amersham (sp. act. 7·8 Ci $mmol^{-1}$). All chemicals were obtained from Sigma unless otherwise stated.

Colony radial growth rate determinations. Colony diameters were measured twice daily up to 96 h after inoculation in two 90 ° planes on four replicate colonies on plates which had been inoculated centrally with a 2·5 μl drop of a 1×10^6 spore ml^{-1} suspension. Colony radial growth rates (K_r) (Trinci, 1971) were calculated from the slope of the line from a plot of colony radius vs time since inoculation, by least squares regression analysis.

Isolation of nitrogen-mustard-resistant (*nim*) mutants. An aliquot (0·1 ml) of a 1×10^8 spore ml^{-1} suspension was placed on Vogel's agar medium containing 100 μg nitrogen mustard ml^{-1}. The *nim* mutants were isolated as colonies that grew vigorously on the plates and appeared between 4 and 8 d after inoculation. The *nim* mutants were subcultured on nitrogen mustard agar to check their validity.

Choline transport assay. Uptake of [^{14}C]choline by *F. graminearum* A3/5 and *nim* mutants was performed as described previously (Robson *et al.*, 1992).

Incorporation of [^{3}H]GlcNAc by mycelia. Incorporation of [H^3]GlcNAc into the fungal biomass was carried out as described previously (Binks *et al.*, 1990). [^{3}H]GlcNAc (0·08 μCi ml^{-1}) was added to an exponentially growing culture of *F. graminearum*. Duplicate 0·5 ml samples were removed at 1, 2 or 3 min intervals and added to 0·5 ml 10% (w/v) trichloroacetic acid. Samples were then filtered through Whatman no. 1 filter papers and washed with three 2 ml volumes of distilled water. The biomass and filters were then added to scintillation vials containing 4 ml scintillation fluid (Optiphase Hisafe) and radioactivity counted using a Packard Tricarb 1500 Scintillation counter. Choline (20 μM final concentration) was added to the culture from a × 100 stock, 15 min after the addition of [^{3}H]GlcNAc.

RESULTS

Effect of choline and related analogues on the morphology of *F. graminearum* A3/5

A typical dose–response curve for the effects of choline on the K_r of *F. graminearum* is shown in Fig. 1a. The maximum stimulation in K_r was calculated as the percentage increase in K_r compared with the control at the point where a further increase in the choline concentration had no additional effect on K_r. Probit transformation of the curve produced a linear relationship and therefore the concentration which gave a 5% (ED_5) or 50% (ED_{50}) increase in the K_r was calculated (Fig. 1b). Using this approach, it was possible to quantify and compare the effects of the other compounds with that of choline (Table 1). With the exception of betaine, propionylcholine,

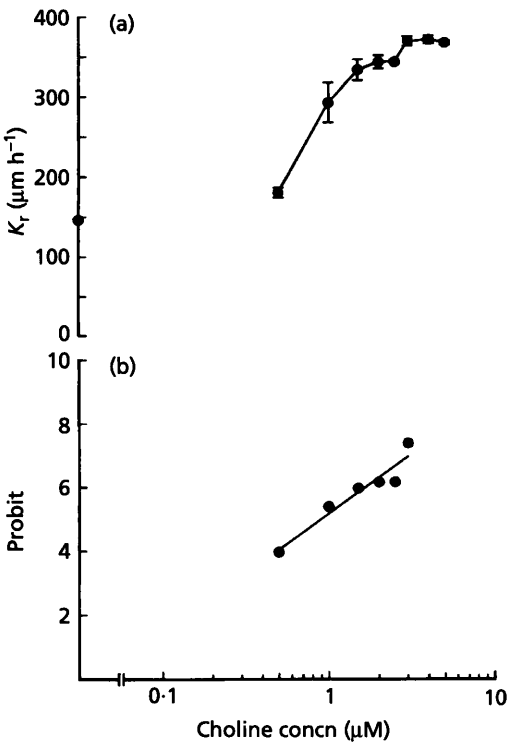

Fig. 1. (a) Effect of various choline concentrations on the colony radial growth rate of *F. graminearum* A3/5. (b) The same data transformed as a probit plot; LD_{50}, 0·9 μM, Max., 154%; LD_5, 0·3 μM.

Table 1. Effect of choline and related analogues on the colony radial growth rate of *F. graminearum*

1-Amino-propan-2-ol, triethanolamine, acetylthiocholine and carbamylcholine had no effect up to a concentration of 1 mM.

