THE MOLECULAR ANALYSIS OF SEXUAL MORPHOGENESIS

IN SORDARIA BREVICOLLIS

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In accordance with the regulations of the University of Edinburgh, I hereby declare that this Thesis has been composed entirely by myself and that all the work described herein was carried out by myself, except where otherwise stated.

S.J. Broxholme

ABSTRACT

The Ascomycete fungus *Sordaria brevicollis* undergoes a transition during its development from a vegetative, mycelial state, to one in which sexual structures, protoperithecia, are formed. In laboratory culture, this switch between developmental modes appears to be mediated by contact of the mycelium with the wall of the containing vessel. This characteristic of *S. brevicollis* has been used in this work to investigate the molecular changes that occur during the transition, and also in the further development of the protoperithecium which is initiated by fertilisation with a strain of the opposite mating-type.

This Thesis describes the analysis of changes in the protein profiles of the fungus during its life cycle, and the changes that occur in the messenger RNA population between the different stages. Also described are some recently discovered features of development of *S. brevicollis* namely facultative homothallism and sclerotial formation, which may have an important bearing on the interpretation of the life cycle in terms of alternative morphogenetic pathways.

ACKNOWLEDGEMENTS

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CHAPTER 1
INTRODUCTION

1.1. The Filamentous Ascomycetes.

The Ascomycete fungi form a group of some of the simplest eukaryotes available for experimental analysis. Ascomycetes are widely used for many different aspects of research, and will doubtless remain popular systems as a consequence of the ease with which they can be manipulated to the needs of individual researchers. They can be cultured with modest requirements for growth and reproduction, and so the numerous aspects of their biology can be conveniently re-created in the laboratory.

In this thesis I shall be concerned with the transition of the fungus from a growing, or vegetative, state – the mycelium – to the phase of sexual reproduction, in which a specialised structure is formed for the production and dispersal of sexual spores.

1.1.1. The Sexual cycle – Physiology and Cytology.

The sexual fruitbodies of filamentous Ascomycetes show great diversity (for example, see figure 1), and this largely forms the basis for their taxonomic classification, as outlined in table 1. However, despite the differences in appearance, and the mechanisms involved in the morphogenesis, there is a unifying sequence of events which can form the basis for a general model of the sexual cycle.

In this thesis I shall be concentrating on the Pyrenomycetes, of which the fruitbody is a perithecium, and in particular, the heterothallic *Sordaria brevicollis*. The first stage in the morphogenesis is the formation of an ascogonium, which develops into a protoperithecium. Proliferation of the protoperithecium results in the formation of a perithecium, which contains the mature sexual products – the ascospores, and is responsible for their dispersal.
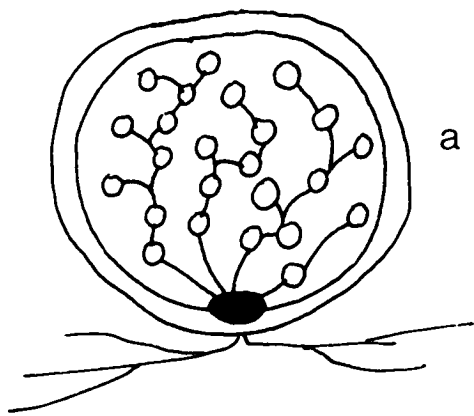
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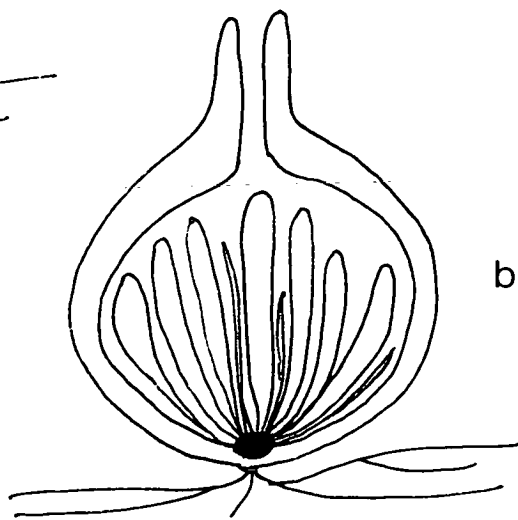
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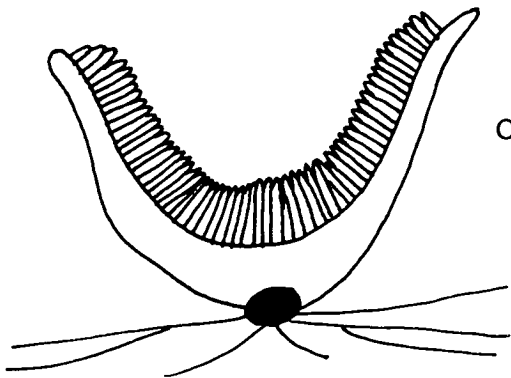
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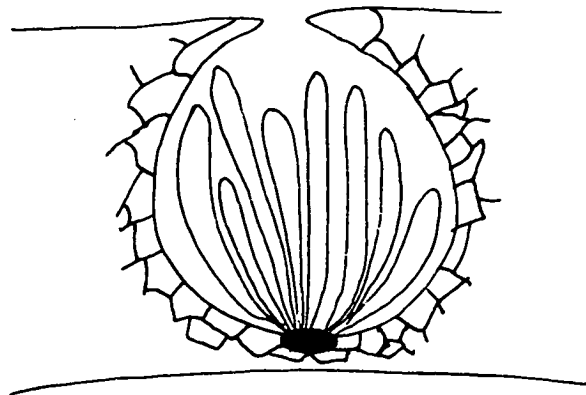
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Table 1. Subdivision Ascomycotina

Class Hemiascomycetes	Asci naked, no ascocarp.
Class Plectomycetes	Ascocarp a cleistothecium, asci evanescent and scattered.
Class Pyrenomycetes	Ascocarp a perithecium, asci inoperculate arising from a hymenium.
Class Discomycetes	Ascocarp an apothecium, asci operculate or inoperculate arising from a hymenium.
Class Loculascomycetes	Asci bitunicate, ascocarp an ascostroma.
Class Laboulbeniomycetes	Exoparasites on arthropods, thallus much reduced, ascocarp a perithecium.

The protoperithecial initial, the ascogonium, forms as a sidebranch of a vegetative hypha. As it coils around itself, it becomes completely enveloped by other hyphae, which may arise either as branches from the parent hypha, or from the base of the coil itself, or from adjacent vegetative hyphae. As these enveloping hyphae continue to grow and branch, the outward branches (fringe hyphae) give a 'hairy' appearance to the protoperithecium. Prominent also at this stage, in heterothallic Ascomycetes, is a fine, branched trichogyne, as shown in figure 2(ii), which will mediate transport of the male protoplasm into the ascogonium.

In homothallic Ascomycetes, such as *Sordaria humana*, the nuclei of the dikaryotic ascogenous hyphae arise from the parent hyphae, so there is no delay necessary before the protoperithecium enters the next stage in its development. However, in *S. brevicollis*, as with other heterothallics, the protoperithecium will remain as such until the trichogyne makes contact with a 'male' cell of the opposite mating type to that of the 'female' parent hypha. This may take the form of a detached spermatium, often a macroconidium or a microconidium, although vegetative hyphae are capable of acting as the fertilising agents.

Following fertilisation, the two parent nuclei undergo several divisions within the ascogenous hyphae, which subsequently undergo septation into binucleate cells. The nuclei within these dikaryons now divide synchronously in such a way that one of each daughter nucleus reaches the tip of a

Figure 2

Scanning Electron Micrographs of Morphogenetic Stages of
Sordaria brevicollis.

(Courtesy of N.D. Read)

(i)   Hyphae of growing mycelium – 1 day–old culture.

(ii)   Protoperithecium in 4 day–old culture.

        Note the fringe hyphae and prominent trichogyne.

(iii)   Maturing perithecium, 3 days after fertilisation.

        Note the size of the fruitbody compared with that of

        the unfertilised protoperithecium.

(i)

100μm

(ii)

100μm

(iii)

100μm
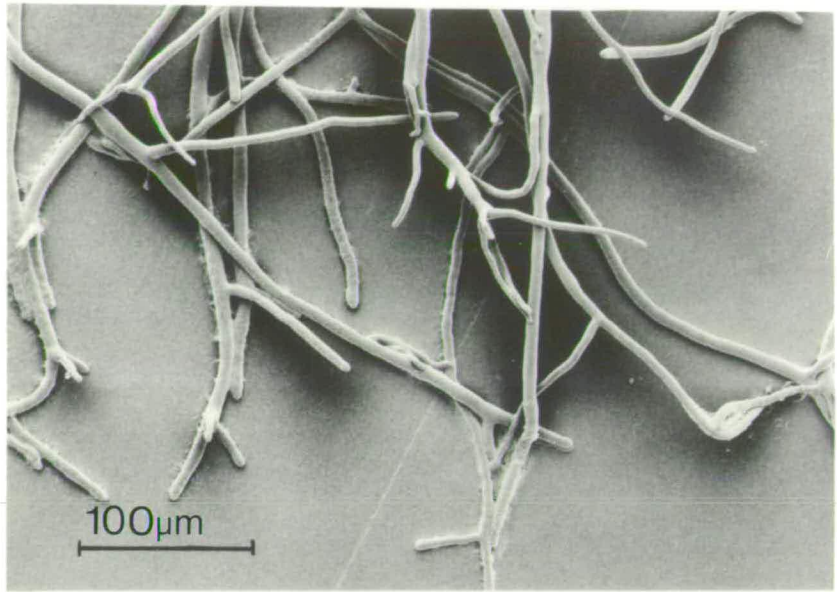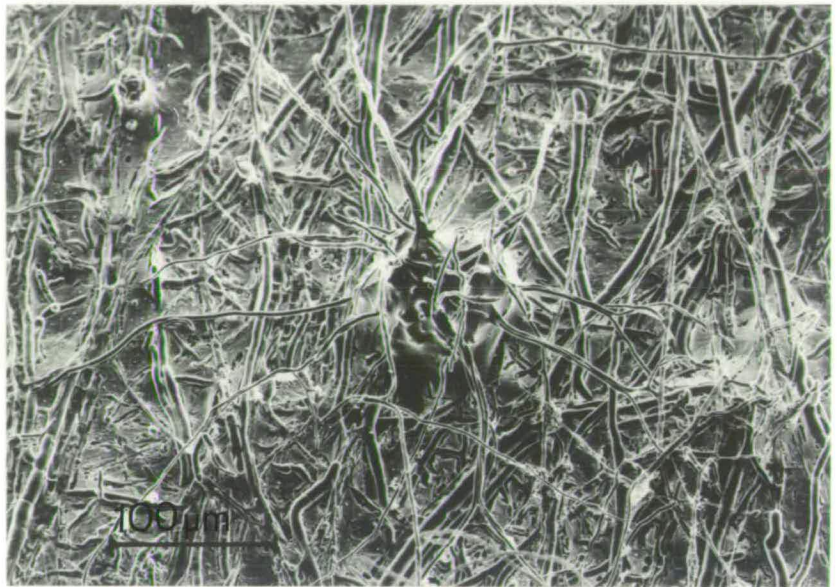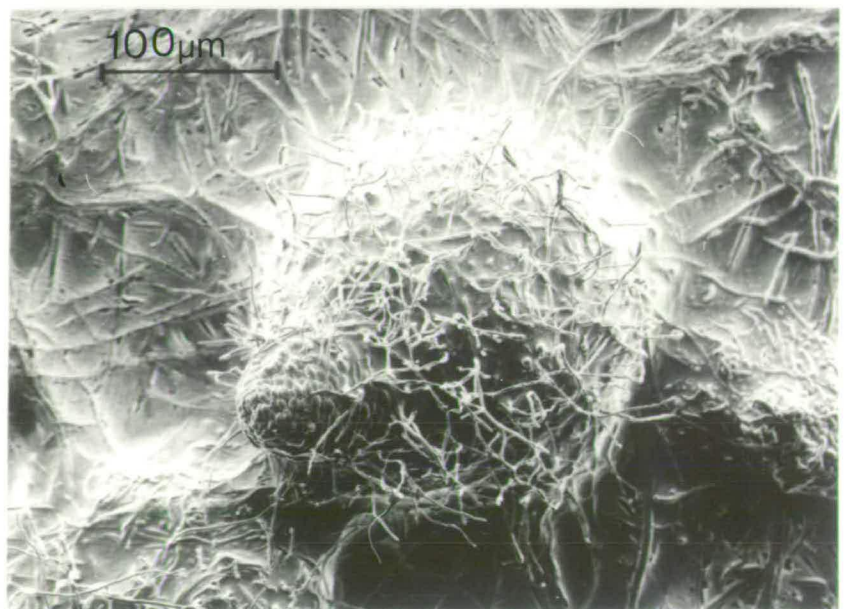
crozier-shaped cell, as shown in figure 3.

These daughter nuclei fuse and the resulting (diploid) nucleus immediately undergoes a meiotic division. Normally there is a single mitotic division following the meiosis, producing eight haploid nuclei, around which ascosporogenesis occurs. In the *Sordaria* genus, and with most species of *Neurospora*, the eight spores are aligned in order with respect to the nuclear divisions (figure 3). This feature has been found to be of great use to geneticists in classical mapping analyses, in analysing the mechanisms of recombination (for example, see Whitehouse, 1982), in the characterisation of chromosome rearrangements (Perkins and Barry, 1977) and in the analysis of different sources of aneuploidy (Fulton and Bond, 1983; Bond and Broxholme, 1983).

There is more to the development of the fruitbody than ascus production. Within the centrum there are also abundant sterile hyphae, paraphyses, which are located between asci and constitute a large proportion of the volume of the centrum. Their precise role is uncertain, but it is possible that they function as space-making and space-filling elements, which allow unrestricted movement of the asci during maturation, and for preventing the formation of intercellular spaces between the centrum elements (Read, 1985).

The external appearance of the fruitbody undergoes a complex sequence of changes following the fertilisation event. The transition of the protoperithecium to the perithecium has been described (Read, 1983), as being the time when there is differentiation of a neck, or the rudiments of one. This observation on *Sordaria humana* holds true also for the heterothallic *S. brevicollis* since neck initials do not normally develop until about one day after fertilisation.

During the course of this work, conditions were found which did allow further development, in *S. brevicollis*, of a small number of protoperithecia in the absence of fertilisation. This phenomenon of facultative, rather than obligate, heterothallism will be discussed later.

At about the same time as the appearance of the neck, (*viz.* within 24 hours after fertilisation), there is a general increase in the size of the perithecium, as seen in figure 2(iii). This, and the increased pigmentation of the

Figure 3

The Sexual Cycle of *Sordaria brevicollis*.

1. Germination of ascospore.

2. Branched, vegetative hyphae.

3. Ascogonium, surmounted by trichogyne.

4. Macroconidia, to fertilise ascogonium of opposite mating-type.

5. Dikaryotic ascogenous hyphae from conjugate nuclear divisions.

6. Differentiation of crozier from ascogenous hypha.

7. Conjugate nuclear divisions.

8. Septum formation.

9. Septum formation.

10. Fusion of nuclei and fusion of terminal with stem cell.

11. Ascus proliferation, with occurence of meiosis.

12. Mitosis and formation of ascospores.

13. Mature perithecium, with ejection of mature ascospores.
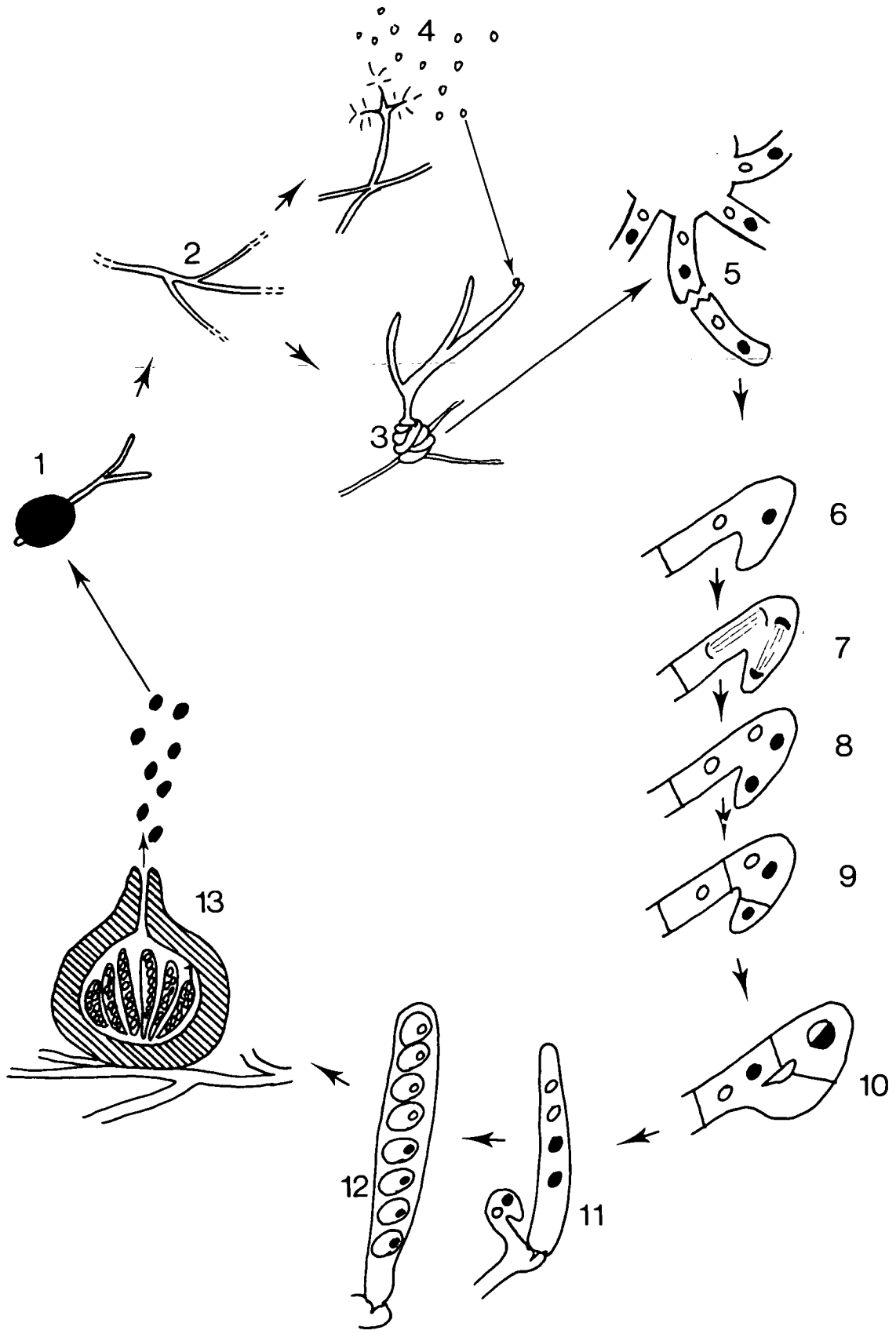
fertilised fruitbody allows it to stand out against the unfertilised protoperithecia in the culture.

While the pattern of cellular movements involved in the growth of the base of the perithecium (enclosing the centrum) is not well documented, there has been a considerable amount of interest in the elongation and maturation of the neck. Read and Beckett (1985) have observed, by the use of scanning electron microscopy, that the major structural tissue of the neck, the peridium, is built up from neck periphyses which have differentiated and adhered together. Periphyses are sterile, hyphal-like elements lining the ostiolar canal (Read and Beckett, 1985). They appear to function in applying pressure on the wall of the ascus as it elongates along the ostiolar canal and ejects its spores. The current description of perithecial morphogenesis from a cytological and histological point of view shows that there is a precise series of changes which appear to be strictly controlled in order to formulate this rather elegant mechanism for the dispersal of sexual spores and the establishment of spread of the species.

Although many of these observations have been made using the finest methods that are available, there are still many questions that must be asked about the mechanisms by which each part of the morphogenetic process is initiated, and how it is carried out and controlled in such a precise manner.

There have been many observations and much work done on features of fungal development which are directly or indirectly related to sexual reproduction. Some are clearly connected with sexual morphogenesis, while others may at first seem less obviously relevant, although they do in fact provide some insight into the process.

Remarkable similarities are observed between some aspects of the behaviour of fungi, both in nature and in the laboratory, and the physiological characteristics of fungi undergoing sexual morphogenesis. There are features that are typical of a mycelium when changing its role from vegetative colonisation to dispersal (fruition) which are not seen in a mycelium that is not fruiting. Use has been made of rather simple observations in such cases, in order to make predictions on the causes, mechanisms, and consequences of the sexual process. These observations have been made over a period of decades in some cases, and generally on different organisms, often with

distant, if any, relation to one another.

In the above, and in the review that follows, studies on different fungi are referred to on the assumption that there are common features in the sexual developments of different species. In this situation, it would be unjustified to make bold analogies and widely generalised models. Instead it would be prudent to confine speculation to fundamental features of the development of one organism which may have some bearing on similar features of another. Of course, one may be less cautious when comparing closely related organisms, but care should still be taken; it would be all too easy to generalise too widely.

1.2. The Initiation of Sexual Morphogenesis.

1.2.1. Nutritional Effects on Sexual Development.

It has long been recognised that the morphological characteristics of a fungus depend to a great extent upon the available nutrients. Early studies, reviewed by Lilly and Barnett (1951), Cochrane (1958) and Hawker (1966), provided a good deal of background knowledge which still stands established as fundamental to fungal studies.

A fungus invariably utilises certain nutrient sources better than others for growth and reproduction, for example, *Neurospora crassa* forms few protoperithecia in the presence of ammonia (Westergaard and Mitchell, 1947; Somm et al 1987). The nature of such favoured nutrients often relates to the natural habitat of the species in question, as will be seen from the differential effects of many nutrient sources on fungi with different lifestyles. Often, certain nutrients will have a different effect on the growth of a fungus compared to that on its reproduction, and again this may relate to its natural habitat.

Synthetic media have been designed for several fungi – sometimes out of necessity, for example when a minimal medium is required for auxotrophy analysis, or perhaps in order to establish the optimal conditions for laboratory culture. A fungus can often grow well on a very simple medium containing carbon and nitrogen sources, and some inorganic ions. From such conditions,

the medium can be changed until an optimal concentration of each of the added nutrients is found in order that the fungus can grow or reproduce. In those fungi studied extensively in this respect, the medium favoured for luxuriant growth is not necessarily the same as that in which sexual reproduction can perform best. For example, in *Neurospora crassa*, Vogel's (1956) medium favours rapid growth, while that of Westergaard and Mitchell (1947) is more suited for crossing. It has generally been found that while vegetative growth depends largely on the availability and form of the carbon source, the effect on fruiting is rather different. Although vegetative growth will increase, up to a point, with increasing sugar, initiation of fruiting is often not observed unless there is a low or exhausted supply of it. A common observation is that fruiting requires a much lower availability of carbon than that for maximum growth, and above this optimum concentration the fruiting capacity drops quite severely (Hawker, 1966; see also review by Moore-Landecker, 1982).

Hawker and Chaudhuri (1946) observed that the quality of fruiting of numerous fungi depends a great deal on the type of carbon source available. Thus, fruiting is better in the presence of polysaccharides; starch and lactose, than monosaccharides for *Podospora sp.* and *Chaetomium cochloides*, whereas *Melanospora zamiae* and *Ceratostomella adiposa* fruit preferentially on glucose or fructose to di- or polysaccharides.

The preferences shown by many fungi to the carbon source for fruiting may relate to that in their natural habitat, and therefore to the substrates to which they have presumably adapted to in their evolution. Another consideration here would be the preference for complex or simple sugars. For example, perhaps *Podospora* and *Sordaria* would preferentially use glucose and fructose for vegetative growth, then when these are exhausted the cellular mechanism switching on polysaccharide reduction may be related to the s witches for sexual reproduction. Such a relationship would provide a useful system for an organism requiring first an efficient colonisation of the substratum, and then on exhaustion, the utilisation of the remaining complex nutrients, and the re-utilisation of mycelial components for the processes of reproduction and dispersal (Zonneveld, 1972).

1.2.2. Light Effects on Sexual Morphogenesis.

Fungal morphogenesis is often affected by light conditions. Light responses may occur at several different stages during the development of a particular fungus (see Russo *et al.* 1985), and the characteristics of different responses vary a great deal, for example wavelength, degree of exposure, and periodicity may all have an effect on the sexual response.

