Sporulation in *Coprinus cinereus*: use of an *in vitro* assay to establish the major landmarks in differentiation

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Basidia of *Coprinus cinereus* continue differentiation when explanted to water agar. Inclusion of various compounds in the explantation medium can inhibit basidium differentiation. Growth is not inhibited but vegetative hyphal tips grow from regions of the basidium expected to produce sterigmata. Ammonium, glutamine and alkali metal salts were effective in tissues exposed at any time after meiotic division I; whereas ionophores, cAMP and wall synthesis inhibitors were effective only if applied during meiosis. This implies that the cell is prepared for sporulation during meiosis so that by the end of the nuclear division sporulation can proceed despite the presence of some metabolic inhibitors. On the other hand, the sporulation process must involve essential components that are sensitive to excess metal ions, ammonium and glutamine, but which do not contribute to the nuclear division. Since both types of inhibitor cause hyphae to grow from positions corresponding to sterigmata, these sites must be specified early in differentiation, and in a way which survives treatment with many different inhibitors.

Key words: Basidium, Development, Meiosis, Commitment, *Coprinus cinereus*.

Basidia of *Coprinus cinereus* (Schaeff.: Fr.) S. F. Gray *sensu* Konr. express developmental commitment, continuing the meiocyte programme of differentiation to which they are determined even when explanted to water agar (Chiu & Moore, 1988*a*). This technique has been modified into a bioassay and used to study the effects of exogenous compounds on the progress of differentiation *in vitro* (Chiu & Moore, 1988*b*). Of a wide range of compounds tested, only ammonium and its immediate structural analogues, and glutamine and some of its structural analogues were able to inhibit basidium differentiation. Growth was not inhibited; instead vegetative hyphal tips grew from regions of the basidium expected to be in active growth during sporulation. Depending on the stage reached at the time of exposure to the inhibitors, vegetative hyphal tips emerged from the four apical sites for sterigmata, from the tips of sterigmata, from partially formed or abnormal spores, and from the basal regions of the basidium from which paraphyses (sterile cells of the hymenium) normally arise.

There are a number of reasons for suspecting that ammonium may act through effects on the ionic balance of the basidium. The ammonium ion has the same dimensions as potassium, and its salts show many chemical similarities with salts of this metal. Uptake of ammonium and/or metallic ions will affect the electrochemical gradient, and excess ammonia could generate a proton sink and influence cytoplasmic pH. In this paper we use the *in vitro* differentiation assay to examine the effects of metal ions, membrane-depolarizing agents, ionophores, exogenous cAMP, and inhibitors which can alter fungal cell wall composition.

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MATERIALS AND METHODS

Organism, culture and assay conditions

The Meathop strain of *Coprinus cinereus* was used. Cultures were grown and fruit bodies produced as described by Moore & Ewaze (1976). Gill lamellae were excized from a fruit body after the cells of the veil had been removed from the cap surface. A segment consisting of two or three gills was explanted to Nunclon Delta SI 24-well 'Multidishes' which have 16 mm internal diam wells. For tests with liquid media, 0·4 ml of the filter-sterilized solution was placed in each well with two sterile 15 mm diam disks of Whatman GF/D glass microfibre filters which were subsequently overlaid with a disk of cellophane (Chiu & Moore, 1988*b*). All of the solutions were buffered to pH 7 with 67 mM Sorensen buffer except that calcium and magnesium salts were prepared and tested in 50 mM Tris/HCl, pH 7. The developmental stage of the tissue at explantation was determined by fluorescent microscopic examination of squash preparations stained with ethidium bromide (Chiu & Moore, 1988*a*).

Dinitrophenol, sodium azide, valinomycin and gramicidin-S were prepared as stock solutions in absolute ethanol and diluted into the medium for the tests. In these cases buffer containing 0·5% (v/v) ethanol was used as control. Stock

Table 1. Minimum effective concentrations (mM) of some metal salts for inhibition of sporulation of *Coprinus cinereus in vitro*

	Cation						
Anion	Ammonium	K	Na	Rb	Cs	Ca	Mg
Chloride	50	> 150	100	75	> 150	100	100
Nitrate	25	100	25	50	75	75	50
Sulphate	25	100	75	75	75	ND	100
Acetate	25	100	100	ND	ND	ND	ND

ND = not determined.