Compound	ED_5(μm)	ED_{50}(μM)	Percentage increase in K_r*
Choline	0·3	0·9	154
Ethanolamine	1·4	5·0	162
Monomethylethanolamine	2·2	85	155
Dimethylethanolamine	0·9	10	146
Phosphorylethanolamine	32	148	162
Betaine	0·3	5·4	106
Acetylcholine	4·4	127	146
Propionylcholine	73	177	110
Butyrylcholine	175	360	109
Phosphorylcholine	4	30	154
Chlorocholine	25	126	90
3-Amino-1-propanol	8	29	54

*Calculated as $100 \times \frac{K_r(+\text{additive}) - K_r(-\text{additive})}{K_r(-\text{additive})}$.

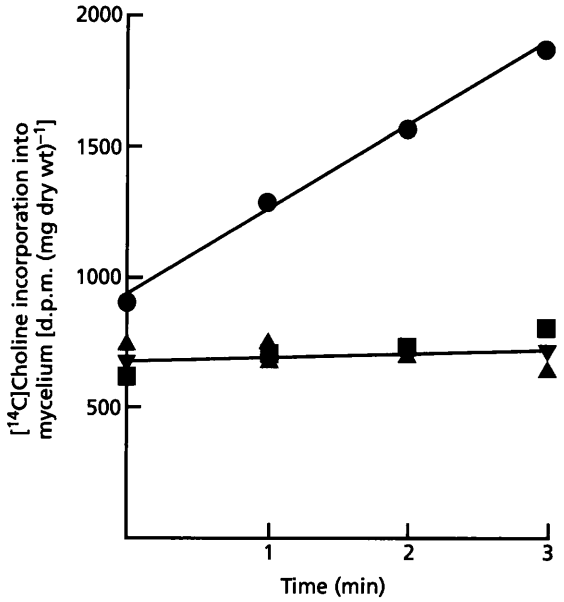

Fig. 2. Uptake of [^{14}C]choline by *F. graminearum* A3/5 (●), *nim1* (■), *nim2* (▲) and *nim3* (▼) mutants.

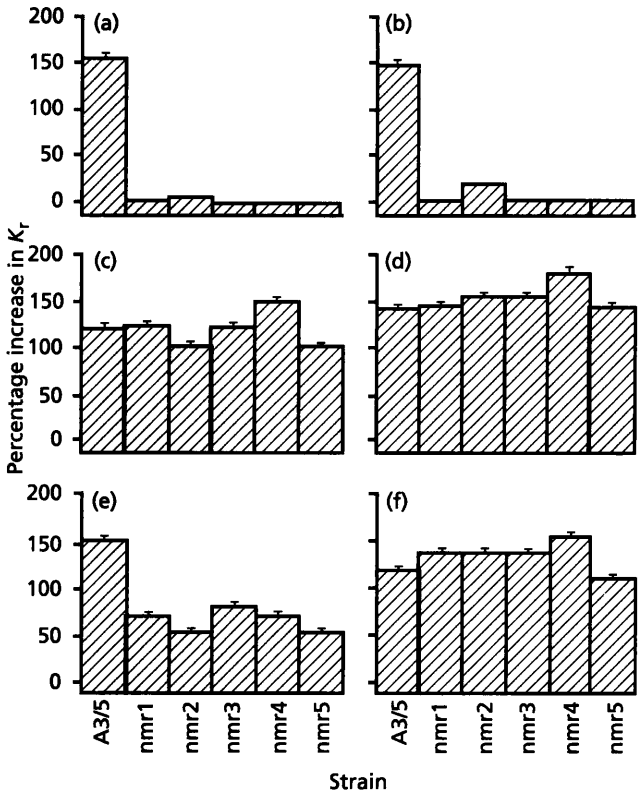

Fig. 3. Percentage stimulation of the colony radial growth rate of wild type and *nim* mutants of *F. graminearum* by 100 μM choline (a), acetylcholine (b), ethanolamine (c), monomethylethanolamine (d), dimethylethanolamine (e) and betaine (f). Percentage stimulation of the colony radial growth rate was calculated as

$$100 \times \frac{K_r(+\text{additive}) - K_r(-\text{additive})}{K_r(-\text{additive})}.$$

Results represent the mean of eight replicates with standard error bars.

butyrylcholine, chlorocholine and 3-amino-1-propanol, all of the compounds which were effective stimulated K_r to approximately the same degree. However, there were substantial differences in the concentrations at which each of the compounds first began to stimulate K_r (ED_5) and in the concentrations which gave half the maximal effect (ED_{50}). Choline and betaine caused morphological effects at the lowest concentrations of all of the compounds examined (0·3 μM) whilst butyrylcholine required the highest concentration (175 μM) to produce an effect. Similarly, the ED_{50} for choline (0·9 μM) was lower than for the other compounds tested and the ED_{50} for butyrylcholine (360 μM) was the highest. In general, those compounds which caused a morphological effect at higher concentrations, also caused maximum effect at the higher concentrations. There was little correlation between the maximum percentage increase in K_r and the ED_5 or ED_{50} values.