Inoue and Furuya (1970) observed that no perithecia were formed in *Gelasinospora reticulospora* under conditions of continuous light, and some perithecia were initiated in total darkness. However, perithecium formation was greatly promoted in younger parts of the mycelium when a culture was exposed to white light after a period of darkness. This light effect was not seen in cultures which had colonised the containing vessel – perithecia formed in the young mycelium irrespective of the light conditions.

Degli-Innocenti *et al.* (1983) showed that blue light can accelerate production of perithecia in *Neurospora crassa* if exposure is above a threshold value. Such a dose effect has also been shown in *Gelasinospora reticulospora* (Inoue and Furuya, 1975), *Pleospora herbarum* (Leach and Trione, 1966) and *Nectria haematococca* (Curtis, 1972). However, light may be required in a specific temporal pattern throughout the development period, as in *Nectria galigena* (Dehorter *et al.* 1980).

Sommer *et al.* (1987) showed that the photomorphogenetic induction of fruiting in *Neurospora crassa* was dependent on a low level of nitrogen in the medium. By transferring a culture from medium containing low nitrogen to one containing high nitrogen at various times after irradiation, they were able to show that the stage at which nitrogen has an inhibitory effect appears to be within 5 hours of photoinduction.

1.2.3. Edge Effects – Physical Means of Initiation.

It has long been established that the initials of fruitbody morphogenesis in fungi are affected by physical contact of the mycelium with external factors (Robinson, 1926; Inoue and Furuya, 1970). A common observation is that fruiting responses are initiated by contact with the wall of the containing

vessel, most easily seen in Petri dishes (Buston and Rickard, 1956; Hawker, 1966). In many species it is not until after the medium has been completely colonised that the first signs of fruiting are observed.

In *Sordaria brevicollis,* there is a pronounced edge effect when a colony is grown on cornmeal agar (MacDonald and Bond, 1976). This conclusion is supported by the following observations:

(i) Protoperithecia are formed only after the mycelium has filled the containing vessel, regardless of the volume of medium in it.

(ii) The correlation between the mycelial front reaching the plate edge and the initiation of protoperithecial production shortly afterwards holds for Petri dishes of different sizes and shapes.

It is not only containment that induces fruiting – Pollock (1975) reported that damage to the mycelium by incision of part of a colony of *Sordaria fimicola* induced a localised fruiting response – there was an increase in protoperithecial production at the site of damage. Pollock also reported the induction of a similar localised response by introducing a barrier to diffusion in the medium, although this also appeared to involve wounding of the mycelium by incision.

Also, contact with other microorganisms – bacteria as well as other fungi – often elicits such a reaction (Asthana and Hawker, 1936; Buston and Khan, 1956) This is commonly encountered when a culture becomes contaminated in the laboratory.

Buston and Rickard (1956) showed that, in colonies of *Chaetomium globosum* which were inoculated eccentrically on a Petri dish, there was an increased production of perithecia in the parts of the colony nearest the edge of the dish. This suggests that the pattern of perithecial initiation is dependent on the relationship between the mycelial front and the wall of the containing vessel.

The precise cause of these so-called 'edge effects' is unknown. Buston and Rickard (1956) showed that in *Chaetomium globosum* perithecial production was coincidental with localised increases in sugar phosphates in the medium. Suggestions that it is the depletion of nutrients in the colonised

medium were refuted by Macdonald (1976) and Macdonald and Bond (1976), who showed that, after a culture of *Sordaria brevicollis* had been sustained on a plate of cornmeal agar to the point of fruit induction, that medium could be re-used without any sign of fruiting until the same interval after the new colony had reached the Petri dish wall.

There is no doubt a more complex mechanism underlying the edge effect. The pattern by which many fungi will react to a region of contamination, often producing masses of fruitbodies in the immediate vicinity of the foreign organism, shows similar characteristics to the culture reaching the plate wall. A similar effect is seen when a colony encounters a region of medium containing an antibiotic, antagonistic to the fungus.

In *Sordaria brevicollis*, the presence of an excess of potassium dihydrogen orthophosphate or potassium nitrate in the medium causes a change in the branching mode, and therefore the growth rate, of the mycelium. Under these conditions, protoperithecia are formed as the colony is still growing, without the prior need for edge contact (Bond, unpublished).

Drinkard *et al.* (1982) showed that a culture of *Phycomyces blakesleeanus* reduced its growth rate by an order of magnitude or more when within one millimetre of another culture of the same species. Three to four hours later, the hyphal tips of confronting cultures of opposite mating-types would swell to form zygophores.

Lysek (1977) monitored protoperithecial production in a colony of *Podospora anserina* grown from a central zone of rich medium to a peripheral region of poor, minimal medium. Protoperithecia were initiated in greater numbers at the point of transition between the two media than on the rest of the colony.

Bond and Broxholme (1987) describe earlier experiments in which a colony of *Sordaria brevicollis* showed a fruiting response at the point of transition from cornmeal agar to a minimal medium with no added nutrients. This effect was also seen on the transition from a double-strength cornmeal agar to a normal cornmeal agar. These results would indicate that the stimulus for fruit initiaion is the growth of the mycelium across the transition point. If this were to be likened to the edge effect, then it would be the disruption of

growth rather than contact with a physical barrier that initiates the response.

Perhaps surprisingly, there has been little work reported, save that in early observations, on edge effects. There is little in the literature of any biochemical or metabolic changes during the transition of a mycelium from a radially growing thallus to the state of the colony after reaching the limits of the available medium. Numerous features make it an interesting phenomenon - not least the change to a sexually active phase.

The edge effect as a mechanism of morphogenetic switching has been neither matched conclusively to, nor discriminated conclusively from, the exhaustion of nutrients or the accumulation of other metabolites as a similar switch (see above). Thus while any combinations of these mechanisms might apply to a particular species, it should be noted that there is no evidence for their being mutually exclusive of one other.

Other observations suggest good potential systems for analysis. Bond and Macdonald (1976) and MacDonald and Bond (1976) obtained a mutant of *Sordaria brevicollis* which showed an interesting change in the pattern of protoperithecial production whereby they were restricted to the margin of the colony when grown on cornmeal agar, whereas a wild-type colony would produce protoperithecia all over the surface. This mutant showed an even more intriguing phenotype when grown on a dialysis membrane laid over the surface of the same medium - the protoperithecia were confined to an area in the centre of the colony, of which the limit was always a specific distance from the plate wall, irrespective of the size or shape of the containing vessel. These observations led to the conclusion that the initiation of protoperithecial production is a response to the relationship between the mycelial front and the plate edge.

On further analysis, this single-locus mutation, *per1*, when combined with a second, unlinked mutation, *per2*, produced a female-sterile colony which, on reaching the plate edge, showed a remarkable change in its metabolic state. The glycolytic activity as measured by sugar phosphate content was reduced to undetectable levels, compared with the wild-type, which showed little change after the edge had been reached. Also, the 'subculturability', as measured by the proportion of subcultures which were viable, dropped dramatically shortly after the edge of the plate was reached. It appeared that

the *per1 per2* strain was capable of surviving only if radial growth was not stopped.

1.2.4. Quiescence and Sexual Development in Podospora.

Podospora anserina is similar to *Sordaria brevicollis* in that it, also, normally undergoes fruiting only after the medium has been colonised. In *P. anserina,* there is an interesting series of mutants which show alterations in the normal pattern of protoperithecial initiation. These mutants were discovered on investigation of a system of protoplasmic incompatibility.

Apart from allelic incompatibility, in which strains carrying different alleles at a particular locus will undergo cell death at hyphal fusion (Bernet, 1963; Rizet *et al.* 1959), there is also a system of genes concerned with non-allelic incompatibility. In this system, a single strain can carry two incompatible alleles at different loci, resulting in the same cell lysis and death seen between two incompatible strains. There are several such systems, discovered by comparing wild-collected cultures (Bernet, 1965; 1967), or by mutagenesis (Delettre and Bernet, 1976).

Modifiers of these self-incompatibility reactions, the *mod* series of mutations, show not only recovery of the 'self-lysis' condition to 'normal' growth, but also most of them show changes in protoperithecial formation and fertility.

The observations on the *mod* mutations are consistent with a model for the developmental pattern of the fungus, summarised in figure 4. The normal developmental sequence, according to the model, requires that the mycelium enters a period of quiescence after it has colonised the medium. Following this, hyphal cells become temporarily totipotent in a 'switch' stage, when their fate in further development to protoperithecia, aerial hyphae, or rejuvenated vegetative hyphae, is determined.

According to the model, the *mod* genes are responsible for the transitions from each developmental phase to the next. They appear to operate either by mediating the transition, or, in the cases of *modE* and *modF,* by blocking a transition which would allow the quiescence phase to be bypassed.

Figure 4

Action of *mod* Mutations of *Podospora anserina*

The model is described fully in the text.

Wild-type alleles at the *mod* loci are

required for normal function of the

developmental steps. *mod E* and *mod F*

appear to control the recovery of the

quiescent cell to developmental totipotency.

mod E and *mod F* suppress the effects

of *mod D* or *mod G*, permitting further

development by bypassing the normal pathway.

(modified from Durrens and Bernet, 1982)

$modA^+, B^+$

$modE^+, F^+$

GROWTH RENEWAL

VEGETATIVE
HYPHA

QUIESCENT
CELL

TOTIPOTENT
"SWITCH" STAGE

PROTOPERITHECIUM

AERIAL HYPHA

$modC^+$

$modD^+, G^+$

DORMANT
ASCOSPORE

GERMINATION
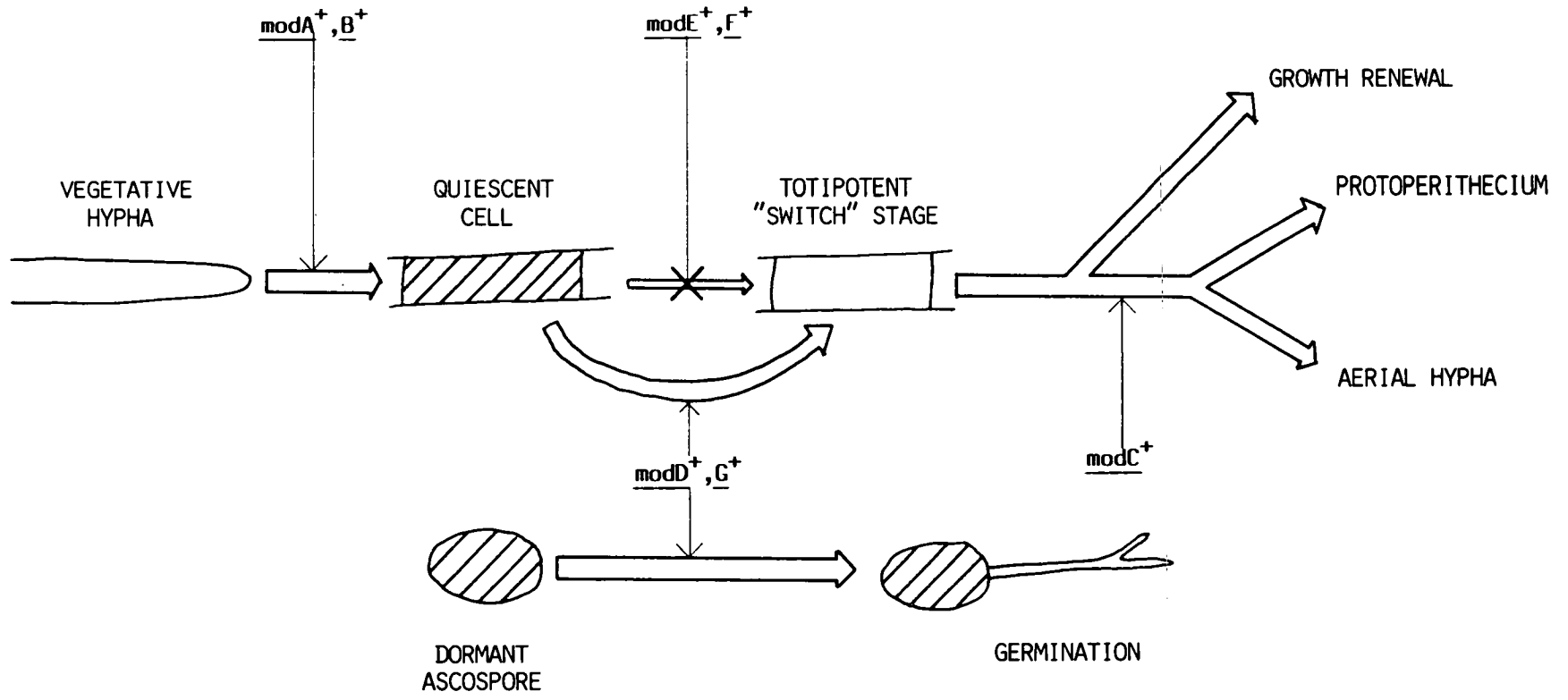
Thus, mutations in the *mod* genes confer the following phenotypes:

modE and *modF* cause unscheduled and abundant production of protoperithecia, increases in the density and size of aerial hyphae, normal growth renewal on subculturing but reduced survival on glucose starvation (Durrens, 1982b; 1983). According to the model, *modE* and *modF* would allow cells to differentiate into hyphal derivatives without the prior need to enter a quiescence phase – this would account for the precocious differentiation observed.

modC mutants are female-sterile, with little or no aerial hyphae (Labarere and Bernet, 1979a;b). This would be the expected consequence of selective blocking of the 'protoperithecia' and 'aerial hyphae' fates from the switch stage.

modD and *modG* mutants show similar phenotypes to those of *modC*, with the addition of having inhibited growth renewal (Labarere and Bernet, 1979a;b; Durrens *et al.*, 1979; Durrens and Bernet, 1982). According to the model, this would be the expected result of no recovery from quiescence. It is interesting also that *modD* and *modG* mutations prevent ascospore germination, and so this could be a transition equivalent to that from quiescence to renewed growth.

modA and *modB* mutations suppress the sexual incompatibility reaction in the ascogonium, but not in the microconidium. Protoperithecial production is also suppressed in *modA modB* double mutants (Boucherie *et al.*, 1976; Boucherie and Bernet, 1980).

There are obvious similarities between the *modD* or *modG* mutations and the *per1 per2* double mutation of *Sordaria brevicollis*. The *per1 per2* strain of *S. brevicollis* appeared to 'shut down' much of its metabolic activity after reaching the plate edge, and thus could have entered a quiescent phase. However, while a wild-type would progress from this state to other modes of development – sexual activity or rejuvenated mycelium – the *per1 per2* strain did not recover and remained quiescent, perhaps died.

1.2.5. Biochemical Action of *mod* Mutations.

The non-allelic incompatibility system described for *Podospora anserina* poses many questions regarding its mode, or modes, of action, and more so on the complex system of switches involved in the life-cycle of the fungus. How is it that a single mutation can abolish the self-incompatibility reaction, and at the same time have deleterious effects on other stages in the developmental process? One way in which this can be investigated is by looking at the mode of action of the incompatibility genes themselves, and thence to find out how they may be suppressed.

Various biochemical changes have been observed in incompatibility reactions. Enzymes found to be induced in self-incompatible strains include proteases III and IV (Begueret, 1972; Begueret and Bernet, 1973; Labarere *et al.* 1974), also a phenoloxidase, malate dehydrogenase, NADH dehydrogenase, and an amino-acid oxidase (Boucherie *et al.*, 1978). A protease inhibitor, β-phenyl pyruvic acid, prevents cell lysis (Delettre *et al.* 1978; Labarere and Bernet, 1979a), and dihydrostreptomycin, which affects translation of mRNA, had a similar effect (Bernet *et al.* 1973). These observations suggest that the self-lysis observed is at least in part due to the action of proteases, and therefore the *mod* mutations might act by affecting the expression of the protease genes. Specifically, it has been suggested that the *modA* gene encodes a ribosomal product essential for some messengers to be translated (Bernet *et al.* 1973). *modC* was shown to suppress production of laccase and proteases III and IV, and to lack a membrane-bound protein of molecular weight 42,000 Daltons (Bonneu and Labarere, 1983).

These observations have led to the idea that the non-allelic incompatibility system is mediated by changes in enzyme production, perhaps mainly proteases (Begueret, 1972). Modification of the self-lysis comes about by the control of these enzymes, which may be at the level of translation or inactivation of the proteins themselves. The cell membrane appears in some cases to play an important role in the modification, and therefore presumably the self-lytic and natural non-self recognition processes (Bonneu and Labarere, 1983; Durrens 1982a).

It is not known if the model for the mode of action of the non-allelic

incompatibility and modifier genes is related to the allelic incompatibility seen in other Ascomycetes, such as the heterokaryon incompatibility systems of *Neurospora crassa*, and indeed the familiar mating-type systems. But it is known that allelic incompatibility in *Podospora anserina* does not have the marked pleiotropic effects on sexual morphogenesis that are seen with the non-allelic incompatibility system (Blaich and Esser, 1971).

1.3. Heterokaryon Incompatibility Systems, and Mating-Type.

Many fungi are homothallic, that is they are capable of producing fertile fruitbodies on an individual mycelium, and two nuclei from any adjacent hyphae can fuse and enter the sexual cycle. However, heterothallic fungi must rely on meeting individuals of opposite mating-type for crossing to occur. Obviously the mating-type genes of heterothallic fungi play a central role in the control of morphogenetic development, but the molecular basis of the action of mating-type has not yet been elucidated. Heterothallic Ascomycetes have a single genetic locus determining mating-type; the alleles are generally *A / a* as in *Neurospora crassa* and *Sordaria brevicollis*, or +/- as in *Podospora anserina*.

In the *A / a* systems of *N. crassa* and *S. brevicollis* two strains of similar mating-type are unable to enter the sexual cycle together, yet two strains of opposite mating-type are unable to form a stable heterokaryon with each other (Beadle and Coonradt, 1944).

1.3.1. Basidiomycete Incompatibility Systems.

In the Basidiomycete fungi there is a variety of different forms of sexuality that appear to be governed by incompatibility systems. While the mode of sexuality of all but a few Basidiomycetes is unknown, some have been shown to possess a multiallelic system involving one or more loci. The multiallelic nature of the Basidiomycete mating type loci is far from well understood, and opinions differ with respect to the evolutionary significance and the consequences of encounter in natural fungal populations. The incompatibility loci do appear to play an important role in recognition between hyphae, and the consequences of contact between them.

In bipolar compatibility, there is a single locus concerned with the ability to

undergo hyphal fusion and so form a dikaryon. This locus is generally multiallelic, unlike the biallelic systems of heterothallic Ascomycetes. In tetrapolar compatibility, as in *Schizophyllum commune* and numerous other Basidiomycetes, mating can only occur when two loci, A and B, each have different alleles, ie $A \neq B \neq$. A feature called hemicompatibility appears when only one of these pairs is unlike; in such a case the sexual cycle is incomplete, because of the mode of action of the system. Heterogeneity at each of the two loci govern two different parts of the process of heterokaryon formation; the 'A factor' controls conjugate nuclear division and clamp cell formation, while the 'B factor' controls nuclear migration first during dikaryon establishment, and second during clamp cell fusion (Casselton, 1978). When two homokaryons that are sexually compatible meet, they are able to undergo cell fusion and nuclear exchange to form a self-fertile dikaryon.

The scheme outlined above is a simplified one. The mating type systems of Basidiomycete fungi are the subjects of much debate and controversy, which would extend beyond the scope of this thesis. Schemes describing possible mechanisms for the incompatibility, recognition and mating processes are common, for example see Burnett (1975), Casselton (1978), Kemp (1980), Raper (1966), Raper (1978).

1.3.2. Ascomycete Incompatibility Systems.

Ascomycetes show rather a different level of complexity from the Basidiomycetes. While it is apparent that the incompatibility systems of Basidiomycetes have functions in controlling steps in the dikaryon formation, such functions have not yet been described in Ascomycetes. It is unlikely that a direct comparison could be made since in Basidiomycetes only 'unlike' hyphae can form a stable heterokaryon, while in Ascomycetes the mating-type loci must carry similar alleles for this to happen. One could speculate that the incompatibility systems described do not function in the same way, the reason being related to the biological differences between the organisms. One function of the Basidiomycete system is to permit cell fusion in undifferentiated, vegetative cells, whereas in Ascomycetes this is not the case, and fusion between cells of opposite mating-type does not lead to extensive heterokaryosis, and normally is only observed in the structures specialised for sexual reproduction.

Although there are two definitive functions of the mating-type system, namely heterokaryon incompatibility and mating ability, they have never been separated by recombination in *Neurospora crassa* (Newmeyer *et al*, 1973). However, one of them has been lost by mutagenesis (Griffiths and DeLange, 1978) - strain a^{m33} is capable of forming a heterokaryon with either *a* or *A* strains, yet it can only mate with an *A* strain. There are species of Neurospora which are pseudo-homothallic; the mycelium is a mixed mating-type heterokaryon that grows as well as a homokaryon (Dodge, 1957). The two mating-types *A* and *a*, of *N. sitophila* can form a stable heterokaryon only when the two strains are from genetically related backgrounds (Mishra, 1971). *N. dodgei*, however, is a true homothallic with one self-fertile mating-type.

An unlinked suppressor of the mating-type-mediated heterokaryon incompatibility of *Neurospora crassa* has been found (Newmeyer, 1970). The mutation *tol*, when homozygous, permits heterokaryosis between *A* and *a* strains, without any effect on the crossing ability. However, *tol* does not suppress any other vegetative incompatibility system.

Griffiths and Delange (1978) found that several isolates of *Neurospora crassa* had mutated so that the *a* mating-type had lost both fertility and heterokaryon incompatibility, but one had retained its fertility and yet was compatible in a heterokaryon with an *A* isolate.

Mutational analysis of the *a* locus was extended to the *A* allele (Griffiths, 1982). However, when attempts were made to find a mutation equivalent to a^{m33}, no such heterokaryon-compatible fertile isolate was found. Furthermore, a confrontation between mutants of *A* did not produce barren perithecium-like bodies as occured with the *a* mutants.

The mechanisms by which the mating-type systems of filamentous ascomycetes operate has not yet been elucidated, but extensive research has yielded much information about that of the yeast, *Saccharomyces cerevisiae*.

Genetic analysis of mutations at the mating-type locus led Strathern *et al.* (1981) to propose that the α gene encodes two regulatory proteins; α_1 and α_2. According to this model, α_1 protein acts as a positive regulator of expression of α-specific genes, while α_2 protein blocks expression of a-specific genes.

Both mating-type alleles - *MATa* and *MATα* - have been cloned and sequenced (Hicks *et al,* 1979; Nasmyth and Tatchell, 1980; Strathern *et al,* 1980; Astell *et al,* 1981), and the α_1-α_2 hypothesis confirmed by *in vitro* mutagenesis (Tatchell *et al,* 1981). The allele-specific region of *MATα* contains two open reading frames, encoding the α_1 and α_2 regulatory proteins. α_1 is required for transcription of α-specific genes, including the α factor (Fields and Herskowitz, 1985) and the a factor receptor gene, *STE3* (Sprague *et al,* 1983). α_2 represses transcription of the genes encoding the a factor (Fields and Herskowitz, 1985) and the α factor receptor gene, *STE2* (Hartig *et al,* 1986).

The allele-specific region of *MATa* also contains two open reading frames; these only function in the a/α diploid (see Sprague *et al,* 1983a).

1.3.3. The Heterokaryon Incompatibility Systems of *Neurospora crassa*

In *Neurospora crassa* there are several loci which determine heterokaryon incompatibility in addition to the mating-type locus. These *het* genes behave in the same manner as the mating-type genes when heterokaryon construction is attempted. However, they have no effect on crossing ability between strains (Garnjobst, 1953). Blaich and Esser (1971) found evidence suggesting that degradation enzymes are released on breakdown of membranes in the allelic incompatibility reaction in *Podospora anserina*.

Heterokaryon incompatibility genes have been found using partial disomics (Mylyk, 1975). Phenotypes of isolates heterogenic for *het* loci are very abnormal; they are inhibited in their initial growth, produce dark pigmentation on certain media, and later 'escape' from the inhibition as a result of somatic mutation, to produce wild-type growth.

In *Aspergillus nidulans,* vegetative incompatibility can have a varied effect on morphology and recoverability of heterokaryons, depending on the particular locus at which there is heterozygosity (Dales *et al,* 1983).

The mechanism by which heterokaryon incompatibility acts is not known, and presents some very intriguing questions, yet there is little in the literature of attempts to describe it in molecular terms.