Table 2. Effects of ionophores and wall synthesis inhibitors on progress of differentiation of basidia *in vitro*

		Stage at which the tissue was first exposed to the agent	
Agent	Concn	Meiosis	Post-meiotic
Dinitrophenol	1, 1·5 mM	Complete arrest	Complete arrest
	0·5, 0·75 mM	+*	—
Sodium azide	1, 1·5 mM	Complete arrest	Complete arrest
	0·5, 0·75 mM	+	—
Gramicidin-S	0·13 to 1 mM	+	—
Valinomycin	0·1 to 0·5 mM	+	—
cAMP	3 to 50 nM	+	—
Tunicamycin	5 to 100 μg ml^{-1}	+	—
Nikkomycin	12·5 to 100 μM	+	—

Entries show the qualitative effects of the agents at the concentrations indicated; 'complete arrest' means that no further development or growth of any sort occurred, ' + ' means that the explanted gills remained white and spore-free and that some basidia (proportion increasing with concentration) produced vegetative hyphal outgowths, ' — ' means that the agent had no effect and the explanted gills continued their development to produce and disperse basidiospores.

solutions of tunicamycin (10 mg ml^{-1}) and the sodium salt of adenosine 3′,5′-cyclic monophosphate (cAMP) (0·016 mg ml^{-1}; 46 μM) were prepared in water and diluted with buffer to produce the test solutions. Nikkomycin was also dissolved in water (50 mg ml^{-1}) and kept at −20 °C until use. The 50% pure mixture of nikkomycin X and Z was a gift from Prof. G. W. Gooday. A working solution (250 μg ml^{-1} in buffer; approx. 500 μM) was diluted from the stock solution and used to prepare test solutions.