Response of *nim* mutants to choline

Ten *nim* strains of *F. graminearum* A3/5 were isolated and found to grow weakly on agar medium when choline was supplied as the sole nitrogen source, compared with the vigorous growth observed with strain A3/5 under these conditions (results not shown). Choline uptake studies confirmed that *nim* mutants were unable to transport choline compared with the parental strain (Fig. 2). When Vogel's agar medium was supplemented with choline or related analogues, *nim* mutants showed a loss of or severely reduced morphological response to choline and acetyl choline and a reduced response to dimethylethanolamine. There was no significant difference in the response to ethanolamine, monomethylethanolamine or betaine between the parental strain and the *nim* mutants (Fig. 3).

Effect of hemicholinium-3 and acetylcholinesterase inhibitors on the morphological response of *F. graminearum* A3/5

Hemicholinium-3 has been shown to act as a mixed non-competitive inhibitor of choline uptake (Kinney & Moore, 1988) and to block choline uptake by *F. graminearum* (Robson *et al.*, 1992). Hemicholinium-3 reduced the morphological response of *F. graminearum* to 5 μM choline by 30 % at 1 μM, 87 % at 10 μM and 95 % at 100 μM hemicholinium-3 concentrations (Table 2). At a concentration of 20 μM choline, hemicholinium-3 was less effective in reducing the morphological response to choline. Hemicholinium-3 had no significant effect on the K_r in the absence of choline. In the presence of the acetylcholinesterase inhibitors neostigmine, 1,5-bis(4-allyldimethyl-ammoniumphenyl)pentan-3-one dibromide

Table 2. **Effect of hemicholinium-3 on the colony radial growth rate of *F. graminearum* A3/5 in the presence and absence of choline**

Values are the mean of eight replicates ± SEM.

Hemicholinium-3 concentration (μM)	K_r (μm h^{-1})		
	No addition	5 μM choline	20 μM choline
0	149 ± 3	367 ± 3	359 ± 6
1	145 ± 4	305 ± 5	360 ± 3
10	148 ± 2	185 ± 2	310 ± 3
100	147 ± 2	154 ± 2	195 ± 4

Table 3. **Effect of various acetylcholinesterase inhibitors on the morphological response of *F. graminearum* to choline and acetylcholine**

Values are the mean of eight replicates ± SEM.

Inhibitor	Concentration (μM)	K_r (μm h^{-1})		
		No additive	20 μM choline	100 μM acetylcholine
None	–	149 ± 2	390 ± 3	301 ± 8
Neostigmine	10	149 ± 2	396 ± 3	270 ± 11
	100	148 ± 6	402 ± 5	258 ± 6
	1000	150 ± 2	399 ± 4	194 ± 6
1,5(4-ADAP)	1	147 ± 4	385 ± 8	180 ± 2
	10	143 ± 2	391 ± 8	155 ± 2
	100	153 ± 2	219 ± 9	147 ± 2
Eserine	10	157 ± 2	–	303 ± 13
	100	153 ± 4	–	306 ± 13
	1000	155 ± 2	–	166 ± 5

[1,5(4-ADAP)] and eserine, the morphological response of *F. graminearum* A3/5 to acetylcholine was progressively reduced with increasing concentrations of inhibitors (Table 3). At a concentration of 1 mM, neostigmine and eserine reduced the morphological response of *F. graminearum* A3/5 to acetylcholine by 72% and 92%, respectively. 1,5(4-ADAP) completely blocked the morphological response to acetylcholine at a concentration of 10 μM. None of the inhibitors had any effect on the K_r of *F. graminearum* A3/5 with the exception of 100 μM 1,5 (4-ADAP).

Effect of choline on the *in vivo* incorporation of [H^3]GlcNAc into the cell wall

Incorporation of [^{3}H]GlcNAc into the cell wall of growing cultures of *F. graminearum* was approximately log linear up to 45 min (results not shown). Addition of choline, 15 min after the addition of radiolabel led to a rapid increase in the incorporation of radiolabel within 30 s, representing a 75% increase in incorporation. Subsequently, incorporation continued at the same rate as that observed prior to the addition of choline (Fig. 4). No such effect was found when choline was added to growing cultures of the *nim1* mutant (results not shown).