Current knowledge of the system could be summarised as follows: Fusion

between *het* pairs of hyphae initiates an irreversible reaction; upon exchange of protoplasm, there is a degradation of all the cellular contents in the vicinity of the fusion (Garnjobst and Wilson, 1956). Thus the reaction observed has also been called protoplasmic incompatibility.

The mating-type genes, which also carry heterokaryon incompatibility function, appear to act in the same fashion during the reaction (Garnjobst and Wilson, 1956) as the *het* genes. It is not known whether the mating-type system arose independently from the *het* systems, or whether it took on the mating function later, perhaps in the evolution of heterothallism.

One observation that may prove useful here is that different species of Neurospora can 'induce' protoperithecial proliferation when used to confront one another in culture. Although these 'perithecia' may contain complete, mature asci they are never true crosses since all of the progeny of one fruitbody are of the same species (Perkins *et al,* 1976; Perkins and Raju, 1986). Metzenberg and Ahlgren (1973) found that the mating-type genes of *Neurospora tetrasperma* behaved similarly to those of the host when introgressed into *N. crassa,* indicating no evolutionary divergence between these species at this locus. Dutta *et al,* (1981) found several lines of evidence supporting the conservation of mating-type specificity within the Neurospora genus.

What does seem a plausible model is the evolution of a protoplasmic incompatibility system as a sort of defence mechanism, while in competition with other organisms. There would be a danger to one species if it had no means of preventing hyphal fusion with another fungus which were capable of destroying the first's cell contents. The consequence of such an encounter would be death of the first individual, and either colonisation of the medium or 'parasitism' of the individual's mycelial system by the second organism. Such disastrous endings would surely be avoidable by the presence of a simple method by which foreign hyphae would instantly be recognised, resulting at most in cell death, perhaps mutual, only at the margin of the colony.

1.4. Genetic Control of Sexual Morphogenesis.

It has been established that the initiation of protoperithecial morphogenesis depends to some extent upon what have been described as 'environmental factors', such as the degree of colonisation of the medium, the presence of other organisms competing for the same substrates, the nutritional state of the medium, and other factors such as light conditions, aeration and moisture. In certain fungi there is also observed a requirement that the mycelium itself should have the necessary constitution of endogenous factors. This is most easily seen in the Basidiomycetes which must have a heterokaryon containing nuclei of different genotypes, before the mycelium can become 'competent' to respond to initiating factors.

Given all these appropriate conditions, fruiting would proceed, from initiation through to maturation and spore discharge, without any further interference from environmental conditions. There are, of course, other, internal factors that influence and mediate sexual development. Although one's first suggestion to this problem would be that the morphogenesis is under genetic control, it is a very different question to demonstrate this.

The following is an account of the numerous attempts, and the knowledge gained, in the search for the genetical control mechanisms concerned with sexual morphogenesis.

There have been numerous descriptions of genetic variation in fertility, although in many cases analysis has progressed little further than to describe the ultrastructural abnormality associated with the genetic defect. Perhaps a model of the order of expression of a set of genes concerned with sexual morphogenesis might be postulated as a result of the aforementioned descriptions, and from this, a more detailed description of the sequence of events involved in production of the sexual structure.

Esser and Straub (1958) undertook the first extensive genetic analysis of sterility, with 23 mutants altered for some part of the sexual cycle of *Sordaria macrospora* By microscopic analysis, and by complementation through crossing from heterokaryons, they gave a detailed report of the genetic effects on normal sexual development. Since Esser and Straub (1958), there has not been another system described so extensively.

Understandably, fertility may be a relatively simple phenotype to score and demonstrate to be a genetic phenomenon, but the biochemical analysis of the underlying cause of a defect would be all but simple.

Early studies of fertility in Ascomycetes succeeded in showing that variation was often due to an inherited factor (Aronescu, 1933; Dodge, 1946). Presumably because the knowledge of gene action was considerably less than as of now, there was a tendency in early work to study the interaction of environmental conditions with inherited factors. The correlation of fruiting with tyrosinase synthesis and melanin formation were well established in *Neurospora* (Westergaard and Mitchell, 1947; Hirsch, 1954). There was such a strong coincidence of the three that it seemed prudent to consider them as parts of the same system, and to look for variation from the 'wild-type' relationships between them. In general the initiation of protoperithecium formation coincided with an increase in tyrosinase and melanin formation, while in 'female-sterile' isolates there was either a concomitant lack of the latter two, or an overproduction of both (Westergaard and Hirsch, 1954).

Since the early work there have been numerous reports of female-sterile mutations, mainly in *Neurospora crassa* (Westergaard and Hirsch, 1954; Fitzgerald, 1963; Tan and Ho, 1970; Ho, 1972; Mylyk and Threlkeld, 1974, Johnson, 1978; Leslie and Raju, 1985), also in *Glomerella cingulata* (Wheeler and McGahen, 1952; Wheeler, 1954), *Sordaria macrospora* (Esser and Straub, 1958) and *Podospora anserina* (Zickler and Simonet, 1980). Many of these were discovered by what were often rather elaborate screening methods, others by chance.

Mylyk and Threlkeld (1974), have shown that female sterility mutations, although frequent, rarely have an effect on the fertility of the mutant as a male parent (Mylyk and Threlkeld, 1974). This might be expected if the morphogenetic pathway for the protoperithecium were different from that for the conidia. Many female-sterile mutations also have effects on vegetative growth (Murray and Srb, 1959; Mylyk and Threlkeld, 1974; Johnson, 1978).

The high frequency of female-sterile mutants obtained by Mylyk and Threlkeld (1974) led them to suggest that the production of protoperithecia is a complex process under an elaborate genetic control. In general, they found a change from the wild-type colony morphology was a feature of those

mutants which were blocked in the early stages of protoperithecial development.

Griffiths and DeLange (1978) and Griffiths (1982) obtained mutants of the *A* and *a* mating-type genes of *Neurospora crassa*, which were incapable of acting either as the female or the male parent in a cross. These 'null' mutants were isolated on the basis of their loss of heterokaryon incompatibility function at the mating-type locus. These mutants showed differences between mating-types in that while many a^m mutants produced abortive mating reactions when used as male parents to wild-type females of either mating-type, A^m mutants failed to do so.

Correlation between colony morphology and fertility serves to reinforce the findings with the *mod* mutations of *Podospora anserina*, that there is frequently an alteration in the membrane structure of the hyphae which has an effect on the ability to form hyphal aggregates.

Furthermore, Kappy and Metzenberg (1967) showed that a mutation at the *un-3* locus, closely linked to mating-type in *Neurospora crassa* has no effect on fertility, but has numerous effects in the uptake of amino-acid analogues, neutral and acidic amino-acids, and potassium ions, but not basic ions, in addition to having altered characteristics in the stability of its protoplasts. Kappy and Metzenberg suggest that the mutation confers a generalised membrane defect, and postulate that the closely-linked mating type gene may have similar functions in membrane structure, and therefore it may be part of the same cluster of genes. A further piece of evidence for this is that the mutant *un-t* is unable to grow above 30°C, at which in wild-type isolates, protoperithecia fail to form.

Rather intriguing also are the mutations which confer 'male-sterility'. Evidence that conidia play more of a part in sexual reproduction than by donating nuclei has been presented by Weijer and Vigfusson (1972), who found among thirty male-sterile isolates, six groups according to the degree of perithecial development permitted. This ranged from 'few small brown protoperithecia, completely sterile' to 'abundant black perithecia with incomplete ostiole, empty or with few spores'.

The mutations did not act by affecting conidium survival, and many

showed different behaviour when present in the female parent. Specifically, those that acted later in the developmental process acted similarly when from either parent, so perhaps the block occurs after karyogamy, when many *of the cells of* the perithecium are heterokaryotic.

On complementation, Vigfusson and Weijer (1972) showed that there were four complementation groups of male-sterile mutations that acted up to the stage of plasmogamy.

Mutations specifically blocking the differentiation of macroconidia in *Neurospora crassa* were assigned to four genetic loci, which corresponded to four specific points that were blocked in their development (Matsuyama *et al*, 1974). Three of these appear to be 'phase-specific' - they have no effect on vegetative or aerial hyphae - although one of the three appears to be female-sterile, producing abundant protoperithecia but never mature perithecia.

Following the differentiation of proconidial chains there are two mutations reported (Selitrennikof *et al*, 1972) which prevent the release of free macroconidia. These mutations are phase-specific and appear to be associated with an absence of cell-wall autolytic activity, normally seen in wild-type cultures.

Raju (1987) has reviewed extensively what is known about ascus development and its genetic control. There are many known mutations which affect ascus development, which can be grouped according to their effects; meiotic alterations, ascus or ascospore morphology, ascospore size, pigmentation or viability, and others. Many of these mutations are lethal, or cause many of the ascospores in a cross to abort, although often such a defect can be carried in a viable spore. One extreme example of this is the 'spore killer' mutation which causes the death of all spores in the same ascus not carrying the mutant gene.

There are frequently reported mutations conferring an altered spore colour, such as those in *Sordaria fimicola* (Olive, 1956), *Podospora anserina* (Picard, 1971) and *Sordaria brevicollis* (Chen and Olive, 1965; Chen, 1965). These generally have a lack of one or more pigments and consequently often have a reduced survival, especially after harmful irradiation such as ultra-violet light.

Zickler and Simonet (1980) have combined mutations of spore development in. *Podospora anserina*, in order to establish their sequential action. They concluded that such mutations vary in their mode of action; some disturb post-meiotic mitosis (PMM) and sporulation, in others the ascospore membrane forms independently of nuclear division, and consequently supernumary PMMs were observed.

Certain mutations proved to be pleiotropic, having very different characteristics when combined with another. For example, two mutations which independently caused a block in the formation of a crozier caused a block later during sporulation when combined as a double mutant. Therefore, while mutations causing the blocked early stages complemented each other, there was at least a second effect of one or both of the mutations which appeared at a later stage in the process.

Mutations expressed during the sexual diplophase, that is the part of the life cycle where diploid cells are present, would rarely be observed unless dominant. However, DeLange and Griffiths (1980a,b) and Leslie and Raju (1985) have designed rather elegant methods for detecting recessive mutations, by making homozygous linkage-group I genes in an otherwise self-fertile disomic strain (DeLange and Griffiths, 1980a) or by crossing wild-collected isolates to their progeny so that a mutation will be homozygous (Leslie and Raju, 1985).

There are many mutations reported which act at the diploid stage of the sexual cycle (reviewed extensively by Raju, 1985). While their characteristics show great diversity, it is still unknown how they act and moreover, the mechanisms by which two haploid nuclei from different mycelia are combined, how they undergo such a complex routine of cellular and nuclear divisions within the ascogenous hyphae, and how this sequence culminates in the production of hundreds of mature asci in each perithecium.

1.5. Biochemistry of Sexual Development

1.5.1. Carbohydrate Metabolism during Sexual Development.

Of the major polysaccharide components of the fungal cell wall, α-1,3-glucan appears to be the only one with differential metabolism during

the sexual cycle of *Aspergillus nidulans* (Zonneveld, 1972). α-1,3-glucanase has been shown to be under strong repression in the presence of glucose (Zonneveld, 1972), although it is also subject to repression by all carbon sources which confer good vegetative growth in *A. nidulans*. Other catabolic enzymes are apparently produced even when glucose is present, although the activity of most of them will increase with glucose exhaustion.

During vegetative growth, α-1,3-glucan is stored in mycelial walls. On depletion of glucose, α-1,3-glucan is broken down to provide an energy source for the production of cleistothecia. α-1,3-glucanase activity is repressed if glucose is kept at a sufficient level (Zonneveld, 1972).

In the yeast Saccharomyces cerevisiae), the presence of soluble glycogen appears to depend upon sporulation – there is a period of glycogen degradation during maturation of ascospores (Kane and Roth, 1974). Colonna and Magee (1978) observed that glycogen is synthesised during meiosis then degraded during spore maturation. The activity of the catabolic enzyme corresponded with the levels of glycogen present.

When Basidiomycete fungi are to be considered, a similar sequence of events is observed. Jirjis and Moore (1976) observed that in *Coprinus cinereus*, the extractable glycogen increased during early vegetative growth then declined concomitantly with a major uptake of carbohydrate from the medium, a major reduction of soluble reducing sugars in the mycelium, and the appearance of mature sclerotia.

Madelin (1956) showed how the gain in weight of the basidiocarp of *Coprinus lagopus* (*sensu* Buller) and *Agaricus campestris* is equal to the loss of weight from the mycelium; this supports the concept of translocation of carbohydrate and protein resources to the actively growing parts of the colony. Robert (1977a;b) described the elongation of the stipe of *Coprinus congregatus* at the expense of carbohydrates accumulated during vegetative growth, and the maturation of the fruitbody cap utilising carbohydrates from the stipe.

1.5.2. Protein Metabolism During Sexual Development.

The correlation between nitrogen limitation and induction of protoperithecia has been described already. Zonneveld (1980) observed that in *Aspergillus nidulans*, nitrate starvation has an effect on cleistothecial formation, irrespective of the presence of glucose or other growth-promoting sugars in the medium (see above). Cleistothecium formation was preceded by high levels of protease activity in such conditions. However, in the presence of excess nitrate, protease activity was low, yet cleistothecia could still be formed. It therefore appears that protease activity is not essentially involved in cleistothecium formation if sufficient α-1,3-glucan is present.

1.5.3. Phenoloxidases and Sexual Development.

Correlations of specific biochemical events with sexual development have been largely concerned with pigmentation, and more specifically with melanin formation and the enzymes associated with it.

It seems unlikely that phenoloxidases have a single role, if all fungi were to be considered. Laccase has been implicated in the production of the dark green pigment of *Aspergillus nidulans* conidia (Clutterbuck, 1972) by the polymerisation of phenols. Butnick *et al.* (1984) found evidence for a phenolic pathway being involved in sporulation of *A. nidulans*, and suggested that one or more products of this pathway participates in the initiation of sporulation. It may also play a role in lignin degradation, for example in white-rot fungi (Ishihara, 1980), and perhaps in the breakdown of other substrates (*e.g.* Wood, 1980a). There is a correlation of melanisation of fungal cell walls and their resistance to degrading or lytic enzymes of other microorganisms (Bloomfield and Alexander, 1967; Kuo and Alexander, 1967).

That phenoloxidases are associated with sexual morphogenesis has been suggested and supported for many years and by many workers. Hirsch (1954) observed that tyrosinase activity was limited to a sexually active colony of *Neurospora crassa*. Barbesgaard and Wagner (1959) proposed a close correlation between melanin formation and protoperithecial production in *N. crassa* by comparing the two in various mutant strains – female-sterile

strains appeared to lack melanin, while a highly fertile strain produced large quantities of melanin.

However, although Horowitz *et al.* (1960) found a similar correlation using other mutants, they found also that when tyrosinase activity was restored by induction with cycloheximide and amino-acid analogues, the female-sterility remained. Therefore it appeared that tyrosinase was necessary but not sufficient for sexual morphogenesis.

Esser (1968) reported a coincident reduction in perithecial size and melanin formation in two allelic mutations of *Podospora anserina.* The altered pigmentation was found to be due to an altered laccase enzyme; this defect was restored at the same time as was the morphogenetic defect in the presence of an unlinked suppressor gene. Esser thus concluded that laccase activity and perithecial morphogenesis are correlated.

Hermann *et al.* (1983) demonstrated the localisation of a laccase, different from the conidial laccase of Clutterbuck (1972), in the cleistothecial primordia of *Aspergillus nidulans* and in the enveloping hulle cells. They suggested that the laccase is largely produced by the hulle cells, and is transported to the cleistothecia. Here it is used to cross-link hyphal walls into cohesive aggregates, according to the mechanism proposed by Bu'Lock (1967), to make a multicellular tissue from the filamentous cells.

Leonard (1971) found a correlation of phenoloxidase activity and fruitbody formation in *Schizophyllum commune,* although he later (Leonard, 1972) found that the two could be separated, and concluded, as did Horowitz *et al.* (1960), that phenoloxidase activity may be essential to, but not sufficient for, fruiting.

Wood (1980a) showed that *Agaricus bisporus* produced laccase constitutively, with no apparent induction by treatment with standard phenolic inducers, although the production varied depending on the carbon source added. 90% of the laccase activity was extracellular, and the remaining activity was in the soluble fraction. Wood (1980b) found a decrease in laccase activity during fruitbody development, which he assigned to be a result of enzyme inactivation and proteolysis. The reverse situation to that seen in *Schizophyllum commune* may still allow a relationship to fruiting, even if the timings of the events do not coincide. The extracellular laccase of *A. bisporus*

accounts for 2% of the mycelial protein, and it may be that such activity would cause enzyme products to be present at levels inhibitory to fruitbody production. Also, the high levels of laccase, which may have been required for decomposition of the substrate, would provide a nitrogen source for the development of the fruitbody.

Leatham and Stahmann (1981), on determining the localisation of laccase activity within parts of the Basidiomycete *Lentinus edodes*, reported association with pigmented primordia and aerial hyphae, and the highest activities were localised in tissues with a high degree of pigmentation, such as the rind of the cap, or with a structural support function, such as the stipe.

Ross (1982) demonstrated the localisation of laccase activity in *Coprinus congregatus* specifically to areas of young mycelium, and not to those parts which were competent to produce primordia, although there was a correlation of overall laccase activity with the appearance of the primordia.

1.5.4. Pheromones in Sexual Development.

There is evidence that there are metabolites excreted by some fungi, which have profound effects upon the behaviour of other individuals. Such interactions often form an intricate mechanism which is part of the complex set of interactions leading to sexual development.

The Oomycetes *Achlya ambisexualis* and *A. bisexualis* secrete a biologically active metabolite - antheridiol - to which a potential male partner will respond by producing many branched antheridial hyphae. The male cells then secrete a second pheromone - oogoniol - which induces the production of oogonia by the female partner. Antheridial hyphae grow towards the oogonium, become appressed to it, and delimit antheridia, from which nuclei migrate to fertilise the female gametes (see Horgen, 1981). Whether pheromones are involved in later stages of sexual development is not known, but the two sex-specific pheromones described have been fully characterised and are known to possess a basic steroid structure, similar to those of some mammalian hormones.

Certain fungi of the Mucorales show a response between mating-types that appears to be mediated by trisporic acid, an 18-carbon terpenoid.

Trisporic acid is the factor responsible for a dramatic increase in β-carotene in mated cultures (Caglioti *et al*, 1964; 1967). It was shown to induce zygophore production in single strains of Mucoraceous fungi, and has been shown to restore fertility to a sterile strain of *Syzygites megalocarpus* (Werkman and van den Ende, 1974).

A characteristic feature of the trisporic acid story is that both partners respond to a single substance, then both participate in a 'collaborative biosynthesis' of the pheromone (see Jones *et al*, 1981).

Interaction between cells of opposite mating-type of the yeast *Saccharomyces cerevisiae* is mediated by peptide pheromones. a and α cells respond in a similar manner to each other's pheromone, but the two peptides bear no resemblance to each other in amino-acid sequence (see Bender and Sprague, 1986). By causing the receptor structural genes to be expressed in cells in which they would normally be silent, Bender and Sprague (1986) have shown that the cell-type specificity is determined solely by the receptor synthesised. Also they have shown that the two receptor-pheromone interactions are interchangeable, and that the signals triggered by the two factors are identical.

Knowledge of pheromonal interaction in the filamentous Ascomycetes is not nearly so advanced as that in certain other types of fungi (see above).

Zickler (1952) reported an attraction of trichogynes of *Bombardia lunata* towards spermatia of the opposite mating-type. Esser (1959) reported a similar situation in *Podospora anserina*, with apparently a second controlling step at plasmogamy. In *Ascobolus stercorarius*, there is interaction between mating-types in that oidia induce formation of ascogonia in mycelia of opposite mating-type, and thereby act as fertilising agents (see Bistis, 1965).

In *Neurospora crassa* trichogyne attractants have been identified for both mating-types (Bistis, 1983). The precise nature of these has not yet been reported, but the characteristics are not inconsistent with those of lipoidal hydrocarbons (Bistis, 1983).

Islam has undertaken extensive work to identify pheromones in the filtrate of a cross of *Neurospora crassa* that stimulate maturation of perithecia,

occasionally with production of ascospores, from a single culture (summarised in Islam, 1981). The work follows on from the observation by Ito (1956) that the filtrate of a mixture of mycelia of different mating-types will elicit such a response, more so than the combined effects of filtrates of individual mating-types. Islam described the activity as being due to two unsaturated hydrocarbons – one from each mating-type – of molecular weights between 354-372 from *a* and between 344-357 from *A* (Islam, 1981). No further characterisation of the pheromones has been reported.

It is conceivable that steroid-like substances are involved in the inductive pathways for sexual morphogenesis of filamentous Ascomycetes, given their roles in other fungi (see above) and the findings of Elliot (1969) that perithecium formation in *Sordaria fimicola* can be prevented by the presence of certain sterol synthesis inhibitors.

1.6. Molecular Biology of Sexual Development.

Analysis of sexual morphogenesis at the molecular level has been little studied; the most prominent work has been in yeast and Basidiomycetes. While sexual morphogeneses in these organisms are not directly relevant to that in the filamentous Ascomycetes, the knowledge obtained from them must be considered to be of substantial significance in terms of the advances that have been made and are possible in other fungi, and perhaps, if not especially, with regard to the findings obtained.

I shall review the state of the knowledge available at the beginning of this project of the molecular biology of sexual development, and I shall outline similar work in other aspects of fungal morphogenesis, again with a view to its relevance to the subject of this thesis.

Sexual morphogenesis in filamentous Ascomycetes

Little has been reported on studies in the molecular biology of sexual morphogenesis in the filamentous Ascomycetes. The most prominent record is the work by Nasrallah and Srb who identified, using gel electrophoresis, a major protein band, migrating at an apparent molecular weight of 20 kDa, specifically associated with perithecia, but absent from mycelia or ascospores of Neurospora. This protein increases in relative concentration after

fertilisation, and apparently different species of Neurospora show different electrophoretic characteristics of the protein, in terms of its migration in the gel (Nasrallah and Srb, 1973).

They later analysed the phenomenon further (Nasrallah and Srb, 1977) to find that there is a good deal of immunological cross-reactivity between the proteins from certain other species in the Neurospora genus. In general, though, there was only partial identity exhibited between the homothallic Neurospora species and the heterothallic or pseudohomothallic species. Other Ascomycetes showed varying degrees of cross-reactivity, although there was no good correlation between cross-reactivity and the presence of the major perithecia-specific band in the electrophoretic profile.

Later, Nasrallah and Srb (1978) used immunofluorescence to localise the protein to the internal cavity and neck of the perithecium. No fluorescence was observed with either the perithecial wall or the ascus cytoplasm.

Sporulation in the yeast *Saccharomyces cerevisiae*

Studies of the molecular biology of the sexual cycle of yeast has progressed rapidly, to the point now where a model has been described and demonstrated, to explain the mechanisms of gene control in the complex pathway of switching from vegetative cells to the sexual phase in which asci and ascospores are produced.

Earlier work observed the changes occuring in macromolecular constituents of cells during this transition. Hopper *et al.* (1974) reported a breakdown of pre-existing vegetative RNA and protein during sporulation, and Elliott and McLaughlin (1978) reported an exponential increase in the rate of RNA synthesis, and a similar pattern in the total protein synthesis which was reflected in the rates of synthesis of a selected number of individual protein species. Magee and Hopper (1975) and Trew *et al.* (1979) reported a number of changes in polypeptides synthesised in cells under sporulation-promoting conditions, although no species were found unique to a/α cells – all were also seen in a/a and α/α diploids.

Petersen *et al.* (1979) found few quantitative changes in total proteins, but several species were synthesised early in sporulation that were not detected

in exponential cultures.

Wright and Dawes (1979) observed 45 changes in the proteins synthesised during incubation in sporulation medium. Of these, 7 were new to, and 11 increased in intensity in, both a/a and a/α diploids, and a further 13 new spots and 7 increases in intensity were observed only in a/α cells, and thus these were 'specifically associated with the sporulation process'. These changes were further analysed (Wright *et al.* 1981) to the demonstration that the changes are sequential and show a characteristic temporal pattern of appearance during the process.

Some of the proteins which show changes in sporulation of yeast have been identified in function. Amyloglucosidase appears in a/α cells at 8-10 hours after initiation of sporulation (Clancy *et al.* 1982), and appears to be a sporulation-specific enzyme. Three histone proteins were synthesised almost exclusively at certain stages of the cell division cycle Lorincz *et al.* 1982). Dawes *et al.* (1983) observed the synthesis of a spore-specific surface antigen in a soluble form before the appearance of the spore surface, and a decrease in the level of this soluble form during spore assembly.