RESULTS

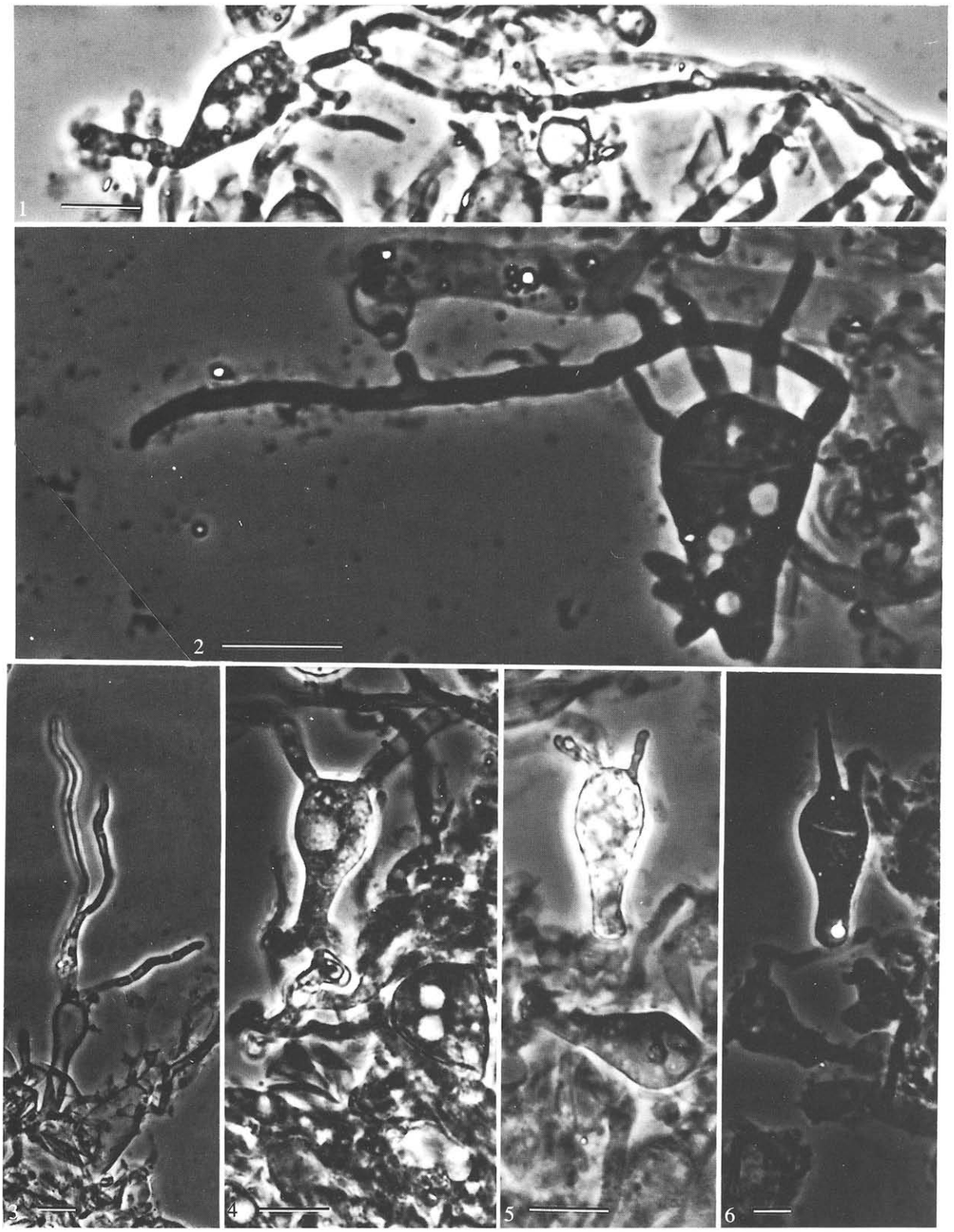
The effects of salts of potassium, sodium, rubidium, caesium, calcium and magnesium were determined using the *in vitro* assay at concentrations over the range 25 to 150 mM. The results are summarized in Table 1 in terms of the minimum concentrations of these compounds required to inhibit sporulation. Meiotic stages of development were marginally more sensitive, but exposure to alkali metal salts at any time from meiosis through to late sporulation stages resulted in premature termination of basidium development and outgrowth of vegetative hyphae.

These results would not support the contention that the ammonium effect is solely due to this ion acting as an analogue of potassium or as an inhibitor of potassium metabolism. If this were so, one would expect the same spectrum of effects to be caused by rubidium but this was not observed. Clearly though, the ionic environment, as affected by both cations and anions, does seem to exert some influence. Nitrate salts of sodium and rubidium were noticeably more inhibitory than any other metal salt. Nevertheless, salts of ammonium were uniformly far more effective than any others in inhibiting basidial sporulation *in vitro*.

Although ammonium is unlikely to act as an analogue of potassium, uptake of ammonium and other ions will affect the electrochemical gradient. As an alternative way of examining this we studied the effects of membrane-depolarizing agents dinitrophenol (DNP) and sodium azide, the sodium channel gramicidin S and the potassium carrier valinomycin. Results are summarized in Table 2.