DISCUSSION

It has been demonstrated previously that intermediates in the pathway from ethanolamine to betaine were capable of stimulating hyphal extension and thus K_r without affecting the overall growth rate of *F. graminearum*, leading to the development of a more rapidly expanding, sparsely branched mycelium (Wiebe *et al.*, 1989). The stimulatory effects of these intermediates on K_r are quantified in Table 1, demonstrating that the active compounds are all components of the pathway from ethanolamine to betaine (Fig. 5). Modifications to these compounds, e.g. the addition of a chloro- or phospho- group to choline or the replacement of a propyl or butyryl group for the acetyl group in acetylcholine, all lead to an increase in the concentration required to elicit the morphological response, probably due to a reduction in the rate of uptake. The relative concentration required to initiate a significant stimulation of extension (we arbitrarily chose the con-

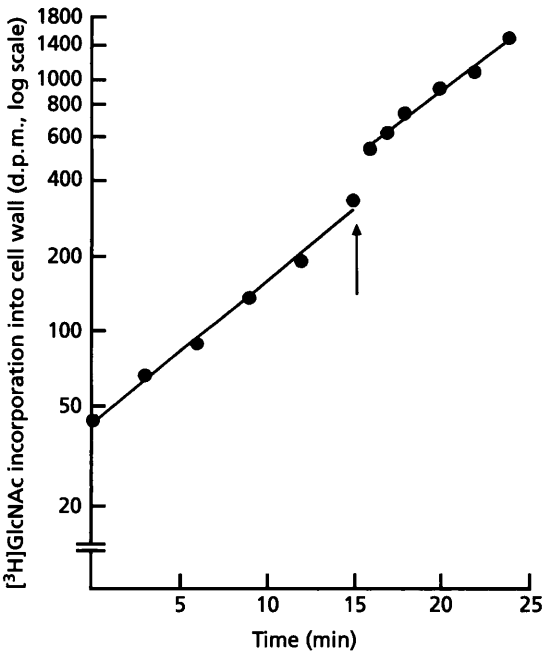

Fig. 4. **Effect of the addtion of choline (arrow, 20 μM final concentration) on the incorporation of [^{3}H]GlcNAc into the cell wall of a growing culture of *F. graminearum*.**

centration causing a 5% stimulation in K_r for comparisons) showed a broad range, with the lowest concentrations required for choline and betaine (0·3 μM) and the highest for butyrylcholine and propionylcholine (175 and 73 μM, respectively). In general, the higher the concentration required to first elicit a morphological response, the higher the concentration required before the maximum response was reached.

F. graminearum A3/5 mutants, resistant to nitrogen mustard (*nim*), produced sparse growth when grown on medium containing choline as the sole nitrogen source. The growth observed was typical of nitrogen-starved mycelia, indicating the loss of choline uptake ability. Transport studies with radiolabelled choline confirmed a loss of choline uptake in these mutants (Fig. 2). *nim* mutants were found to have lost the morphological response to choline (Fig. 3), suggesting that the morphological effects are not due to external receptor-binding but that choline or a product thereof, acts inside the hypha. This view is further supported by the reduction of the response to choline by addition of hemicholinium-3, which acts as a non-competitive inhibitor of choline uptake (Table 2).

In addition to loss of sensitivity to choline, *nim* mutants also lost sensitivity to acetylcholine. Acetylcholine does not appear to be transported by the choline permease (Robson *et al.*, 1992), suggesting that acetylcholine may first be hydrolysed to choline by an acetylcholinesterase located on the outside of the hypha, with the choline released eliciting the observed morphological effect. This conclusion is further supported by the loss of sensitivity