Weir-Thompson and Dawes (1984) observed changes in the translatable mRNA of a/α, a/a, and α/α diploids on transfer to sporulation medium. 36 mRNA species underwent changes in all three strains, 46 changes only occurred in a/α cells, and one was unique to α/α cells. Four of the sporulation-specific changes were due to appearance of new mRNA species; two of these became predominant species of the total population.

In a similar approach, Kurtz and Lindquist (1984) observed the induction of two sets of mRNA species in a temporal order such that the first set is degraded as the second appears. The second set persists in a highly stable form within the ascospores for long-term storage.

Steps forward in the analysis of yeast sporulation at the molecular level were made by Clancy *et al.* (1983) and Percival-Smith and Segall (1984) who isolated genomic sequences specifically transcribed during the process. Both groups hybridised genomic libraries with cDNA from the different developmental states, and selected and analysed those clones showing different signals.

Clancy *et al.* (1983) observed that of 4 of the sequences, all appeared initially between 7-10 hours after transfer to the sporulation medium, and decreased in abundance by 11-13 hours. Two of these clones each hybridised to two mRNA transcripts of different sizes, which may have implications for a level of expression other than at transcription, or perhaps for some clustering of the genes. Percival-Smith and Segall (1984) also found that expression of their sporulation-specific sequences began about 10 hours after transfer to sporulation medium, and that some of their clones hybridised to more than one transcript size.

Fruiting in *Schizophyllum commune*

Early work on the molecular analysis of fruiting in the Basidiomycete *Schizophyllum commune* by deVries *et al.* (1980) involved the investigation of polypeptides synthesised by cultures with various combinations of mating-type alleles, in order to see the degree of control by these incompatibility genes on protein synthesis. While the monokaryons differed by only 2% of their polypeptides, the dikaryon formed from their fusion showed many new species, of which most could be described as either switched on or switched off in the dikaryon. Mutations at the mating-type loci showed a considerable degree of overlap in the polypeptides affected by the individual (*A* or *B*) mating-type genes.

In a more extensive analysis, deVries and Wessels (1984) compared total, wall-bound, and extracellular proteins in the monokaryons, dikaryons, and fruitbody primordia. By a more rigorous extraction method, 30 polypeptides were detected in the dikaryon which were absent from either monokaryon. Ten additional novel polypeptides were observed in the developing primordia. Of the polypeptides specifically synthesised by the fruiting dikaryon, nine were excreted into the medium by surface-grown cultures.

Comparisons of monokaryons and dikaryons by nucleic acid hybridisations revealed little differences between the mRNA populations (Zantinge *et al.* 1979; 1981; Hoge *et al.* 1982a). However, the same mycelia grown on the surface showed that about 5% of the complex RNA from the fruiting dikaryon was absent from vegetative dikaryons and monokaryons. About 35 mRNA species were specific to the fruiting dikaryon (Hoge *et al.* 1982b).

Dons *et al.* (1984) have isolated a cDNA clone which hybridises to a mRNA species unique to the fruiting dikaryon of *Schizophyllum commune*. The gene (1G2) is specifically expressed in a dikaryon, but on fruitbody initiation there is a 20-fold increase in its abundance compared to the non-fruiting level. Further results implied that transcription of gene 1G2 is conditioned by the presence of heteroallelic incompatibility factors, but full expression only occurs at the time of fruitbody initiation.

Conidiation in *Aspergillus nidulans*

The formation of asexual spores (conidia) by *Aspergillus nidulans* has been analysed at the molecular level and rapid progress has been made. Timberlake (1980) reported that a significant proportion of the mRNA from conidiating cultures and purified conidia contains sequences absent from somatic hyphae.

The presence of state-specific mRNA sequences, and a technique for purifying phase-specific cDNAs, allowed Zimmermann *et al.* (1980) to select several genomic clones containing developmentally regulated sequences. Their initial results indicated that there is a non-random distribution of developmentally regulated coding sequences within the genome. Timberlake and Barnard (1981) further analysed a cluster of genes within one of these clones, and established that it contained the coding sequences for six mRNA species which were all expressed only in conidiating cultures, and which all accumulated in the conidia themselves. The genes within this cluster are expressed simultaneously, although the cellular concentrations of their transcripts vary considerably.

To investigate the chromosomal distribution of these phase-specific genes in a more general way, Orr and Timberlake (1982) took 30 random genomic clones containing spore-specific sequences, and determined the number of spore-specific genes in each. In another approach, they made several chromosomal recombinant DNA libraries, each with a different average insert size, and determined the proportion of clones containing spore-specific sequences in each library. In both cases there was evidence that there is a non-random distribution of the spore-specific sequences in the genome. The clustering observed by Timberlake and Barnard (1981) appeared to be more frequent than expected.

Another approach to the investigation of spore-specific genes is first to identify the gene of interest, then to isolate it by complementation of an auxotrophic strain of *Escherichia coli* with a genomic sequence containing the gene. This was performed successfully by Yelton *et al.* (1983), who cloned the *trpC* gene of *Aspergillus nidulans* by complementation of a *trpC* strain of *E. coli* with fragments of genomic *A. nidulans* DNA. The identity of the cloned gene was verified by subcloning and complementation of *E. coli* with the fragments of the clones, and also by comparison of the nucleotide sequence with that of the *trp-1* gene of *Neurospora crassa*. Analysis of transcription levels during the conidiation process showed a large increase with time, and this was consistent withthe previous observation (Yelton *et al.* 1983) that tryptophan is required in higher concentration for normal conidiation than for vegetative growth.

1.7. This Thesis

The knowledge of sexual development in the filamentous Ascomycetes, while comprehensive in certain respects, has wide gaps that could be bridged by collaboration between different schools of research. Such an inter-disciplinary approach would realise the benefits of understanding the mechanisms from a variety of different viewpoints. Historically, physiological and biochemical studies on sexual morphogenesis have concentrated on similar problems, but by and large, these approaches have been made separately rather than co-operatively. In this respect, also, biochemistry and genetics have a great deal to say to each other, yet in respect of sexual development little progress has been made towards achieving a synthesis of these disciplines.

Recently the molecular biology of filamentous fungi has increased in popularity as a research discipline, and it naturally has a great deal of bearing on genetics and biochemistry, yet again sexual development has been somewhat left behind, perhaps in favour of more accessible systems.

The purpose of this project is to establish a basis for a line of research which could unite the classical schools, by analysing the problem of sexual development from different viewpoints.

The approach taken was to study the molecular changes occuring during

the transition from the vegetative, mycelial state of *Sordaria brevicollis* to a new mode of development, culminating in the formation of structures specialised for the process of sexual reproduction. These changes would be observed with a view to describing molecular events occuring coincidentally with the characteristic physiological events of the morphogenetic process.

The major physiological events could be regarded as initiation of the fruitbody initial (the protoperithecium), conjugation of the parent nuclei and the mechanisms mediating this, maturation of the fertilised fruitbody (the perithecium), and maturation of the ascospores within the ascus.

Molecular events would be investigated, first with a view to characterisation of the developmental pattern of the fungus by means of a description of its molecular constitution, then a more extensive analysis could be made with descriptions of individual molecular events involved in the morphogenetic process. Ultimately, this molecular description could be extended to include the mechanisms governing individual morphogenetic steps, and perhaps more widely a model for the complex interactions involved in the sexual process.

CHAPTER 2
MATERIALS AND METHODS

Abbreviations are listed in Appendix 1

Formulae for media, buffers etc. are given in Appendix 2

2.1. *Sordaria brevicollis* – maintenance and manipulation.

All isolates of *Sordaria brevicollis* used in this work are descendants of an unknown number of ascospores isolated from a wild cross found growing on zebra dung in a New York zoo by Olive and Fantini (1961).

2.1.1. Isolation.

Wild-type isolates of *Sordaria brevicollis* were obtained by random spore plating of spores from a wild-type stock cross (G523). This stock cross was made by intercrossing wild-type isolates which had had no exposure to mutagens.

Ascospores from a stock cross were spread on Dissecting agar + acetate (DA+) to isolate pure cultures from single spores. After overnight incubation at 25°C, germinated spores were transferred on a block of DA+ to a slant of Vogel's (1956) N minimal medium, where they were allowed to grow for 2-4 days during which assessment of normal wild phenotype was made. The mating-type of individual isolates was determined by a standard method of scraping a small amount of the mycelium to be tested onto the surface of each of two wild-type cultures which had been grown for 4 days – after 1 – 2 days, perithecia formed where a cross of different mating types had been made.

Female-fertility was tested by inoculating onto a plate of Cornmeal agar (CMA) on which had been placed a cellulose membrane, and checking after 4 days for abundant, even distribution of protoperithecia. A further check was made by fertilising with a male of the opposite mating-type, and checking 1-2 days later for abundance of perithecia, evenly distributed.

2.1.2. Maintenance of stocks.

Isolates were maintained as colonies on CMA at room temperature, after colonisation was allowed at 25°C. Isolates were kept for approximately six months, after which time the fertility was sometimes reduced and viability also was reduced. Therefore fresh isolates were obtained at approximately six-month intervals in the same manner as above.

2.1.3. Crossing.

Crosses were made on CMA + cellulose membrane, a method used routinely for its enhancement of fertility and the ease by which perithecia can be harvested. The 'female' parent was incubated for 4 days at 25°C to allow formation of protoperithecia, then crossing was performed by making a conidial suspension from the 'male' parent in sterile dH_2O, and pouring this over the female parent. After several minutes, the conidial suspension was drained and the culture replaced lid-down at 25°C. After a few hours excess water was removed from the lid and the cross replaced, lid uppermost, at 25°C in a circulating air incubator. Care was taken that water did not form a seal between the lid and base of the Petri dish, since this was found to have an adverse effect on the maturation of the cross.

As a routine measure, an *A* isolate was used as the female parent in all crosses, and the same mating-type was used for unfertilised mycelium.

2.1.4. Harvesting techniques.

Mycelium.

Mycelium was harvested by scraping off a cellulose membrane using a sterile microscope slide. 48 hour old mycelium was used routinely since this had not yet reached the plate edge and therefore had not yet begun to produce any protoperithecia.

Protoperithecia.

Protoperithecia were purified from 4-6 day old mycelium grown on cellulose membrane, by scraping gently across the surface of the culture with a sterile coverslip.

For preparation of mRNA from the protoperithecial stage of development, whole mycelium from a 4 day-old culture was harvested as above. The reasons for this choice of material are described later.

Perithecia.

Perithecia harvested in the same way as protoperithecia were easier to remove, although when older mycelium was present it proved to be relatively fragile and perithecia were contaminated with mycelial fragments which were removed at the time of harvesting.

For large-scale purification of perithecia, the female parent was grown on surgical grade muslin, placed over the surface of cornmeal agar without cellulose membrane. This appeared to give added strength to the underlying mycelium during harvesting of perithecia, which tended to sit above the surface of the muslin. Consequently, a preparation of perithecia was considerably purer, and harvesting was made much easier.

cellulose discs were prepared by washing twice in distilled water, then making a 'sandwich' of the discs between circles of filter paper (Whatman No.1) in Pyrex Petri dishes. This was then autoclaved at 15 lbs in^{-2} for 15-20 minutes and allowed to cool before opening.

Muslin discs were prepared by sandwiching individually between washed cellulose discs in Pyrex Petri dishes. Care was taken to ensure the muslin was kept dry until positioned on the cellulose, and that it was placed flat with no folds or wrinkles. The discs were sterilised as for the cellulose discs, above.

2.2. Assay of phenoloxidase activity.

Various methods were used for detection of phenoloxidases:

<u>Dihydroxyphenylaniline (DOPA);</u>

5 mM DOPA in 30 mM sodium phosphate pH6.5, was used to flood a culture of *Sordaria brevicollis* (method from Leonard and Phillips, 1973).

<u>4-amino-2,6-dibromophenol (ADBP);</u>

10 volumes of ADBP (ICN Biochemicals Ltd.; 4 mg ml^{-1} in 95% ethanol) were added to 0.1 volume of dimethylaniline (DMA; Aldrich). This mixture was diluted with 2 volumes of laccase assay buffer (37 mM citric acid, 126 mM Na$_2$HPO$_4$, pH6), and used to flood a plate culture. After 5 min, the solution was decanted and the plate was incubated at 25°C for up to 30 min.(method from Hermann et al., 1983.)

<u>3,3',5,5'-Tetra-methyl benzidine (TMB);</u>

A stock of TMB (Sigma; 0.1% in 90% ethanol), was freshly diluted in 0.1 M sodium phosphate pH5. A plate culture of *Sordaria brevicollis* was flooded with the working strength TMB solution, and decanted after 5 min. The culture was observed for appearance of a blue colour, indicating oxidation of the TMB, and therefore presence of phenoloxidase (method from O.M.H. deVries, personal communication).

A colorimetric assay could be performed by adding the reagent to a homogenate of *Sordaria brevicollis* cells. Protoperithecia from half a 6 day old mycelium were homogenised in 1 ml of 0.1 M sodium phosphate pH5. 0.2 ml of this was added to 1 ml of the diluted reagent, mixed, and absorbance at 650nm was monitored.

2.3. Preparation and analysis of proteins.

2.3.1. Labelling of proteins *in vivo* with ^{35}S-methionine.

Preliminary experiments were carried out in order to determine the optimum conditions for labelling *Sordaria brevicollis* proteins *in vivo*. These are described in the results (section 3.3.2).

A small amount of material (*e.g.* 25 mm^2 of 2 day-old mycelium, or perithecia from about 4-5 cm^2 of a cross, or protoperithecia from about 5-10 cm^2 of mycelium) was placed at the bottom of a 10 mm x 75 mm test tube. To this was added 20 µCi of ^{35}S-methionine (Amersham) in 10 µl dH$_2$O. The tube was incubated at 25°C for 1-2 hours, then 50 - 100 µl of sample buffer was added to stop the reaction.

2.3.2. Preparation of soluble protein extract.

The material in sample buffer was homogenised for 5 - 10 seconds at full speed using a Tri-R homogeniser with an 8 mm pestle, and the extract was cleared by centrifugation (1 min in microfuge). Up to 50 µl was used for polyacrylamide gel electrophoresis.

2.3.3. Estimation of protein concentration.

Soluble protein extracts were assayed for protein content by the method of Lowry *et al* (1951). Accurate quantification of the protein concentration of samples in IEF sample buffer was made impossible by the presence of ampholytes, so an optimal volume for loading onto gels was determined by a number of pilot experiments.

2.3.4. Polyacrylamide gel electrophoresis (PAGE) of proteins.

Proteins were separated on the basis of molecular weight alone using SDS-PAGE, or by a combination of Iso-electric focussing (IEF) and SDS-PAGE. This latter method, called Two-dimensional electrophoresis, or 2D-PAGE, enables proteins to be separated according to the nett charge on the molecule

as well as the molecular weight. It has the advantage that a more extensive analysis can be made on a complex mixture of proteins. The methods used are based on those of O'Farrell (1975), with some modifications.

Iso-electric focussing (IEF).

For early experiments, the method used was that of O'Farrell (1975). Gel dimensions were 5 mm x 12.5 cm, ampholines used were 'Ampholytes' pH 3.5 - 10 and pH 5 -7 (LKB), in the concentrations used by O'Farrell (1975). For the later experiments, a modification of O'Farrell's was used: The initiators used for polymerisation were, per 5 ml of gel mixture, 25 µl of 0.1% riboflavin, 7.5 µl 10% ammonium persulphate (APS), and 2.5 µl N,N,N',N'-tetramethyl ethylene diamine (TEMED). Gels of 3 mm x 13 cm were cast and overlaid with 8 M urea, then left to polymerise overnight. Before loading, these gels were pre-focussed according to O'Farrells procedure. The catholyte used was 0.1 N NaOH, and the anolyte was 0.06% H_3PO_4.

Running conditions for all IEF was 400 V constant voltage for 16 hours, then 500 V for 2 hours.

SDS-PAGE;

The method used for SDS-PAGE was that of O'Farrell (1975), using the discontinuous buffer system of Laemmli (1970). 10% acrylamide gels (mixture of 9.73% acrylamide, 0.27% N',N'-methylene bis-acrylamide), 1.5 mm thick were used throughout. For non-2D gels, wells were formed in the stacking gel layer, while for 2D gels, no well former was used, and gel rods were attached to the top surface of the stacking gel with 1% agarose in equilibration buffer.

Running conditions in early experiments (*in vivo* labelling) were overnight at 15 mA constant current per gel. In later experiments, 25 mA per gel was used until the marker dye reached the separating gel, then 35 mA was used until it reached the bottom edge of the gel (total time about 4.5 hours), with water being circulated to cool the gel plates.

2.3.5. Detection of proteins in polyacrylamide gels.

Staining with coomassie blue.

Gels were shaken in at least 500 ml of staining solution – 45% ethanol, 10% acetic acid, 0.2% coomassie brilliant blue (Sigma) – overnight at room temperature. Gels were destained by gentle shaking in 45% ethanol, 10% acetic acid for 2 hours, then overnight in 10% acetic acid.

The addition of pieces of foam rubber to the second destaining solution increased the efficiency of destaining.

Silver staining.

The method of Sammons *et al.* (1981) was used for silver staining. After staining, gels were stored in clear polythene bags containing 5 ml of 7.5 g l^{-1} sodium carbonate for convenience.

The silver stain was reduced where necessary by using Farmer's reagent; 1 part of solution A (37.5 g potassium ferricyanate in 500 ml dH_2O) and 4 parts of solution B (480 g sodium thiosulphate in 2 litres dH_2O) were added to 27 parts dH_2O. Gels were shaken in this solution until the colouration was reduced to the required level, then the reaction was stopped by immersion in 7.5 g l^{-1} sodium carbonate.

This method of reduction could not be used for quantitative analysis of patterns in gels, since it is impossible to control in a repeatable manner, as was observed also by Poehling and Neuhoff (1981).

Fluorography.

The methods of Bonner and Laskey (1974) and Laskey and Mills (1975) were used. After staining with coomassie blue, gels were soaked in 500 ml of dimethyl sulphoxide (DMSO) for a total of one hour, with one change, then in 100 ml of 25% 2,5-diphenyl oxazole (PPO) for 3 hours. Finally the gels were washed with several changes of dH_2O overnight.

Gels were dried under vacuum prior to fluorography. In early experiments, (*in vivo* labelling), Kodak Ortho-G X-ray film was pre-flashed before exposure at -70°C. For later experiments (*in vitro* translations), Fuji RX-100 film was not pre-flashed; exposure was also at -70°C.

2.4. *Escherichia coli.*

Plate stocks of *E. coli* were made by streaking out single cells onto a suitable agar medium, and growing overnight at 37°C, before storage at 4°C.

E. coli strains WL87 and WL95 were used as hosts for bacteriophage λ. WL95 is essentially the same as WL87 except that it has a P2 prophage integrated into the genome. Only λ phage lacking the *red, gam* and *delta* functions – those that have lost the 'stuffer' fragment by replacement with a foreign DNA insert – are capable of normal growth in the P2 lysogen, WL95. Thus, such recombinant phage can be selected on the basis of their Spi⁻ phenotype (Zissler *et al*. 1971; Loenen and Brammar, 1980).

Bacteriophage plating cells were prepared by inoculating 100 µl of an overnight culture of cells into 100 ml of T broth supplemented with 0.2% maltose, and growing at 37°C with shaking for 6 hours. The cells were then recovered by centrifugation (5,000 x g for 10 min), and resuspension in 5 ml of 10 mM $MgSO_4$. Maltose added to the T broth induces the *lamB* gene of *E. coli*, encoding the λ receptor within the maltose operon (Thirion and Hofnung, 1972).

Transformation-competent cells were prepared using *E. coli* strain DIH101, a derivative of HB101. 5 ml of an overnight culture of cells were used to inoculate 100 ml of L broth and grown, with shaking, at 37°C to an A_{600} of 0.5 (approx. 5×10^7 cells per ml). The cells were chilled on ice for 10 min before centrifugation (4,000 x g, 5 min, 4°C). The pellet was resuspended in 0.5 volumes of ice-cold 50 mM $CaCl_2$, 10 mM Tris-Cl pH 8, incubated on ice for 15 min, then centrifuged as before. The pellet was resuspended in 7 ml of 50 mM $CaCl_2$, 10 mM Tris-Cl pH8, and 200 µl aliquots were stored at 4°C for 12 – 24 hours.

To obtain transformants, up to 40 ng of plasmid DNA in TE was added to an aliquot of cells, mixed and incubated on ice for 30 min. After a 2 min heat

shock at 42°C, the cells were cooled on ice and added to 1 ml of L broth containing an appropriate antibiotic. This was then incubated at 37°C for 30 min (tetracycline) or 1 hour (ampicillin) for the antibiotic resistance to take effect, before spreading onto a plate of L agar containing the antibiotic.

2.4.1. Plasmids.

pMF2, containing the entire genes for the 17S, 5.8S and 25S ribosomal RNAs of *Neurospora crassa*, inserted into the *Pst*I site of pBR322 (amp^S tet^R), were a gift from Dr. R.L. Metzenberg.

A set of plasmids; pSC-1, 2, 4, 5, 7, 9 and 14 – a gift from Prof. J.G.H. Wessels – contained cDNA copies of transcripts that are specifically expressed in fruiting cultures of *Schizophyllum commune*. The cDNAs had been cloned into the *Pst*I site of pGEM-1, therefore the plasmid conferred resistance of transformed cells to ampicillin. Transformed cells of *E. coli* strain JM83 were obtained containing the plasmids, and grown in L broth containing 20 µg ml^{-1} ampicillin.

2.5. Bacteriophage λ.

The vector chosen for construction of a genomic DNA library was λEMBL3 (Frischauf et al., 1983). EMBL3 has the typically large capacity for inserted DNA fragments (9 - 22 kb), and the added benefits of a 'polylinker' providing a number of restriction sites for the insertion and excision of the isolated DNA fragment. Another feature of EMBL3 is the Spi⁻ phenotype of phage that have lost the 'stuffer' fragment in accepting the insert. Thus, recombinant phage can be selected on the basis of their ability to grow in cells lysogenic for the P2 prophage. Plate stocks of bacteriophage λ were made by first allowing 100 - 500 phage to adsorb to 0.1 ml of WL87 plating cells. After incubation at 37°C for 15 min, 3 ml of molten L top agar at 45°C was added to the cells and mixed before pouring over the surface of a plate of L agar. The plate was incubated for 8 - 16 hours at 37°C before storage at 4°C.

Small scale lysates of bacteriophage λ were made by inoculating 10 ml of L broth + Mg with 0.1 ml of plating cells and infecting with a single plaque. With vigorous shaking at 37°C, lysis was typically complete within 6 - 8 hours,

producing 10^{10} plaque-forming units (pfu) per ml. 100 µl of chloroform was added and the lysate was centrifuged (10,000 rpm, 10 min, Sorvall SS-34 rotor), before storage at 4°C.

2.6. Preparation of Ribonucleic acid (RNA).

2.6.1. Preparation of total RNA.

Several different methods for preparation of RNA from *Sordaria brevicollis* were used in this project. Initially the method of Thomas (1983) was tried. This method required material to be freeze-dried before re-suspension in extraction buffer, then several extractions with a phenol-chloroform. While good results were obtained with RNA from mycelium, the freeze-drying of perithecia proved to be somewhat unreliable, and perhaps this was the reason for the low yields of RNA for the effort involved in its preparation.

A second method of RNA preparation (Chirgwin *et al*, 1979), involves the use of guanidinium thiocyanate to denature the protein material. The method was originally designed for mammalian cells, but it was found to be unsuitable for fungal material, at least in the large quantities required for this work; certainly for perithecia, the yield of RNA obtained made the method far too time-consuming and expensive.

The method of choice was a modification of that of Chambers and Russo (1986). This method allowed immediate extraction, without the prior need for freeze-drying, and mycelium from about 15 plates, or perithecia from about 150 plates, required only enough extraction buffer to fill eight 30 ml Corex centrifuge tubes (the capacity of a Sorvall SS-34 rotor). Another advantage of this method is the reduced need for repeated extractions with phenol-chloroform compared with that of the first method, in which they made the preparation a laborious and time-consuming task.