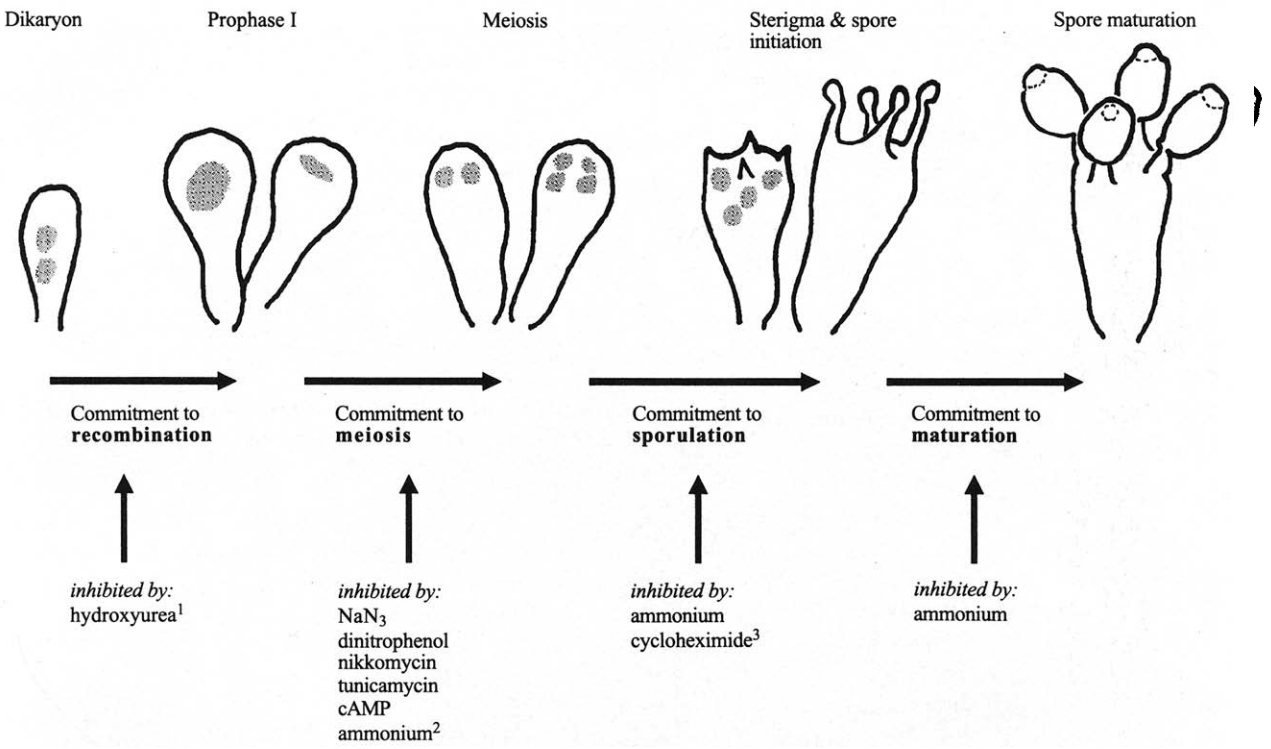
At DNP and NaN_3 concentrations of 1 mM and above, excized tissues were completely arrested in development; no reversion to hyphal growth occurred. Tissues excized when in meiotic stages were inhibited by exposure to 0·75 mM DNP or NaN_3; though many basidia completed sporulation and the explanted gills autolysed, some showed reversion with hyphae growing out in place of sterigmata. In addition, the bodies of some basidia were swollen and mis-shaped (Figs 1,2). Much the same result was obtained with gramicidin-S (Fig. 3) and valinomycin. With all ionophores, gills excized at sporulation

Figs 1–6. Effects of various chemical agents on sporulation in *Coprinus cinereus*. **Fig. 1.** 0·75 mM dinitrophenol. **Fig. 2.** 0·75 mM sodium azide. **Fig. 3.** 0·75 mM gramicidin-S. **Fig. 4.** 50 nM cAMP. **Fig. 5.** 25 μg ml^{-1} tunicamycin. **Fig. 6.** 12·5 μM nikkomycin. Scale bars = 20 μm.



stages were not affected but those excized during meiosis were sensitive and in each case an increased concentration of the agent increased the proportion of basidia affected rather than increasing the severity of the effect. Since dinitrophenol can raise the cAMP level in *C. cinereus* (Uno & Ishikawa, 1981) and other membrane-depolarizing agents are also known to affect the cAMP level in various fungi (Trevillyan & Pall, 1979; Thevelein *et al.*, 1987) we tested the effect of exposure of explanted lamellae to cAMP (Table 2). Here again, tissues excized during meiosis were inhibited, some basidia being

Fig. 7. Summary of the sporulation pathway in *Coprinus cinereus*. References to the effects of the inhibitors are indicated with superscripts: [1], Lu (1982), Raudaskoski & Lu (1980); [2], Chiu & Moore (1988*b*); [3], Lu & Chiu (1978).



arrested but others reverting to hyphal growth. The higher the concentration of cAMP used the greater the number of basidia affected (Fig. 4).