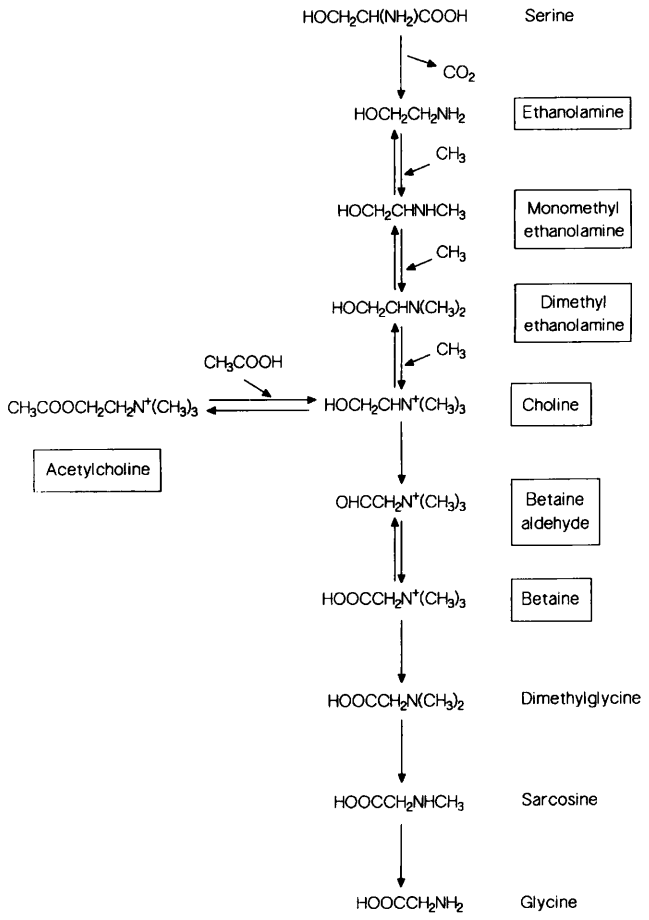

Fig. 5. **Biosynthetic pathway of choline and betaine synthesis. Boxed compounds are capable of eliciting a morphological effect in *F. graminearum*.**

to acetylcholine, but not to choline, in the presence of various acetylcholinesterase inhibitors (Table 3).

The morphological response of *nim* mutants to betaine was similar to that observed in the parental strain, confirming our previous report that betaine uptake occurs by a transport mechanism independent of choline (Robson *et al.*, 1994). Similarly, there was no reduction in the degree of stimulation of *nim* mutants in the presence of ethanolamine, which we previously found did not compete for either the choline or betaine permease. A third permease capable of transporting ethanolamine may therefore exist. If it is also capable of transporting monomethylethanolamine and dimethylethanolamine, this third permease would explain why the response of *nim* mutants to these compounds, although reduced, was not blocked completely, despite evidence suggesting that these compounds are capable of being transported by the choline permease (Robson *et al.*, 1992).

We have shown previously that [^{3}H]GlcNAc is incorporated primarily into the chitin fraction of the cell wall when added to growing cultures of *F. graminearum* (Binks *et al.*, 1990). Thus, incorporation of [^{3}H]GlcNAc into the mycelium of *F. graminearum* is a measure of the *in vivo*

chitin synthase activity. Incorporation of the label was found to be approximately logarithmically linear. Addition of choline caused a 75% increase in the incorporation of radiolabel within 30 s, after which the rate of incorporation continued at the same rate as that prior to choline addition. This rapid increase in *in vivo* chitin synthase activity may reflect the morphological transition induced by choline. Choline causes an increase in the hyphal extension rate of the mycelium and a reduction in branching frequency without affecting overall growth rate (Wiebe *et al.*, 1989). Consequently, as choline does not affect overall growth but only the spatial distribution of the biomass, *in vivo* chitin synthase activity should not be affected by choline. Indeed, we found that the rate of *in vivo* chitin synthase activity was not significantly different after the addition of choline (Fig. 4). The transient increase in the *in vivo* chitin synthase activity immediately following the addition of choline may result from an initial increase in the extension rate of the hyphae induced by choline before the inhibition of branch frequency. The subsequent inhibition in branch frequency would then establish the rate of *in vivo* chitin synthase to that exhibited prior to choline addition.

The results presented in this study support the hypothesis that choline and related analogues are transported by a number of permeases and act within the hypha, probably via a common active component which then mediates the morphological response. The morphological effect of acetylcholine appears to involve an acetylcholinesterase, presumably acting at the cell surface. The choline produced would then be transported via the choline permease. The choline-induced alteration in mycelial morphology is elicited very rapidly (within 30 s) and may involve an increase in hyphal extension rate before inhibition of hyphal branching. The mechanisms involved in regulating hyphal branching and hyphal extension by choline are unknown, although we have previously shown that cGMP elicits a similar morphological response in *F. graminearum* and acts synergistically with choline (Robson *et al.*, 1991). In addition, we have shown that the inhibition of branch frequency by choline occurs independently of the stimulation of hyphal extension rate (Wiebe *et al.*, 1992). In *Neurospora crassa*, mutants blocked in choline synthesis require exogenous choline for growth. Growth can also be supported by the addition of betaine aldehyde but not betaine. As betaine aldehyde is an intermediate between choline and betaine, it appears that the oxidation of betaine aldehyde to betaine is irreversible (Richardson & Speed, 1969). If this is also true in *F. graminearum*, then betaine may act as a common intermediate in eliciting the morphological effects of choline and related analogues.

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