2 day-old mycelia from 15 - 20 plates, or 4 day-old 'protoperithecial' mycelia from 20 plates, or 3 day post-fertilisation perithecia from 200 plates were harvested, dried if necessary, and frozen in liquid nitrogen. The material was ground in a pre-cooled mortar and pestle to a fine powder, then suspended in 240 ml of 6 M urea, 3 M LiCl. This suspension was blended for 60 seconds in an 'Ato-mix' blender, and then centrifuged at 12,000 rpm in a

Sorvall SS-34 rotor for 30 min at 4°C. The supernatant was decanted into siliconised Corex centrifuge tubes and stored at -20°C, overnight. This was centrifuged as before, and the supernatant discarded. The pellet was resuspended in 10 ml of 0.5% SDS, 10 mM Tris-Cl pH7.5, 1 mM EDTA, extracted with phenol/chloroform until no interface was seen, then with chloroform until no interface remained. The aqueous phase was precipitated with NH$_4$OAc to 2.5 M and 2 volumes of ethanol, for 2 hours at -70°C. The RNA was recovered by centrifugation as above, washed twice with 70% ethanol, and stored in this until required.

2.6.2. Purification of messenger RNA (mRNA).

Two methods for purification of polyadenylated (messenger) RNA (mRNA) were used in this work. Initially, a column of cellulose-bound oligodeoxythymidylate (oligo-d(T) cellulose; BRL) was used according to the method of Thomas (1983). The method of choice is the use of 'Hybond' mAP (messenger activated paper) from Amersham. The method is derived from Wreschner and Herzberg (1984), and the protocol is that provided by Amersham for the product.

1 cm^2 of mAP was moistened with 20 μl of 0.5 M NaCl, then air-dried on Whatman No.1 filter paper. Meanwhile, the RNA in 20 μl of dH$_2$O was heated to 65°C for 5 min, chilled on ice, and made to 0.5 M NaCl. The RNA was spotted onto the mAP and left to be absorbed for 10-15 min, before the mAP was transferred to filter paper to air-dry for 2 min. The mAP was then washed in 5 ml of 0.5 M NaCl for 5 min, twice, then washed in 70% ethanol for 2 min, twice, and finally air-dried on filter paper.

To resuspend the mRNA, the mAP was heated to 70°C for 5 min in dH$_2$O, vortexed briefly and removed from the RNA solution. mRNA was purified twice in this way; after resuspension in dH$_2$O it was precipitated with 0.2 M NaOAc and 2 volumes of ethanol at -70°C for 2 hours, then resuspended in 20 μl of dH$_2$O and the purification was repeated.

2.6.3. *In vitro* translation of messenger RNA.

In vitro translation of messenger RNA was performed using a rabbit reticulocyte lysate (Pelham and Jackson, 1976). A complete lysate, supplemented with amino-acids, was obtained from Amersham (code N90). A preliminary experiment was performed in order to establish the optimum amount of mRNA to be added to the lysate for the highest level of incorporation of radioactive methionine. 8 μl of lysate was incubated with up to 4 μg of RNA in 1 - 2 μl and 2 μl of ^{35}S-methionine for one hour at 30°C.

Incorporation of label was estimated by adding 1 μl of lysate to 15 μl of 100 vol. hydrogen peroxide in a siliconised glass tube, followed by 0.25ml of 1 M NaOH. After incubation on ice for 30 min., protein was precipitated by the addition of 0.5ml of 25% TCA, and keeping on ice for a further 30 min., before recovery on Whatman GFC filters. The filters were washed with several changes of 10% TCA, then air dried. A drop of 1 M NaOH was added to neutralise the TCA, before scintillation counting in approximately . 1 ml of toluene containing 0.3% PPO and 0.03% POPOP.

Results of the preliminary experiment are shown in figure 5. For the final experiments, 1 μg of mRNA was included in the reaction, which was stopped after 1 hour by the addition of either 11 μl of SDS sample buffer or 40 μl of IEF sample buffer.

2.7. Preparation of DNA.

2.7.1. Preparation of *Sordaria brevicollis* genomic DNA.

2 day-old mycelium from two plates was ground in a mortar and pestle in 5 ml of 10 mM Tris-Cl pH7.5, 100 mM NaCl, 10 mM EDTA. To this was added an equal volume of 200 mM Tris-Cl pH9, 30 mM EDTA, 2% SDS, and proteinase K to 0.25 mg ml^{-1}. The suspension was incubated at 37°C for 1 hour, then centrifuged at 6500 rpm for 15 min in a Sorvall SS-34 rotor. The supernatant was extracted twice with phenol, once with phenol/chloroform, and once with chloroform. The aqueous phase was precipitated with 0.7 M NH$_4$OAc and 2 volumes of ethanol, centrifuged for 10 min at 10,000 rpm. The

Figure 5 ·

In vitro Translation of mRNA:

Effect of amount of added mRNA

on incorporation of ^{35}S-methionine

into the acid-soluble fraction.

2 day mycelium ×————×
3 day perithecia o------o

mRNA added (µg)
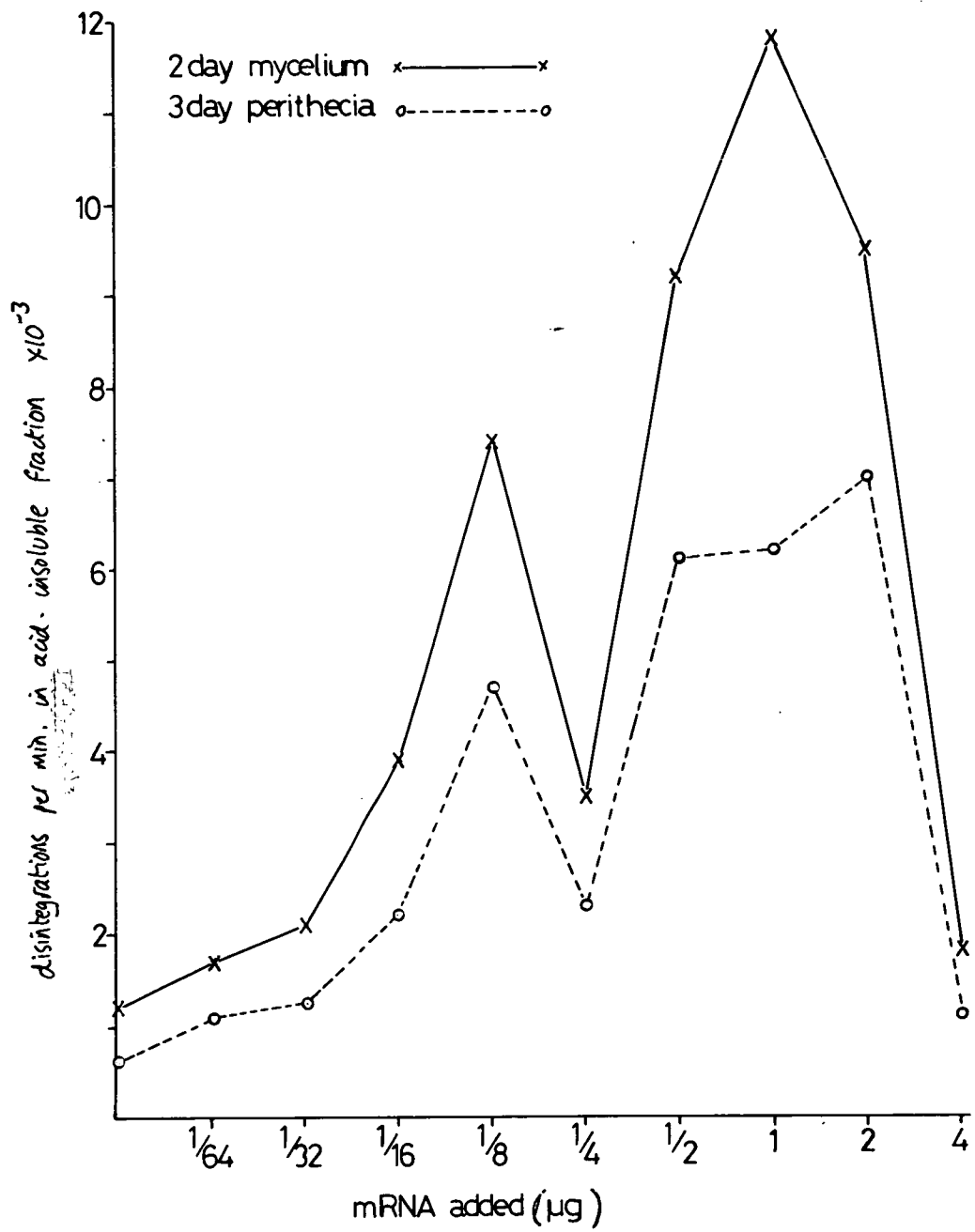
pellet was washed with 70% ethanol, resuspended in TE and re-precipitated. The second pellet was dried well, suspended in TE, then aliquoted and stored at 4°C.

2.7.2. Preparation of bacteriophage λ DNA.

Mini-preparation of λ DNA.

A single plaque was suspended in 200 µl of PSB, and 100 µl of this was added to 10 ml of L+Mg containing 100 µl of plating cells. After shaking at 37°C until lysis occured, 100 µl of chloroform was added and the lysate was centrifuged (10,000 rpm, 10 min) before storage at 4°C.

λ DNA was prepared from the lysate by adding to 0.8 ml in an eppendorf tube, DNase to 10 µg ml^{-1}. After incubation at room temperature for 15 min, 200 µl of 0.3 M Tris-Cl pH9, 0.15 M EDTA pH7.5, 1.5% SDS was added, mixed by rocking, and heated to 70°C for 15 min., then quenched on ice. 135 µl of 8 M potassium acetate was added, mixed by rocking, and the lysate was placed on ice for 15 min. before centrifugation in a microfuge for 1 min. To 800 µl of the supernatant was added 480 µl of *iso*-propyl alcohol; this was mixed for 2 min. at room temperature, then centrifuged for 1 min. The pellet was washed in 70% ethanol, drained and dried, then resuspended in 50 µl of TE. 2-5 µl was used for a restriction digest, in which RNAse at 10 mg ml^{-1} was included.

Large scale preparation of bacteriophage λ DNA

5 ml of an overnight culture of host cells was added to 500 ml of L+Mg, prewarmed to 37°C, and grown at 37°C until the density reached an A$_{600}$ of 0.1 - 0.2, when the cells were infected with λ at a m.o.i. of 0.1. After adsorption was allowed for 15 min., vigorous shaking was resumed until lysis occured.

Purification of bacteriophage λ was by the method of Maniatis *et al.* (1982), after Yamamoto *et al.* (1970). λ DNA was prepared as follows:

The CsCl$_2$ solution containing the λ particles was dialysed against 2 litres of TMN for 2 hours at 4°C, then overnight at 4°C against 2 litres of 10 mM Tris-Cl pH 8, 10 mM MgCl$_2$, 5 mM NaCl, 1 mM EDTA. The suspension was heated to 70°C for 15 min., allowed to cool to room temperature, then potassium acetate was added to 1 M. After 30 min. on ice, the suspension was centrifuged at 10,000 rpm for 10 min. and to the supernatant was added 2 volumes of ethanol. DNA was recovered by centrifugation (10,000 rpm, 10 min.), washed in 70% ethanol and dried. λ DNA was resuspended in TE at 50 μg ml^{-1}, and aliquoted for storage at 4°C.

2.7.3. Preparation of plasmid DNA.

Minipreparations of plasmid DNA.

10 ml of L-broth containing the appropriate antibiotic was inoculated with a single bacterial colony, and incubated overnight at 37°C with vigorous shaking. 1.5 ml of this was then centrifuged to recover the cells, which were then resuspended in 200 μl of STET with 1 mg ml^{-1} lysozyme, and vortexed well.After boiling for 40 sec., cell debris was removed by centrifuging for 10 min. and discarding the gelatinous pellet. The supernatant was extracted once with phenol/chloroform, then precipitated with 25 μl of 7 M NH$_4$OAc and 500 ml of ethanol, for 30 min at -70°C. DNA was recovered by centrifugation for 10 min and the pellet was washed with 70% ethanol, dried and resuspended in 30 μl TE. 3 - 5 μl was used for a restriction digest.

Maxi-preparation of plasmid DNA.

2 x 500 ml of L-broth + antibiotic were each inoculated with a 10 ml overnight culture of cells, and grown overnight at 37°C, with shaking. Cells were recovered by centrifugation in a Sorvall GSA rotor at 7,000 rpm for 5 min, the pellet drained well, and resuspended in a total of 40 ml of 50 mM glucose, 25 mM Tris-Cl pH8, 10 mM EDTA, and 4 mg ml^{-1} lysozyme. This was incubated for 10 min at room temperature, then 80 ml of freshly-made 0.2 M NaOH, 1% SDS was added, mixed, and left on ice for 5 min. 80 ml of ice-cold

5 M KOAc pH 4.8 was quickly added, and the mixture centrifuged (GSA rotor, 8,000 rpm, 5 min). The supernatant was poured through a tea strainer into a measuring cylinder, then the DNA was recovered by precipitation with 0.6 volumes of *iso*-propyl alcohol and centrifugation at 8,000 rpm for 5 min. The dried pellet was resuspended in 5 ml of TE, and 5.5 g of CsCl was added and dissolved. 0.8 ml of 10 mg ml^{-1} ethidium bromide was added, mixed, and the plasmid DNA was purified by centrifugation in a Beckman Type 40 rotor at 36,000 rpm for 40 hours.

Supercoiled plasmid DNA in the lower band in the tube was collected using a siliconised pasteur pipette, and ethidium bromide was removed by chromatography through Dowex AG50X8 resin.

2.8. DNA manipulations.

2.8.1. Restriction endonucleases.

Procedures for restriction digests of DNA were exactly as described in Maniatis *et al.*, (1982), with the exception of *Sau*3AI, for which the incubation buffer used was that recommended by the makers, Boehringer Mannheim: 6 mM Tris-Cl pH 7.5, 50 mM NaCl, 6 mM MgCl2.

Generally a 3 – 5 fold excess of enzyme was used to ensure complete digestion, where required.

2.8.2. Calf-intestinal phosphatase (CIP).

Calf-intestinal phosphatase (Boehringer Mannheim) was used according to the supplier's recommendations; incubation buffer was 50 mM Tris-Cl pH 8, 0.1 mM EDTA. The reaction was incubated for 1 hour at 37°C, and stopped by addition of EGTA to 10 mM, and heating to 65°C for 45 min. The DNA was extracted with phenol, then phenol/chloroform, and precipitated with ammonium acetate to 2M and two volumes of ethanol.

2.8.3. T4 DNA Ligase.

Conditions for ligation of DNA were as recommended by the supplier, Boehringer Mannheim. Ligation buffer was 20 mM Tris-Cl pH 7.6, 10 mM MgCl2, 10 mM DTE, 0.6 mM ATP. DNA was pre-annealed by incubation at 42°C for 1 hour, then ligase was added and the reaction was incubated at 14°C overnight.

2.8.4. *In vitro* packaging of λ DNA.

Preparation of *in vitro* packaging extracts was carried out as described in Maniatis *et al.* (1982), from Scalenghe *et al.* (1981).

2.9. Analysis of nucleic acids.

2.9.1. Agarose gel electrophoresis of DNA.

Agarose gels were made using Type I (low EEO) agarose (Sigma), in Tris-borate buffer (as in Maniatis *et al.* (1982). For rapid analysis of DNA size and integrity, a 'minigel' apparatus was used – this employed gels 11 x 13cm and running conditions were typically around 200 V for 0.5 – 2 hours. For more crucial analysis, larger gels of 20 x 13.5cm were used – these were generally run overnight at 30 – 35 V. Sample buffer was Type II (Maniatis *et al*, 1982), using bromophenol blue as tracking dye. Samples were heated to 65°C for 5 min before loading.

For mini-gels, DNA was visualised by incorporating ethidium bromide 0.5 µg ml^{-1} into the gel mixture, for rapid analysis. Larger gels were immersed in 0.5 µg ml^{-1} ethidium bromide for 30 min, then destained in TBE until DNA bands were easily seen.

2.9.2. Formaldehyde-denaturing gels for electrophoresis of RNA.

Agarose gels containing formaldehyde were prepared as in Maniatis *et al*, (1982), after Lehrach *et al* (1977) and Goldberg (1980). As with DNA

electrophoresis, rapid analysis of RNA could be made using a mini-gel apparatus. RNA was visualised by immersion of the gel in 10 mM sodium phosphate pH 7.0 containing 50 μg ml^{-1} acridine orange.

2.9.3. Recovery of DNA fragments from agarose gels.

Specific fragments of DNA were isolated initially by agarose gel electrophoresis, then by rotating the gel through 90°, a particular band could be recovered onto a small piece of DEAE-cellulose paper (Schleicher and Schuell) inserted adjacent to the band, by continuing electrophoresis in the second dimension. The paper was then rinsed several times in TE before incubation at 65°C for 30 min in 50 μl of 1 M NaCl in TE.

2.9.4. Southern transfer of DNA.

DNA from agarose gels was transferred to nitrocellulose (Schleicher and Schuell) or Hybond N (Amersham) filters by the method of Southern (1975) as modified by Wahl *et al.* (1979).

2.9.5. Radioactive labelling of nucleic acids.

Labelling of DNA by primer extension.

The method of primer extension (Feinberg and Vogelstein, 1984) was used to prepare radioactive DNA probes for filter hybridisation. Typically 50 ng of DNA was denatured by heating in dH$_2$O to 100°C for 90 sec., then ice. When plasmid DNA was used it was first linearised by cutting with a restriction enzyme. The DNA solution was brought to 0.1 mM dATP, 0.1 mM dGTP, 0.1 mM dTTP, 25 mM MgCl$_2$, 250 mM Tris-Cl pH 8, 50 mM BME, 1 M HEPES and 0.3 mM mixed hexadeoxyribonucleotides (Pharmacia). To this was added 1 unit of DNA polymerase (Klenow fragment), and 50 – 100 μCi of α-^{32}P dCTP. After incubation for 1 – 3 hours at room temperature, typically 70 – 90% of the radioactive nucleotide had been incorporated.

End-labelling of RNA with T4 polynucleotide kinase.

RNA at approximately 1 µg / 4 µl was subjected to mild alkaline hydrolysis in 50 mM Na_2CO_3 in degassed dH_2O at 55°C for 60 min. This provided an increased number of 5'-hydroxyl ends for labelling with the enzyme. The hydrolysis was stopped by addition of NaOAc pH5 to 0.2 M and 2 volumes of ethanol.

For labelling, up to 3 µg of RNA was suspended in 14.5 µl of kinase buffer I (10 mM Tris-Cl pH 7.5, 1 mM spermidine, 1 mM EDTA), heated to 60°C for 3 min and chilled on ice. To this was added 2.5 µl of kinase buffer 2 (50 mM Tris-Cl pH9, 100 mM $MgCl_2$, 50 mM DTT), 1 µl of 0.1 mM ATP, 5 µl (50 µCi) of γ-^{32}P ATP, and 7 units (2 µl) of T4 polynucleotide kinase (Amersham). The reaction was incubated at 37°C for 60 min, and stopped with the addition of 1 µl of 0.4 M EDTA pH7.5.

Preparation of labelled cDNA.

Complementary DNA (cDNA) was made using the Amersham cDNA synthesis kit, and the protocol supplied with it.

Estimation of nucleotide incorporation.

A fraction (usually 1 µl) of the nucleic acid solution was spotted onto a small (0.5 cm^2) piece of nitrocellulose paper. After air-drying, radioactive emission was estimated using Cerenkov counting (measurement in the ^{3}H channel of a scintillation counter). After two washes in 5% TCA, and a final rinse in ethanol, the filter was air-dried and counted again. Incorporation was estimated by acid-soluble counts divided by total counts.

Removal of unincorporated nucleotides.

Removal of unincorporated nucleotides from labelling preparations was by

the spun-column procedure (Maniatis *et al*, 1982) using a 1 ml column of Sephadex G-50 in a 1 ml syringe. The column was spun for 1.5 - 2.5 min at 3,000 rpm in a MSE bench centrifuge.

2.10. Genomic library.

A library of random 15 - 20 kb fragments of *Sordaria brevicollis* genomic DNA was constructed in a bacteriophage λ vector. The random genomic fragments were prepared by partial digestion with a 4-base cutting restriction enzyme, *Sau*3AI, and multiple insertion was prevented by phosphatase treatment.

The vector chosen was λEMBL3 (Frischauf et al, 1983) which has several features making it suitable for library construction. The capacity for inserted DNA is sufficiently large (23 kb) for most purposes. The 'stuffer' fragment of EMBL3 is easily removed by cutting with a single restriction enzyme, in this case *Bam*HI, and can be prevented from re-insertion by trimming the ends with a second restriction enzyme, *Eco*RI, for which there is a site just a few bases from each end. The short linker between the *Bam*HI and the *Eco*RI sites can be removed from the preparation by *iso*-propanol precipitation. The stuffer fragment is bounded at each end by a polylinker, which provides an array of combinations of sites to be used for insertion of foreign DNA and inactivation of the stuffer. In this case, a fragment inserted into the *Bam*HI sites can be cut out intact by *Sal*I, if required.

An additional feature of EMBL3 is that it incorporates a system by which recombinants can be selected on a genetic basis, by resistance to the inhibition caused by a P2 lysogen. The stuffer fragment of EMBL3 confers the phenotype Spi[+], that is, sensitivity to P2 inhibition, and therefore intact EMBL3 will not grow in a host lysogenic for P2. When the stuffer is replaced by a different piece of DNA, however, the phenotype becomes Spi[-] and thus only recombinants can grow in a P2 lysogen.

2.10.1. Preparation of EMBL3 arms.

EMBL3 DNA was digested to completion with a three-fold excess of *Bam* HI. Complete digestion was checked, first on an agarose gel, then by attempting *in vitro* packaging of the digested DNA. The digestion buffer was adjusted to that for *Eco* RI, a three-fold excess of this enzyme was added, and a fraction (containing 0.45 units of the enzyme) was added to 300 ng of intact EMBL3 DNA in the same buffer, to check for digestion by *Eco*RI. After the second digestion, the reaction was stopped by addition of EDTA to 15 mM, extracted with phenol/chloroform, and precipitated with *iso*-propanol. The DNA was washed with 70% ethanol, dried well and resuspended in TE at 0.5 mg ml^{-1}.

2.10.2. Preparation of *Sordaria brevicollis* genomic DNA fragments.

A pilot experiment was conducted, as in Maniatis et al (1982) to establish the digestion conditions required to obtain a maximal proportion of 15 - 20 kb fragments.

40 µg of *Sordaria brevicollis* DNA was mixed in a total of 400 µl *Sau*3AI buffer, on ice, with 3.5 units of the restriction enzyme. The mixture was incubated at 37°C, and aliquots of 130, 140, and 130 µl were removed after 5, 10, and 15 min respectively. After phenol/chloroform extraction and ethanol precipitation, the pooled DNA was resuspended in 180 ml dH$_2$O, then phosphatased in a total of 200 µl.

Phosphatase activity was checked by attempting to re-ligate the digested DNA, and analysis on a mini-gel. After phosphatase treatment, DNA was re-suspended in TE at 0.5 µg ml^{-1}.

2.10.3. Ligation and packaging.

Optimum ligation conditions were determined to obtain the maximum number of recombinant phage from insert DNA.

1.25 µg of *Sordaria brevicollis* DNA fragments was mixed with 5 µg of

EMBL3 arms and 2 units of T4 DNA ligase, in a total of 15 µl. This was prepared and incubated as described previously, and the ligated DNA was packaged in six parts. The entire contents of each packaging was plated with E. coli WL95 to enrich for recombinant phage.

2.10.4. Amplification of the library.

The genomic library was amplified by covering each of the plate lysates with 4 ml PSB, and shaking gently at room temperature for 1 hour. The phage suspension was transferred to a polypropylene centrifuge tube, made to 5% chloroform, and shaken gently for 15 min at room temperature. After centrifugation (SS-34 rotor, 6,000 rpm, 5 min at 4°C), the supernatant was transferred to a glass tube and made to 0.3% chloroform.

The titre of the amplified library was 10^7 pfu ml^{-1}, so 10 µl was sufficient to represent a full complement of the library.

2.10.5. Transfer of plaque DNA to filters for hybridisation.

Bacteriophage DNA was transferred from the plate lysates to Hybond N discs by the method of Maniatis et al (1982), with the exception that neutralisation of the denaturing solution was in 1 M Tris-Cl pH 7, 1.5 M NaCl, and the filters were rinsed in 2 x SSC.