As ammonium seemed to interfere with the close control of wall growth in basidia, we used the *in vitro* assay to examine two selective inhibitors of wall synthesis, tunicamycin and nikkomycin.

Tunicamycin is a specific inhibitor of protein glycosylation (Olden *et al.*, 1985) which inhibits sporulation and growth in yeasts and filamentous fungi by inhibiting wall synthesis (Weinstock & Ballou, 1987; Wheals, 1987; Elorza *et al.*, 1987; Katoh *et al.*, 1978). Nikkomycin inhibits chitin synthase of *Coprinus cinereus in vitro* by acting as a substrate analogue of UDP-*N*-acetylglucosamine (Brillinger, 1979), and inhibits chitin synthesis in various fungi (Muller *et al.*, 1981; Gow & Selitrennikoff, 1984; Elorza *et al.*, 1987). Neither of these inhibitors influenced the progress of development *in vitro* of *Coprinus cinereus* lamellae which were excized from the parental basidiome during sporulation stages (Table 2) but tissues excized during meiosis were affected (Figs 5,6).

DISCUSSION

Exposure of immature *C. cinereus* gill segments to many compounds *in vitro* can cause cessation of basidial differentiation and diversion of effort into the formation of vegetative hyphae. From the way in which the experiments are conducted we presume that exposure to inhibitory agents overloads one or more metabolic aspect(s) of a normally-developing meiocyte, causing it to revert to the vegetative hyphal condition. The number of agents considered so far covers too wide a range of metabolism for specific conclusions to be drawn about the exact nature of the metabolism which is affected but a number of generalized conclusions can be drawn.

In view of the wide range of compounds which have been used, from substrate-analogue enzyme inhibitors like nikkomycin to inorganic salts like $NaNO_3$, it is remarkable that where inhibition of differentiation does occur the response is uniformly one of outgrowth of hyphal tips at the basidial apex and often from positions corresponding to those expected to produce sterigmata. Thus the pattern of sterigma-sites must be specified in a way which survives many different catastrophic metabolic interventions. Of the *in vitro* treatments reported, only the chilling and electrical field experiments of McLaughlin (1982) consistently altered the number and location of sterigma-sites. The sites at which sterigmata will form must be specified from within the plasmalemma and yet they must be firmly located on the basidial wall. A structure something like the focal contacts (Geiger, 1983) by which fibroblasts adhere to their substratum would satisfy these requirements.

Although the diverse activities of the agents tested preclude identification of their targets at the moment, it is clear that they fall into two groups. Ammonium and glutamine and their analogues and those other inorganic anions and cations which were effective inhibited further differentiation but promoted hyphal outgrowths in tissues exposed at any time after meiotic division I; whereas the ionophores, cAMP and wall synthesis inhibitors were effective only if applied during meiosis. The differential sensitivity of basidia between meiotic

and sporulation stages implies that during the nuclear division the cell is prepared in advance for sporulation, rather like the egg cell of an animal or plant, so that by the end of the cytologically recognizable nuclear division sporulation can proceed despite treatment with ionophores and wall synthesis inhibitors.

Similarly, the effects of ammonium, etc. are presumably exerted against essential components of the sporulation process which either do not exist during meiosis or do not contribute to the nuclear division, since in tissue exposed to such compounds after meiotic division I nuclear division progressed although reversion to hyphal growth was later observed at the sterigma-sites. Basidial differentiation in *C. cinereus*, therefore, can be seen as a sequence of integrated steps of commitment (Fig. 7) consisting of the following landmarks: (1) commitment to recombination (requires completion of DNA synthesis; Lu, 1982); (2) commitment to meiosis (at prophase I; Lu & Chiu, 1978; Chiu & Moore, 1988*a*); (3) commitment to sporulation (at or after meiotic II division; Raudaskoski & Lu, 1980; Chiu & Moore, 1988*a*, *b*); (4) commitment to maturation, which we assume to be a specific step since ammonium treatment causes hyphal outgrowth from partly formed spores (Chiu & Moore, 1988*b*). Further development of the *in vitro* assay will provide a tool for the identification and characterization of components contributing to these steps.

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