2.11. Hybridisation of labelled nucleic acids to filter-bound DNA.

2.11.1. Nitrocellulose-bound DNA.

Filters were wetted with 2 x SSC, then washed for 30 min each at 65°C in 0.3% low-fat dried milk (LFDM; Sainsbury), 1 x SSC, then in 0.3% LFDM, 2 x SSC, 0.5% SDS, 50 µg ml^{-1} HSDNA, and finally in 0.3% LFDM, 2 x SSC, 0.5% SDS, 50 µg ml^{-1} HSDNA, 10% Dextran sulphate. The last two washes were performed in a heat-sealed polythene bag, and after the last wash the probe was added to the bag. Hybridisation was at 59°C, overnight.

After hybridisation, filters were washed for 30 min at 59°C in 2 x SSC, 0.5%

SDS, preheated to 59°C. This was repeated until the background radioactivity (non-specific binding of probe to filter) was at an undetectable level, as measured with a mini-monitor.

Filters were blotted dry between sheets of tissue paper and then placed in thin polythene bags prior to autoradiography at -70°C with intensifying screens. Film used was Kodak Ortho-G X-ray film.

2.11.2. Hybond N filters.

This method of hybridisation is adapted from that of Church and Gilbert (1984). Filters were wetted with dH$_2$O, then placed in polythene bags with 0.5 M sodium phosphate pH 7.2, 7% SDS for 30 min at 65°C. The wash was then replaced with 0.5 M sodium phosphate pH 7.2, 7% SDS, 1 mM EDTA and the denatured probe was added. After hybridisation overnight at 59°C, filters were washed at room temperature with 40 mM sodium phosphate pH 7.2, 1% SDS, preheated to 59°C, with changes until the background radioactivity was at an undetectable level.

After washing, filters were prepared for autoradiography as with nitrocellulose.

Hybond N filters could be re-hybridised to a second probe; first the filter was stripped of existing probe DNA by washing in 0.4 M NaOH for about 30 min. at room temperature, then neutralised in 1 M Tris-Cl pH 7, 1.5 M NaCl, and finally rinsed in 2 x SSC. If necessary, the filters were stored between sheets of tissue paper at this stage.

CHAPTER 3
RESULTS

3.1. Sexual development of *Sordaria brevicollis*

Cornmeal agar

Cornmeal agar has been used traditionally for *Sordaria brevicollis* since it allows luxuriant growth and excellent crossing. Vegetative growth is rapid, with aerial hyphae appearing within two days. On reaching the walls of the Petri dish there is a marked edge effect with abundant protoperithecia appearing with an even distribution all over the surface of the medium.

The advantages that cornmeal agar has to offer are easily seen when compared with Vogel's (1956) N medium, a defined medium originally designed for growth of *Neurospora crassa*, but frequently used for slant cultures of *S. brevicollis*. Growth of *S. brevicollis* on Vogel's is rapid and luxuriant, but the hyphal density is very high, so observation of protoperithecium formation is not easy because of the thick mycelial mat. On CMA, however, the hyphal density is sufficiently low that protoperithecium formation can be monitored with ease, with little interference from the mycelial cover.

Fertilisation of *S. brevicollis* on Vogel's medium also presents problems – the high hyphal density appears to cause the mycelium to be rather absorbent, so a cross fertilised by a microconidial suspension is not easily drained. However, the mycelial lawn on CMA is relatively non-wettable, and this overcomes problems associated with water-logging of the culture.

An interesting feature of *S. brevicollis* grown on Vogel's medium is that when inoculated onto large (140 mm) Petri dishes, there are protoperithecium-like bodies formed near the centre of the colony before the edge of the plate has been reached. The pattern of appearance of these structures is such that they form in a wave from the centre outwards, as the colony grows radially (Bond, unpublished). More recent observations have shown that they do not form if the colony is grown in the dark, and only a brief exposure to light is necessary for their appearance.

A recent observation on *S. brevicollis* grown on CMA is that when grown in continuous light, there appear structures similar to the hyphal aggregates seen on Vogel's medium, described earlier. They are considerably larger than protoperithecia, and they have an even distribution across the culture surface. The function of these structures is not known, but the possibility that they are similar in function to sclerotia has not been ruled out.

Under the conditions used routinely in this project, that is, with intermittent exposure to light, none of the sclerotium-like structures described above were observed. Therefore it is assumed that the major morphogenetic pathway analysed in this work is the sexual morphogenesis involving the initiation of protoperithecia and culminating with the maturation of perithecia.

Analysis of sexual development.

The use of cornmeal agar for growth and crossing of *Sordaria brevicollis* allows easy monitoring of the sexual morphogenesis, and a marked edge effect with the production of protoperithecia in abundance makes it a convenient system for the study of sexual morphogenesis. In addition to this, practical techniques have been developed which allow efficient harvesting of the various stages in the developmental process. The use of a cellulose membrane placed over the surface of the agar allows rapid and efficient harvesting of mycelium, without affecting either the growth rate or the pattern of sexual morphogenesis. Thus, mycelium can be easily obtained which is free of sexual structures, and free also of the nutrient agar.

Protoperithecia normally form only after the mycelial front has reached the wall of the Petri dish, and then their formation is well synchronised, with an even distribution across the entire colony surface. When *S. brevicollis* is grown on a cellulose membrane, the protoperithecia are situated above the surface of the mycelium, and thus they can be harvested with relative ease by scraping across the surface with a coverslip. This harvesting method is efficient, but the yield of protoperithecia is rather small, and hundreds of plates are needed to obtain microgram quantities of messenger RNA, for example. Therefore, for mRNA preparation from the protoperithecial stage, purification of protoperithecia was abandoned, and 4 day-old mycelium, which is actively producing protoperithecia, was used instead.

While perithecia could be harvested with high efficiency using the same technique as that for protoperithecia, this proved to be a rather laborious task when large numbers of plates were involved. Also, the perithecia were often contaminated with fragments of mycelium. Efficiency is not maximal since many perithecia must be left in the culture, otherwise pieces of mycelium are torn away with them. This was more evident on harvesting of perithecia from older cultures; it appeared that the mycelium becomes relatively fragile with age, and is possibly due to its degradation. The cellulose membrane also became more fragile in older cultures, this could be due to the degradation of the cellulose by the fungus.

A more rapid method was developed in which a circle of muslin was placed directly onto the agar surface before inoculation, instead of the cellulose membrane. This preparation allowed the hyphae to grow into the agar, and therefore the mycelium did not disintegrate as it could with the cellophane disc. The fibres of the muslin appeared to give the mycelium and agar extra strength during harvesting, presumably because the mycelium had ramified throughout the muslin by this time. In addition, the perithecia were situated mainly above the surface of the muslin, and therefore they could be harvested with very high efficiency, partly aided by the rigidity of the underlying material.

3.2. Analysis of Phenoloxidase Activity

As reviewed in the Introduction (section 1.5.3), it has long been established that there may be a connection between phenoloxidase activity and sexual morphogenesis in many fungi. Phenoloxidase activity was investigated in *Sordaria brevicollis*, initially to see how it might be related to that in other fungi. An initial experiment was carried out using the method of Hermann *et al.* (1983), in which the reagent ADBP-DMA was used as described in Materials and Methods. *In situ* staining of phenoloxidases was easily visualised by lifting a section of the culture, on its underlying cellophane membrane, onto a microscope slide before adding a phenoloxidase indicator.

No phenoloxidase activity was detected in undifferentiated mycelia of *S. brevicollis*. When mycelium of 4 days after inoculation, that had initiated protoperithecium formation, was flooded with the ADBP-DMA reagent, light blue foci appeared which corresponded with the positions of the

protoperithecial initials, as seen in figure 6(a). On further incubation in the presence of the reagent, these foci appeared darker and more distinct from the background, and dark blue crystals, possibly of the oxidised ADBP, appeared to accumulate within the protoperithecial hyphae. There did not appear to be any localisation of the colouration to any specific part of the protoperithecia, although it was restricted to the part bounded by the peripheral hyphae, and did not 'escape' to any of the surrounding mycelium.

Wild-type perithecia were not tested for their phenoloxidase activity, on the assumption that the pigmentation of the perithecial wall would mask the effect of the stain. Therefore a cross was made using a mutant, *hyaline* (*hya*), which has the phenotype of colourless protoperithecia, perithecia and ascospores. The colourless perithecium is dependent on the genotype of the female parent, while spore colour is an autonomous character, and is determined by the allelic status of the *hya* locus of that particular spore.

A cross was made of *hya A* x *hya a* (*hya A* as the female parent) and perithecia from this cross were examined for phenoloxidase activity, one and three days after fertilisation. Photomicrographs are shown in fig. 6(c-d).

It is quite obvious that most of the phenoloxidase activity is located to the neck of the perithecium, and more specifically to the upper part of the neck (note the intense staining), in the region where neck periphyses would be undergoing the transition into the peridial hyphae, according to the model by Read and Beckett (1985). This observation, while not entirely conclusive, does hold implications for the role of phenoloxidases in the cohesion and aggregation of hyphae in the formation of the perithecial neck.

When a similar experiment was carried out using *hyaline* perithecia one day after fertilisation, the appearance of the stained preparation was similar to that of the unfertilised protoperithecia. There was a general staining of the whole fruitbody, and no localisation to any specific part of the perithecia was observed. However, it should be noted that these perithecia showed no sign of neck initials at this stage.

The dark blue crystals, mentioned earlier, which appeared in the protoperithecia some time after staining, also appeared in the perithecia. There was no obvious pattern to their localisation, and it may be that the

Figure 6

Phenoloxidase Activity in *Sordaria brevicollis*.

Stained using the ADBP-DMA reagent of Hermann *et al.* (1984).

a.   Wild type culture, grown in continuous dark

for four days (protoperithecium). Mag. x84

b.   Wild type culture, grown in continuous light

for 3 days (sclerotium). Mag. x270

c.   Hyaline x Hyaline cross; one day after

fertilisation. Mag. x336

d.   Hyaline x Hyaline cross; three days after

fertilisation. Mag. x210
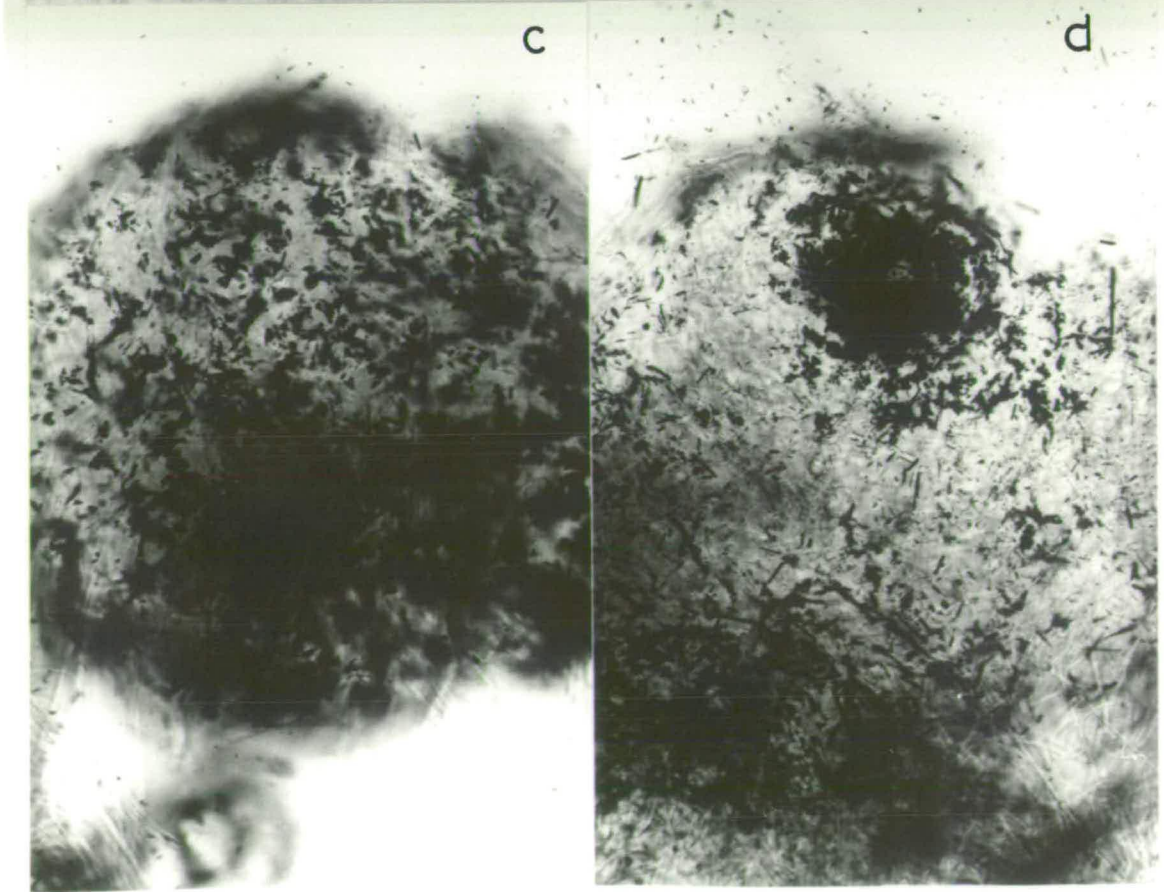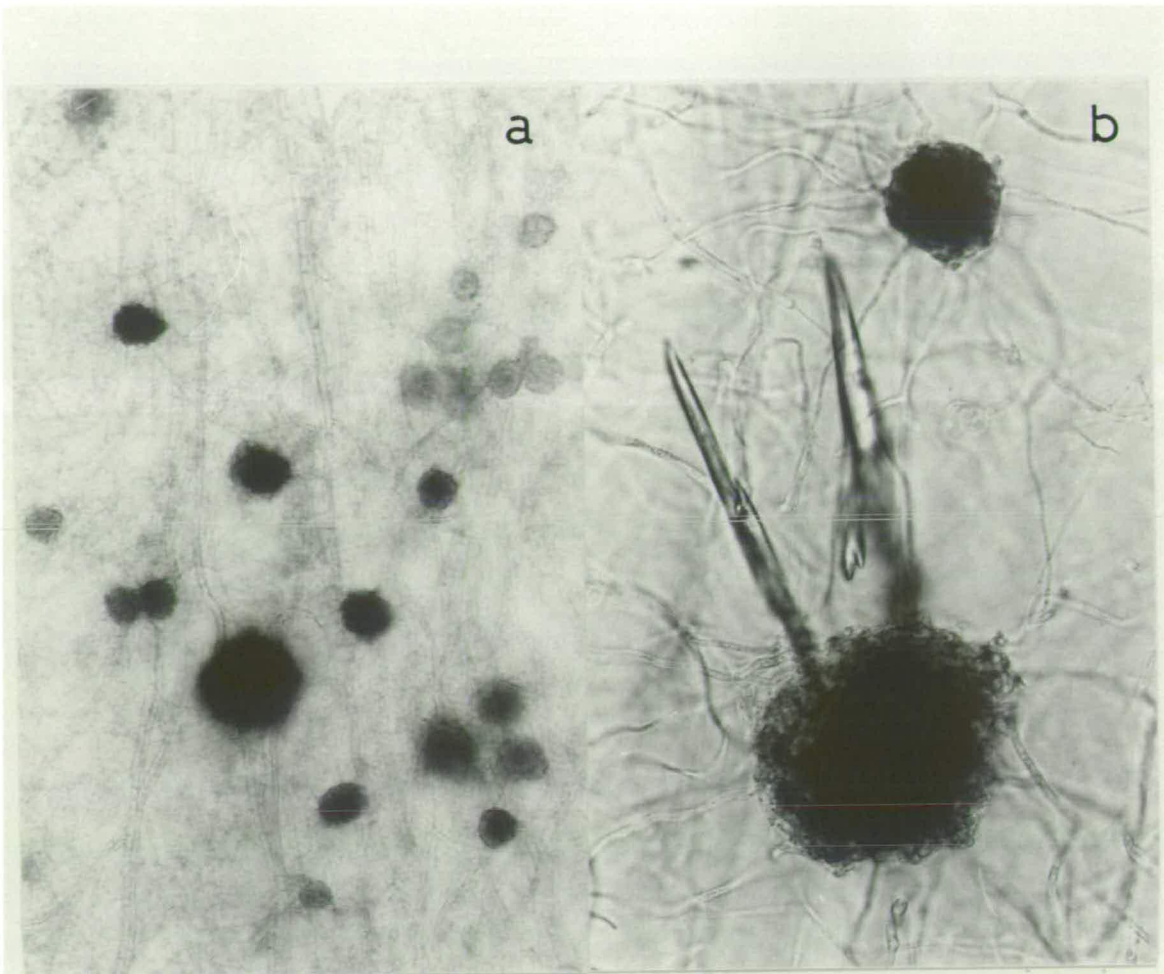
crystals are merely products of the oxidation pathway or due to the evaporation of the solvent.

A further experiment was carried out, using a wild-type culture that had been grown in continuous light for 4 days. The culture had been grown at 29°C instead of the 25°C used routinely for maintenance of *S. brevicollis*. The sclerotium-like bodies, described previously (page 63), that appear in continuous light conditions, were present, as were protoperithecia (formed after the mycelium had reached the plate edge). On application of the ADBP-DMA reagent, there was staining of these 'sclerotia' to about the same intensity as the protoperithecia, as seen in figure 6(b).

It is interesting to note that while protoperithecia have a general, even staining throughout, these sclerotia appear to be stained with greater intensity in the centre, and less so in the periphery. This correlates well with the unpublished observation of N.D. Read that the peripheral hyphae of these sclerotia are more loosely arranged than those of protoperithecia, so there may be further evidence here for a role of phenoloxidases in the aggregation and / or cohesion of hyphal elements in morphogenesis.

There are two disadvantages with this method of analysis of phenoloxidase activity that should be mentioned here. One is that the dark blue crystals which appear late on in the reaction increase in number and size, and consequently make visualisation of the staining more difficult with time. The second point is that later still, there appears a 'halo' of blue colouration in the vicinity of the stained material, consisting of lighter crystals, perhaps of the same substance.

Experiments are in progress to establish a method for controlling the colour reaction, perhaps by fixation of the material after a certain time. This would also confer the advantage of the possibility of a quantitative study of the phenoloxidase activity in the developmental process.

A second method (communicated by O.M.H. deVries) was to use a non-carcinogenic analogue of benzidine - 3,3',5,5'-tetramethyl benzidine (TMB). When a culture was flooded with TMB there was no staining of 2 day old, undifferentiated, mycelium but protoperithecia in 4 day old cultures showed as blue foci after a few seconds, increasing in intensity with time, and with no

background colouration in the underlying mycelium. A disadvantage of this method is that the blue colouration appears to fade with time if kept in the light, and the intensity is less than that with ADBP-DMA, so presumably the sensitivity is lower.

Dihydroxyphenylalanine was also used in an attempt to see phenoloxidase activity. When used in the same way as for TMB, protoperithecia appeared to form light brown foci, distinct in colour from unstained protoperithecia.

An attempt was made to quantify the activity of phenoloxidase on a colourimetric basis. Protoperithecia from half a plate of 6 day old mycelium were homogenised in 0.5ml of NaP pH5. 200μl of this was added to 1ml of 'working' TMB reagent, and absorbance at 650nm was monitored. An almost linear increase in absorbance with time was observed the rate being 0.055 A_{650} units per min. However, when the homogenate was cleared by centrifugation, the activity was only 0.0022 A_{650} units per min. Thus it appeared that 96% of the activity was located in an insoluble fraction, and so a quantitative analysis would undoubtedly prove unreliable.

3.3. Molecular Analysis of Sexual Morphogenesis.

Characterisation of sexual morphogenesis in molecular terms was approached at three levels: by differences in the protein populations present at different stages in the morphogenetic sequence, by the differences observed in the patterns of protein synthesis, and by differences in the messenger RNA populations.

3.3.1. Analysis of 'Cold' Proteins.

An initial experiment was carried out in order to examine the populations of proteins that exist in the different developmental stages of the fungus. Nasrallah and Srb (1973) identified a major phase-specific protein in the perithecia of *Neurospora crassa*, and Bond and McMillan (unpublished) detected a major phase-specific protein band in perithecia of *Sordaria brevicollis* with a similar molecular weight - around 20 kDa - to that reported by Nasrallah and Srb. These observations were made using SDS-PAGE.

Protein profiles of mycelium and perithecia of *S. brevicollis* were compared using SDS-PAGE and coomassie blue staining. No convincing differences could be identified between the two preparations.

Following these experiments which indicated that the protein mixtures were too complex to be analysed usefully using one dimension only, two-dimensional gels were used. While it was apparent that many more species could be identified using 2D-PAGE than with SDS-PAGE alone, the efficiency of staining using coomassie blue was too poor for the resolution required. The method of 'silver staining' provided a much more sensitive system for the analysis of these complex protein spectra.

The pattern of proteins present in perithecia, as shown in figure 7, showed a great deal of similarity with that of mycelium. Certain 'spots' were easily used as 'landmark' proteins that allowed one gel to be aligned with the other, and so comparisons could be made with relative ease. There were obviously many spots that simply added to the background colouration, and therefore could not be discerned because of the numbers present.

One advantage of this particular method of silver staining is that there is often differential colouration in the staining of different proteins. Thus the identification of a particular protein species was greatly facilitated by the way in which its colour was different from those in its immediate vicinity in the gel. Red, yellow and green spots, and others in various shades of brown, enabled simple matching of species between gels, and thereby eased analysis.

Because of the numbers of proteins apparent, together with the heavy background staining, differences between the gels are generally only convincing in protein species with spots of different colour from that of the background. In addition, the conditions used in this investigation only allow those proteins that are easily extracted, and those that lie within the particular pI range used, to be analysed. Furthermore, the nature of the silver-based colour staining technique is not fully understood. It is possible that many factors may influence the colour and degree of staining, for example, the concentration, secondary or tertiary structure of the protein, and perhaps other protein modifications such as side-chains may contribute to the silver-staining characteristics (see Sammons *et al*, 1981). Quantitation of the differences observed would therefore be not only difficult, but also of limited accuracy, so

Figure 7

'Cold' Protein Changes in the Sexual Morphogenesis of
Sordaria brevicollis

Silver stained polyacrylamide gels of protein

extracts from two developmental stages are shown.

The lower diagram is a tracing of the perithecial

gel, to indicate the phase-specific species

(arrowed and numbered).
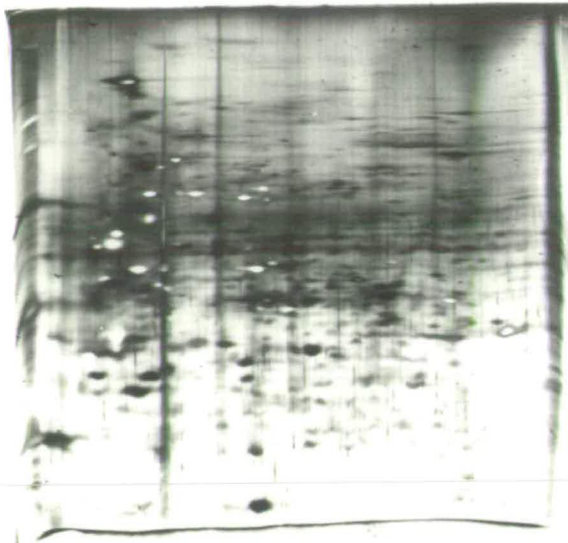
MYCELIUM
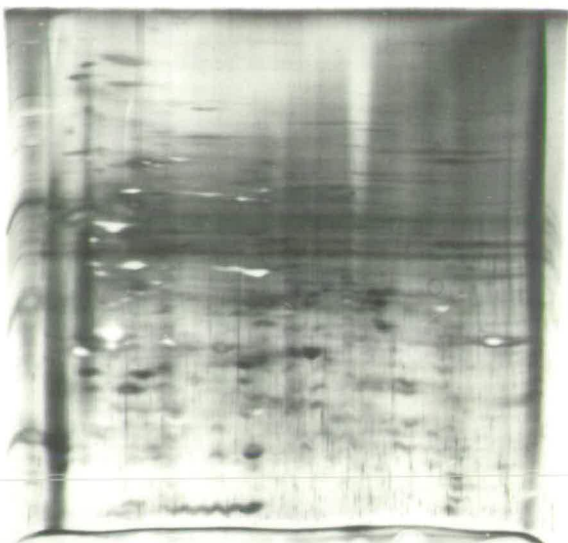
PERITHECIA
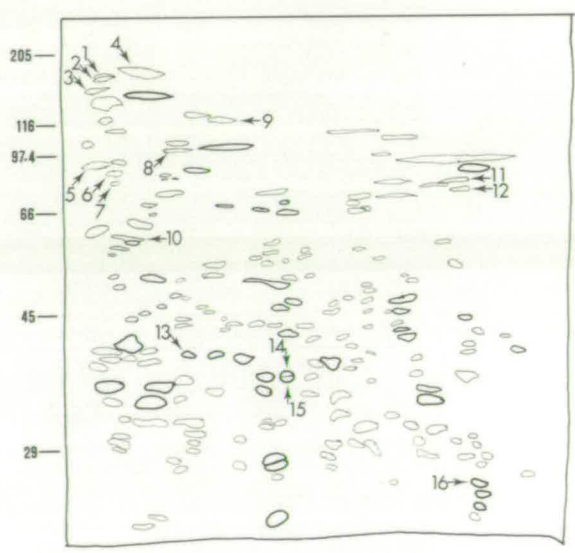
a qualitative description is all that can be made with certainty.

I have identified 16 major differences between the mycelial and perithecial patterns, such that a protein is present in perithecia and absent in mycelium. This number is obviously a minimum estimate, since many more would be expected if the minor spots could be analysed more thoroughly. Of particular interest in this set of perithecia-specific polypeptides are the species (numbered 1 – 4) which are grouped in the molecular weight region around 200 kDa. The separation in this area is sufficient to make these proteins quite easy to recognise. Polypeptide species with similar characteristics were also found in later experiments (see *in vivo* labelling).

Unfortunately, monochrome representation of the silver-stained gels could not do justice for the results, and so a line copy is added to aid interpretation.

That there are many more spots present than are seen in the gel as shown was observed by reduction of the silver stain by photographic reducing agents as detailed in Materials and Methods. During this reaction there was a brief time in which the background cleared to show a similar distribution of spots in all parts of the gel. Unfortunately, there was no simple way of stopping the reaction with any degree of control, in order to analyse the gels further.

3.3.2. *In-vivo* Radiolabelling of Proteins.

The detection of 'cold' proteins by silver staining provides a sensitive way of demonstrating differences in protein profiles, but it offers no indication of the patterns of protein synthesis of the various protein species. In order to investigate this aspect, proteins synthesised during a brief interval can be analysed by exposure to a 'pulse' of radiolabelled amino-acid. Labelling with ^{35}S-methionine allows the methionine-containing proteins to be detectable on a gel by fluorography.

Preliminary experiments to determine the optimum conditions for the labelling of proteins were undertaken. The optimum pulse duration, that which allowed the most convenient time with high incorporation, was determined by varying the pulse time for both mycelial and perithecial samples. As can be seen from the results in figures 8 and 9, the incorporation increased

Figure 8

In vivo Labelling of Proteins of *Sordaria brevicollis.*

Effect of Pulse Time on Incorporation.

Vertical axis is acid-precipitable ⟶ disintegrations per minute

per mg wet weight of material.

2 day mycelium ——————— x/x
3 day perithecia ------------- o/o
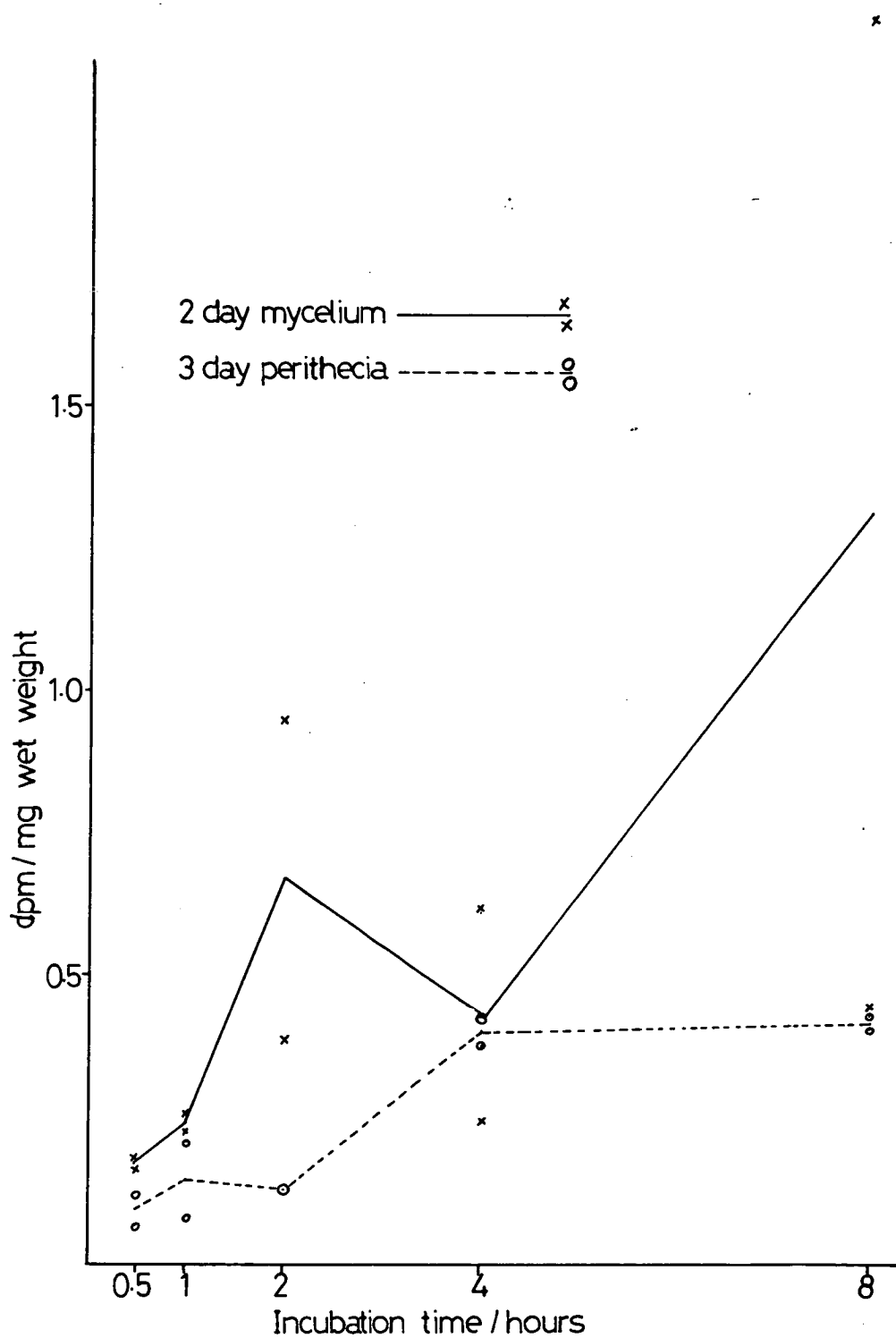
Figure 9

In vivo Labelling of Proteins of *Sordaria brevicollis*.

Effect of Pulse Time on Incorporation II.

Vertical axis is acid-precipitable *disintegrations*

per minute per µg protein, as estimated

according to Lowry (1951).
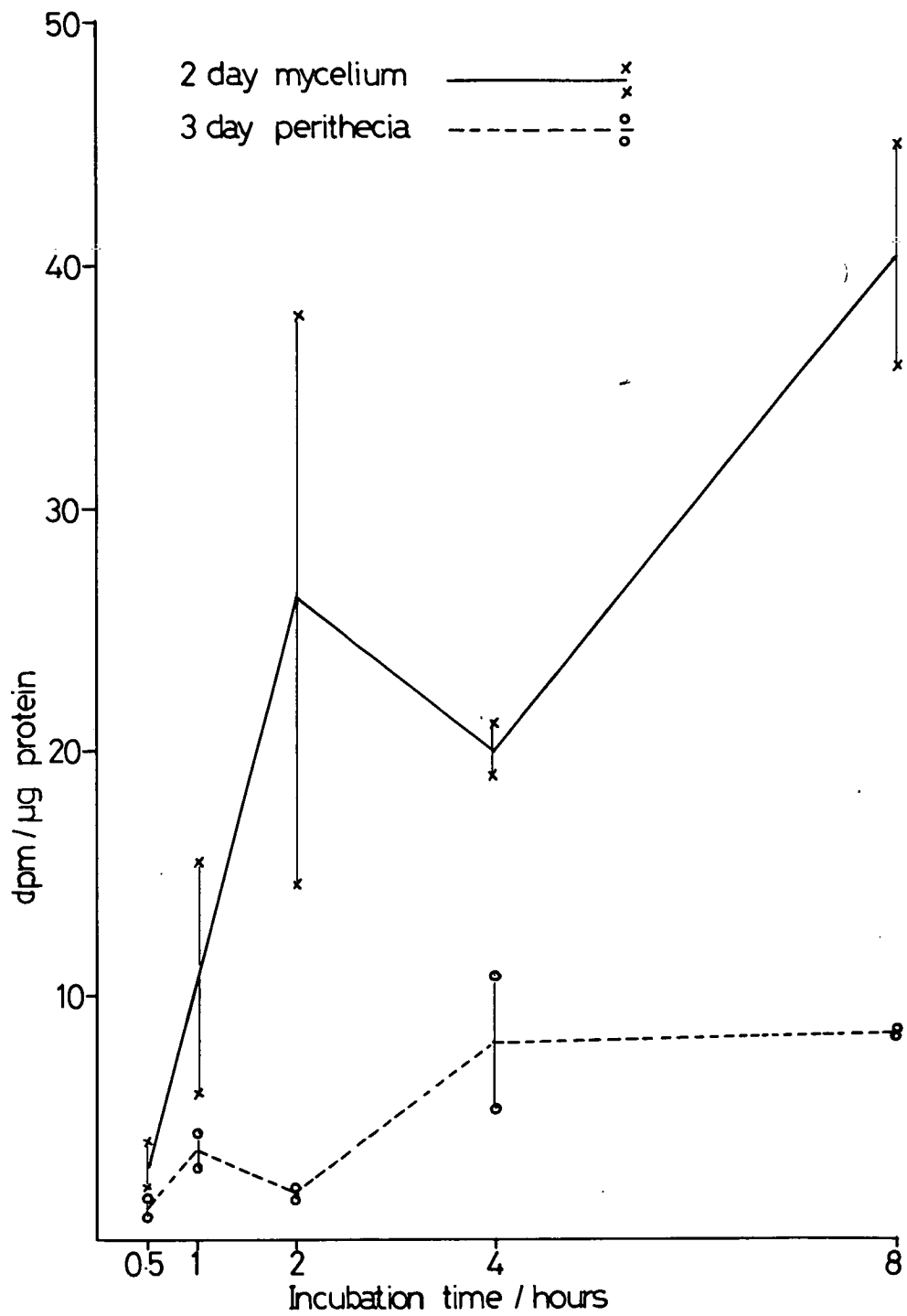
significantly for the first 1 – 2 hours, then appeared to level off. The method used for measuring specific incorporation (either cpm/mg wet weight or cpm/μg protein), gave a high degree of variability. This could well be due to inaccuracy in the method of counting incorporation, either by the TCA washing, or as a result of a fault in the scintillation counter (the latter explanation was supported by observations of several other users). In any case, an arbitrary time of 1.5 hours was chosen for the *in vivo* labelling experiments, to minimise any adverse effects due to protein degradation during a prolonged period.

A second experiment to compare the incorporation by different developmental stages of the fungus was performed; the results are depicted in figure 10.

As can be seen from the graphs, there is a great deal of variation between the results from different experiments. This may be due to real differences in the uptake or incorporation by the fungus at the various timepoints, or perhaps more likely, this represents an artefact of the method for counting the incorporation of label.

This 'pulse-labelling' analysis has its limitations – only proteins synthesised within the brief exposure to the radiolabelled methionine can be detected, so proteins synthesised irregularly or according to some biological (or circadian) rhythm might not be detected. Therefore, differences should be described with some degree of caution.

Comparisons between different proteins are difficult owing to the different methionine contents of individual species, so each protein spot can be compared only with the corresponding spot in other gels.

The fluorograms obtained from these radiolabelling experiments are depicted in (figs. 11 – 13).

As with the silver-stained gels, there are numerous major spots which are present in all of the protein profiles, and these can be used to align the fluorograms for ease of analysis. These 'landmark' spots are marked by a square in the figures, for reference.

Results are shown for the following developmental stages of the fungus:

Figure 10

In vivo Labelling of Proteins of *Sordaria brevicollis.*

Incorporation of Radioactivity with Morphogenetic Stage.

Both graphs show the same data;

the upper graph has indicated the mean values for

incorporation over several experiments. The lower

graph shows the variation seen within one experiment.

(i)

(ii)
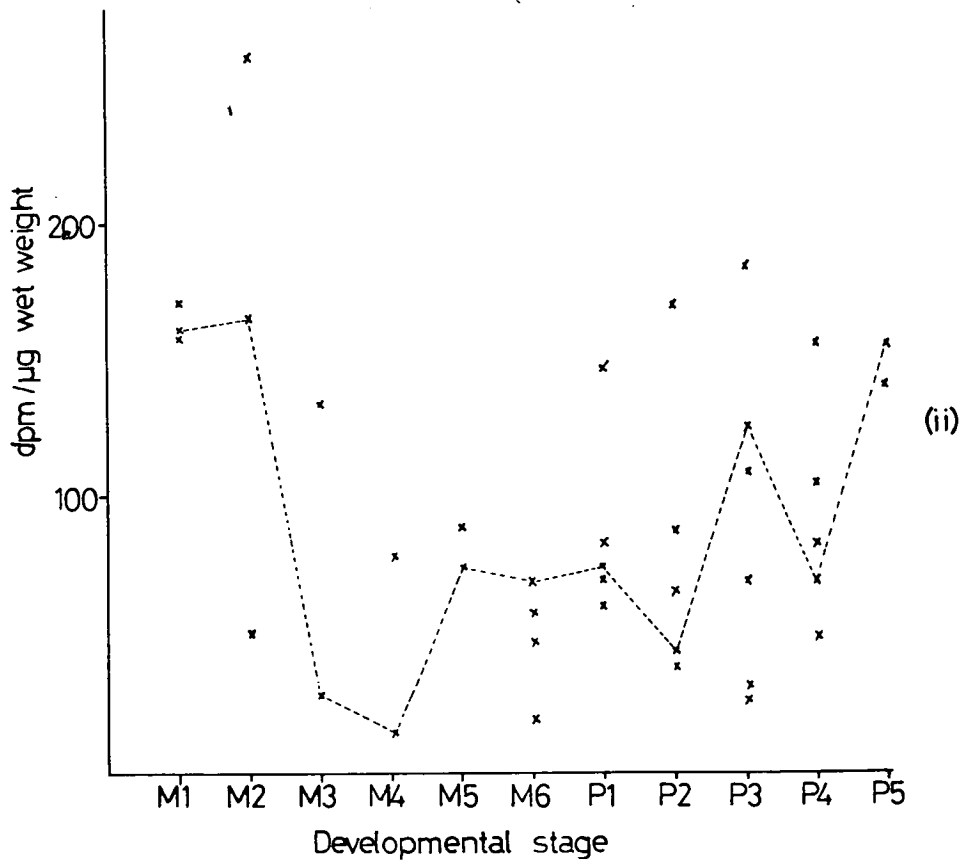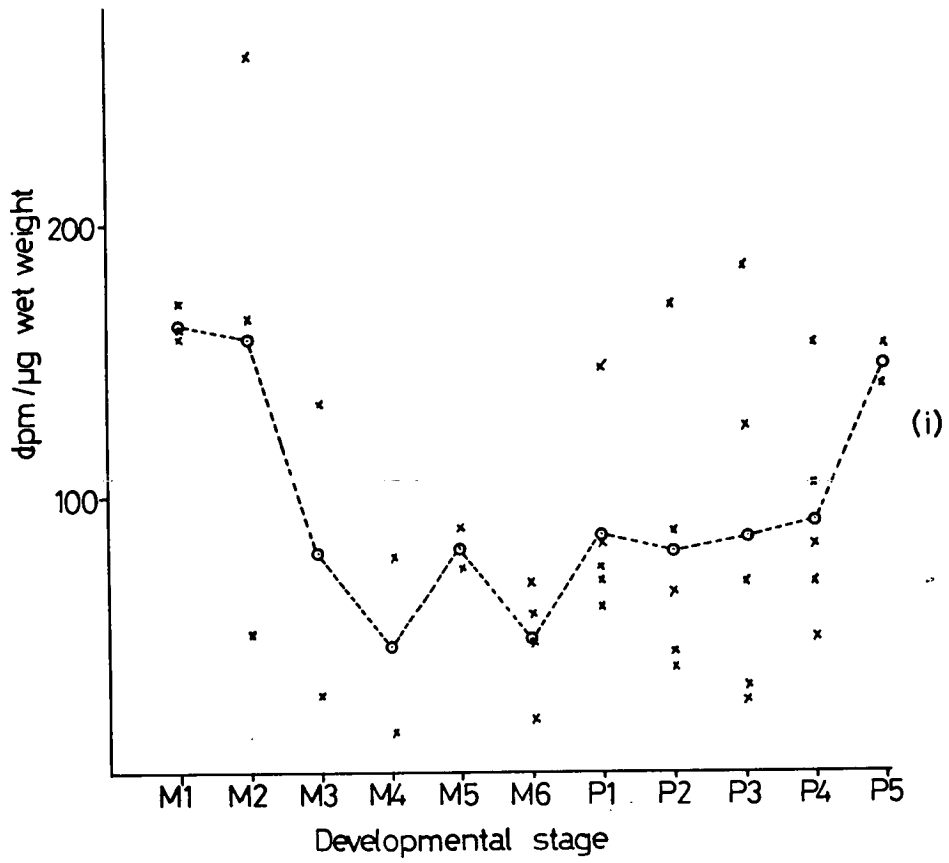
Figure 11

In vivo Labelling of Proteins of *Sordaria brevicollis.*

Proteins synthesised by:

(i)   Mycelium

(ii)   Protoperithecia.

Phase-specific polypeptides in figures 11–13

are numbered according to their first appearance

in this set of fluorograms.

Thus P1 – P121 are first detectable in protoperithecia,

115 – 121 in 1 day-old perithecia,

222 – 223 in 2 day-old perithecia,

324 – 329 in 3 day-old perithecia,

430 – 431 in 4 day-old perithecia.

The set of spots indicated with 'V' arrowheads show

how there is also reduction in synthesis of certain

polypeptides during the developmental program.
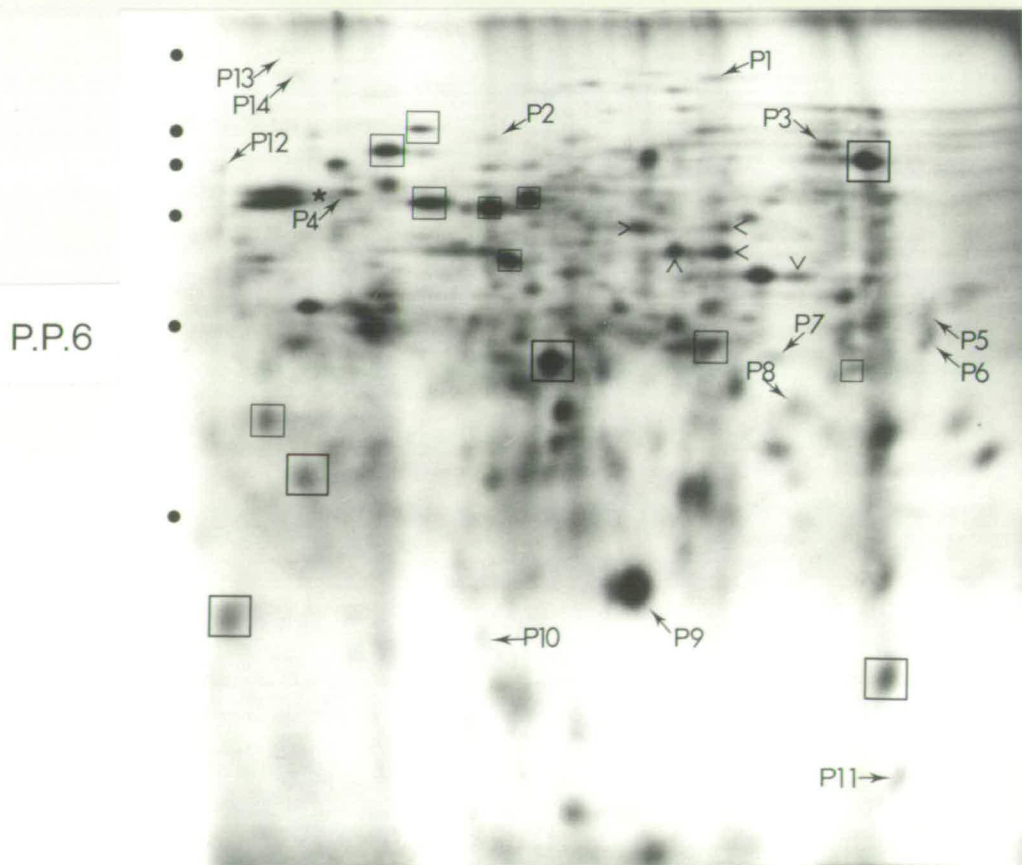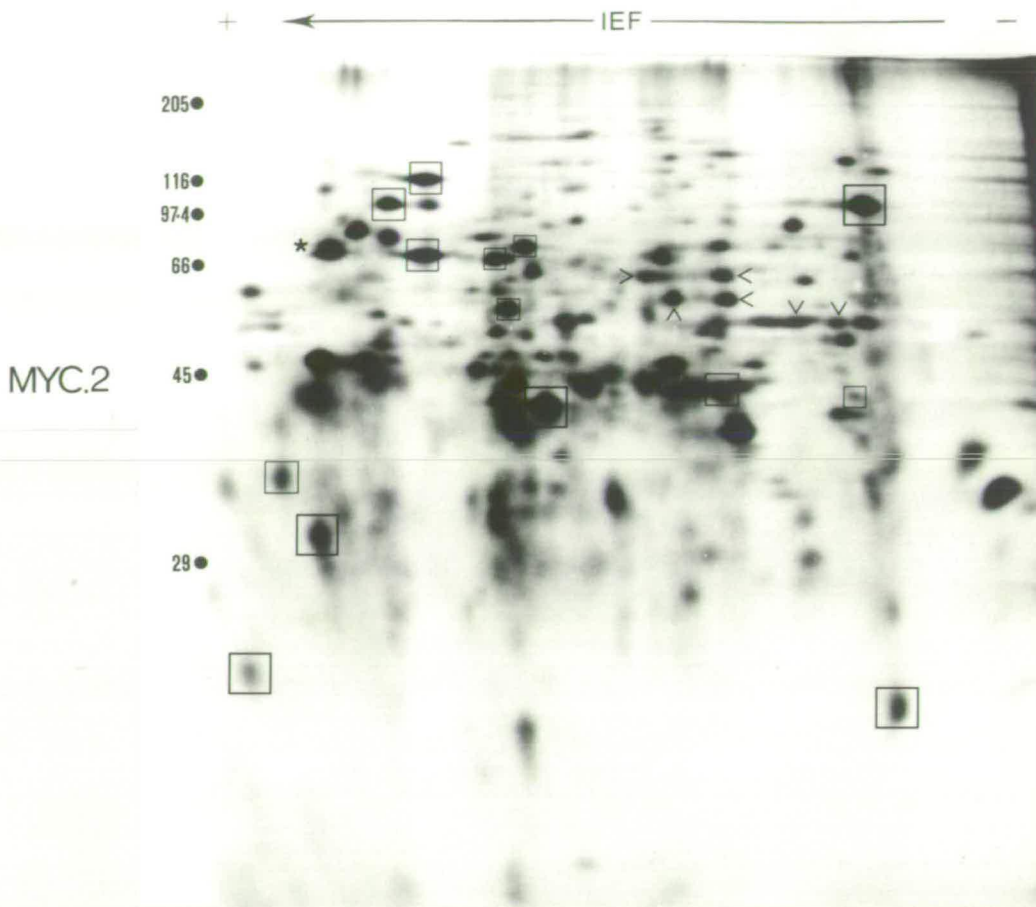
Figure 12

In vivo Labelling of Proteins of *Sordaria brevicollis*

Proteins synthesised by:

(iii) 1 day-old perithecia

(iv) 2-day-old perithecia
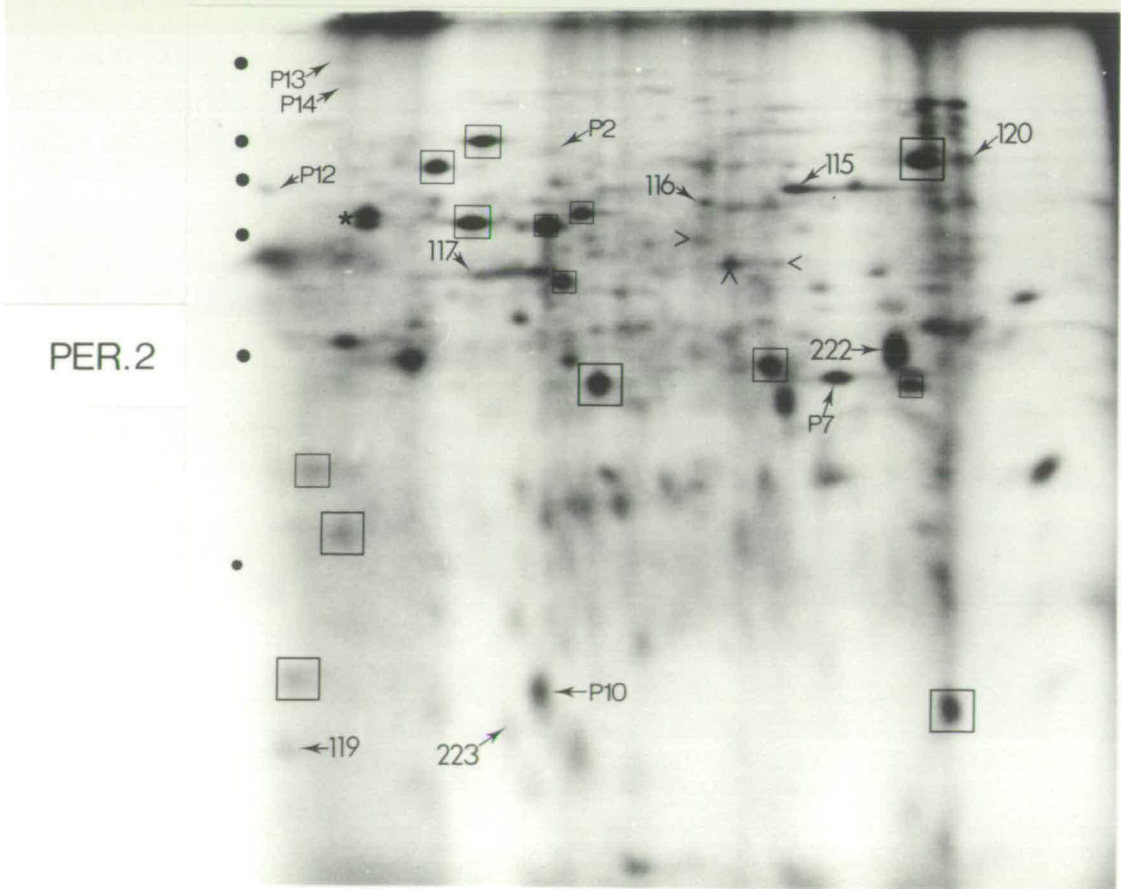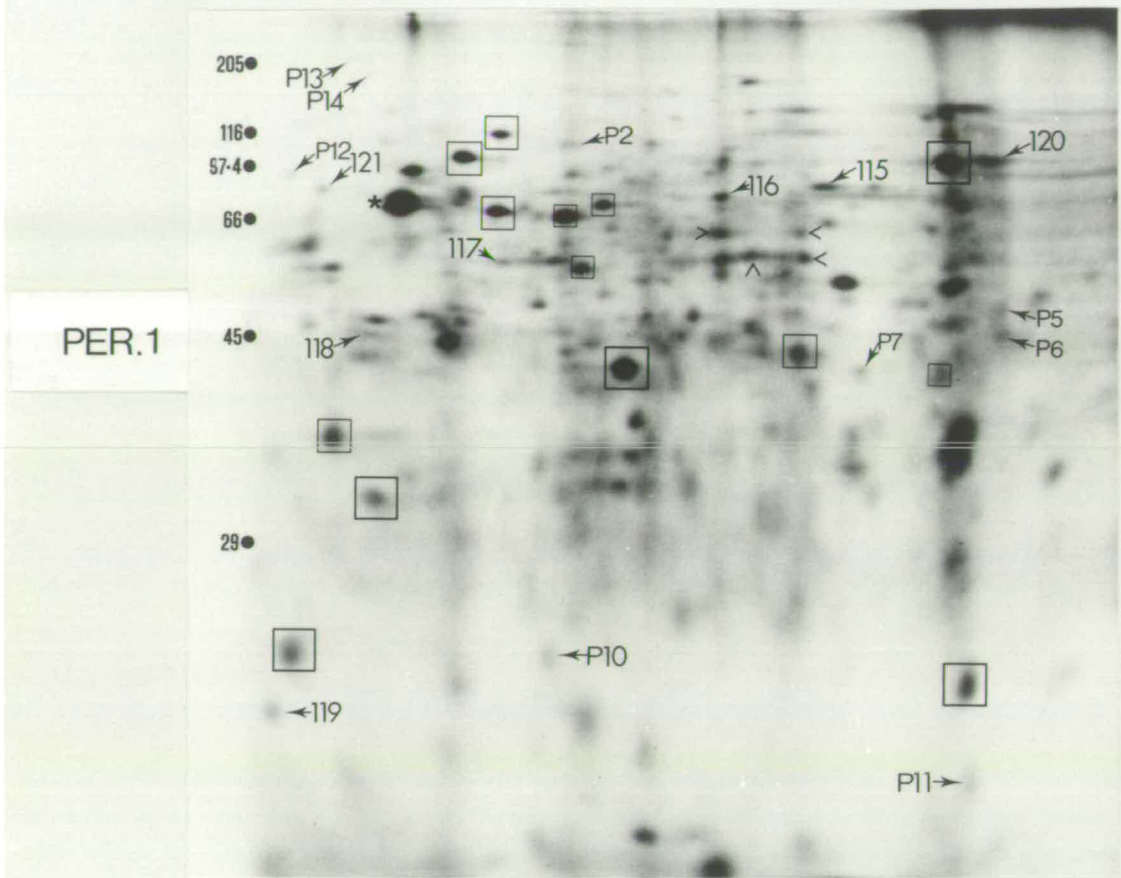
Figure 13

In vivo Labelling of Proteins of *Sordaria brevicollis.*

Proteins synthesised by:

(v)   3 day-old perithecia

(vi)   4 day-old Perithecia.

PER.3
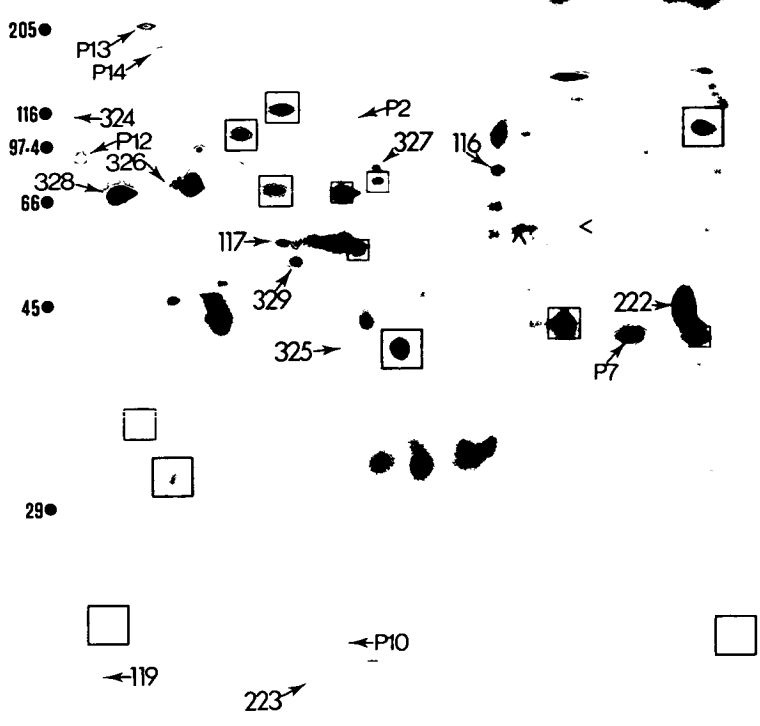
PER.4
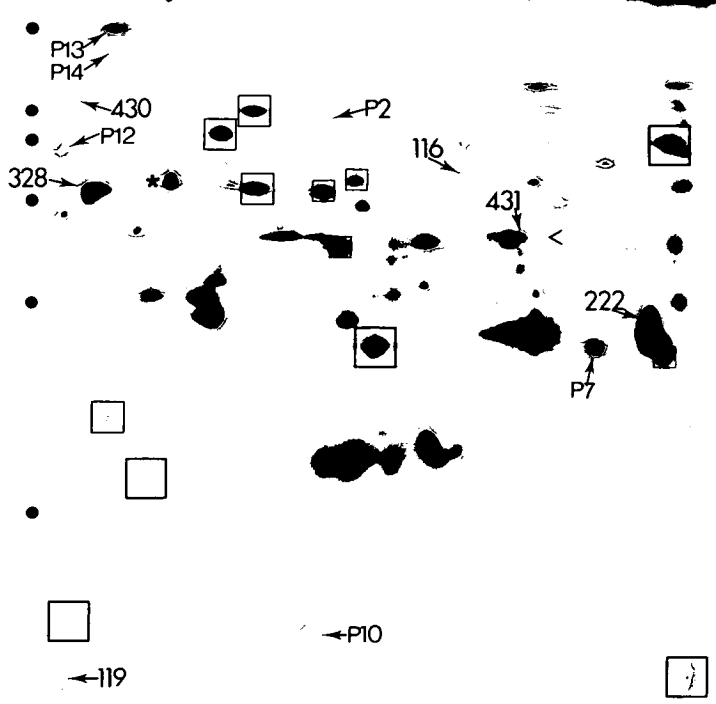
two day-old mycelium, protoperithecia from 6 day-old mycelium, and perithecia from 1, 2, 3 and 4 days after fertilisation. Protein species are designated as developmentally regulated on the basis of their absence in vegetative mycelium, and presence at some other stage.

A total of 31 proteins have been identified as being developmentally regulated and are numbered according to the time of first appearance in the set of fluorograms. Thus a prefix 'P' for a numbered spot indicates the first appearance in protoperithecia, and the first digit of each other spot refers to the day after fertilisation in which it first appears. For example, spot 115 first appears one day, and 222 first appears two days after fertilisation. Table 2 shows the pattern of appearance, and the estimated level of expression (according to the intensity of the spot on the fluorogram), of each of these 31 polypeptides.

Differences in expression during the sexual morphogenesis, of each developmentally regulated protein can be described in a number of ways. Apart from the time of first appearance, there are differences in the pattern of appearance after this time, during the rest of the morphogenesis. If this pattern is taken into account, then there are many such patterns observed, some of which are very interesting.

Some spots appear at the protoperithecial stage, then never re-appear (for example, P1, P3, P4 and P9). A similar pattern is seen for spots 115 and 121 which appear only at one day after fertilisation, and 324, 325, 326, 327 and 329 which appear only at three days after fertilisation.

Other spots appear at low levels from their first appearance, and remain at a similar level until they cease to appear. Spots P2 and 117 show this pattern of expression. Spot 222 first appears at a high level, then the intensity remains at the same level. Spot 328 behaves in a similar fashion but first appears a day later.

Spot P7 appears at low levels in protoperithecia, then the intensity increases to a constant, higher level one day after fertilisation. Spots P10 and P12 have similar patterns but the increase does not occur until two days after fertilisation.

Table 2

Patterns of appearance of phase-specific

polypeptides synthesised *in vivo*.

The patterns of synthesis of the 31 phase-specific polypeptides identified

in figures 11 - 13. The level of synthesis is estimated from the

intensity of the spot compared with the same spot in other fluorograms.

	PP6	P1	P2	P3	P4
P1	+	−	−	−	−
P2	+	+	+	+	+
P3	+	−	−	−	−
P4	+	−	−	−	−
P5	+	+	−	−	−
P6	+	+	−	−	−
P7	+	++	++	++	++
P8	+	+	−	−	−
P9	+	−	−	−	−
P10	+	+	++	++	++
P11	+	+	−	−	−
P12	+	+	++	++	++
P13	+	+	+	++	+++
P14	+	+	+	++	++
115		+	+	−	−
116		++	+	+	+
117		+	+	+	−
118		+	−	−	−
119		+	+	++	++
120		+	+	−	−
121		+	−	−	−
222			+	+	+
223			+	+	−
324				+	−
325				+	−
326				+	−
327				+	−
328				+	+
329				+	−
430					+
431					+

The area of the gel in the high molecular weight, low pI range shows the appearance of two clearly visible spots (P13 and P14) that are reminiscent of the proteins seen in the same area of the silver-stained gels. From the silver-staining, it was apparent that these spots (numbered 1 – 4 in fig. 7) are present at high levels in perithecia, three days after fertilisation. From these fluorograms, assuming that spots P13 and P14 represent part of the same group of polypeptides, it appears that there is a gradual increase in the rate of synthesis of these proteins during the morphogenetic process. Further down the gel, spot 119 may behave in a similar manner but it could not be detected in protoperithecia.

Spots 116 and 120 first appear at a high intensity, then they decrease in time to a lower level.

An interesting observation on comparing the protoperithecial proteins with all the other stages concerns the 'doublet' of spots marked with a star. During the protoperithecial stage the doublet undergoes a shift to a higher pI value, then on fertilisation the spots move back to their original position. That this is not an artefact due to distortion of the rod gel is seen by the exact superimposition possible with other spots of the same pI range.

Also shown on the fluorograms is a set of six proteins that are seen in the vegetative mycelium, then disappear during the morphogenesis. This is to show an example of how there are reciprocal changes to those described above, that is, expression is repressed during the transition from vegetative growth to sexual reproduction. The spots shown here are merely an example of the reverse situation to that of the numbered spots. Of the six spots arrowed in the mycelial gel, all but two are lost during the morphogenesis, and the two remaining are at a much lower level. Of course, there are undoubtedly more such proteins 'switched off' in this manner, but these six are shown since they appeared in an easily compared group, and the pattern of expression is clearly seen.

3.3.3. *In vitro* Translation of Messenger RNA (mRNA).

In order to investigate differences in the populations of mRNA extracted from the different morphogenetic states of the fungus, a cell-free translation

system was used to synthesise polypeptides using the added mRNA as a template, then these were separated by PAGE and analysed by comparison.

Comparisons were made between the translation profiles of mRNA from three developmental states; 2 day-old (vegetative) mycelium, 4 day-old (protoperithecial) mycelium, and perithecia from three days after fertilisation. 4 day-old mycelium was used rather than purified protoperithecia because the yield of mRNA from the latter was so poor – protoperithecia from over 200 plates yielded just 1-2 μg of mRNA, sufficient only for 1-2 translations.

As a pilot experiment, SDS-PAGE was used to separate the synthesised polypeptides – the fluorogram is shown in figure 14. There are numerous differences between the translations when comparing 2 day-old mycelium with 4 day-old mycelium and 3 day perithecia (arrowed), although when the two perithecial translations are compared, there are also differences seen. The general lack of polypeptide bands seen in the second perithecial track is perhaps related to its overall diffuseness, which may in turn be due to poor quality mRNA, or some artefact of gel preparation. However, some of the developmentally regulated protein species seen in the 4 day-old mycelial and the first perithecial tracks are repeated in the second perithecial track.

From this result it is clear that the one dimension of SDS-PAGE alone has insufficient resolving power for the comparison required in this analysis. The polypeptide mixtures are evidently too complex for such a crude analysis. Therefore, 2D-PAGE was once again employed. The fluorograms from these gels are shown in figures (15 – 16).

It is evident from the numbers of spots apparent that the protein mixtures were indeed too complex for analysis by SDS-PAGE alone. As with the results of the silver staining and the *in vivo* labelling, there is a great deal of similarity between the three fluorograms. Most of the patterns of spots are exactly superimposable, and there are ample 'landmarks' (labelled with a square surround) to allow alignment and to facilitate comparison.

As before, spots were identified which appeared in the protoperithecial and perithecial stages of development, and which were absent from the vegetative mycelium. A total of six polypeptide species were first synthesised at the protoperithecial stage, and of these, three are still clearly seen in the

Figure 14

In vitro Translation of mRNA:

Analysis of translation product by SDS-PAGE.

Possible candidates for phase-specific

polypeptides are indicated.

M2 = 2 day-old mycelium

M4 = 4 day-old (protoperithecial) mycelium

P3 = 3 day-old perithecia.

M2 P3 M4 P3 BI

●205

● 116
● 97.4

● 66

● 45

● 29

MYC.2

pI ← 6.0   6.5   7.0 ← pI → 7.5   8.0

−205
−116
−97·4
−66
−45
−29
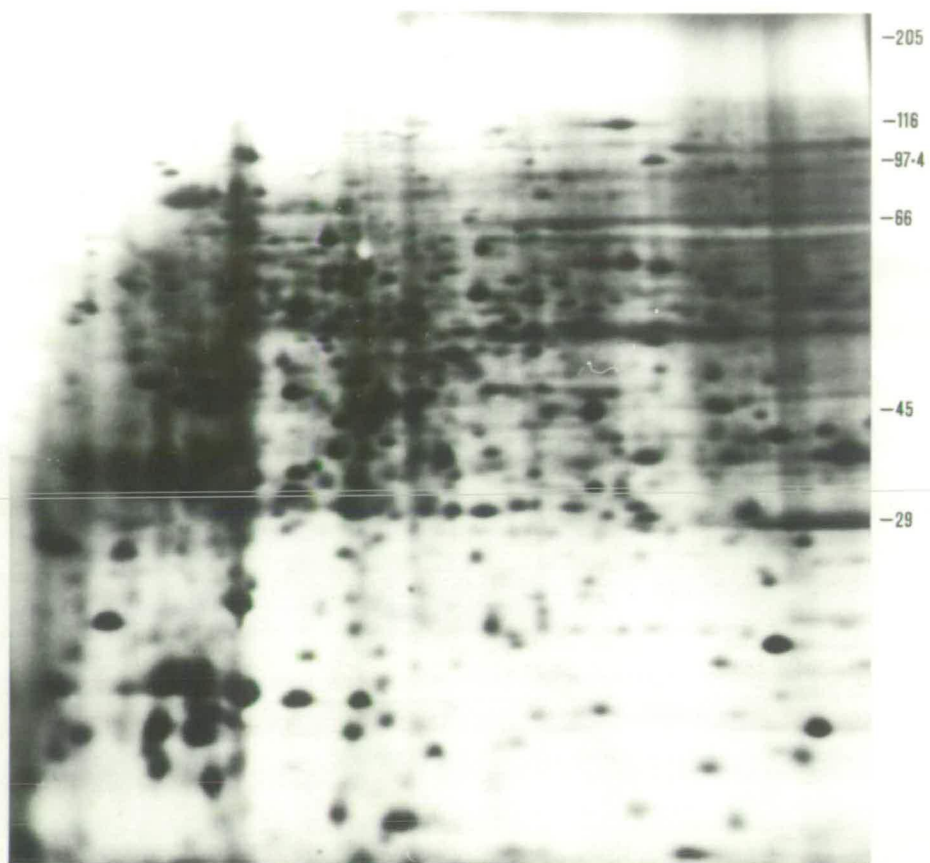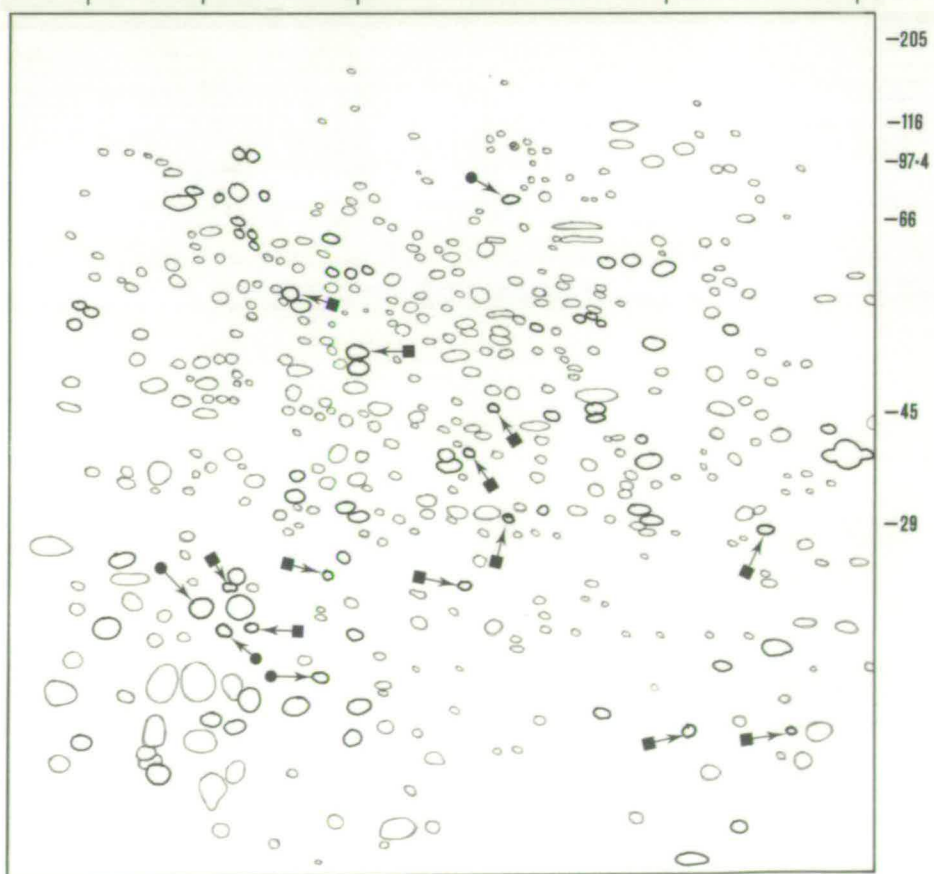
Figure 16

In vitro Translation of mRNA:

Proteins Synthesised from:

'Protoperithecial' Mycelium

3 day—old Perithecia.

MYC.4
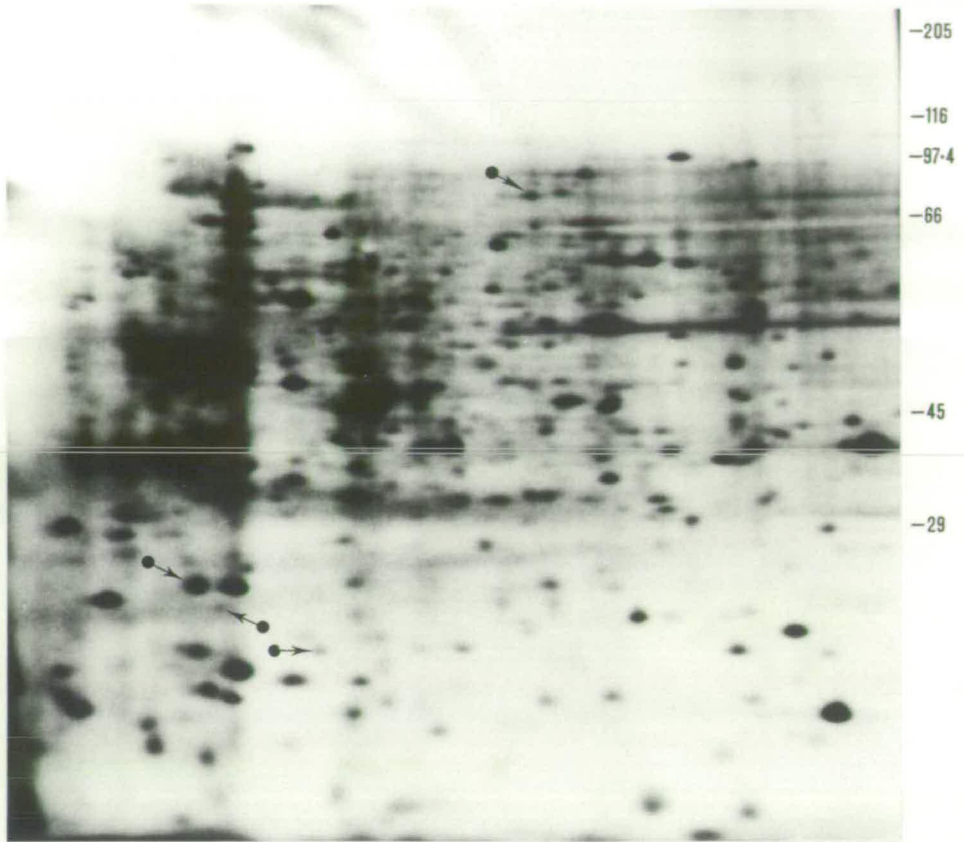
PER.3
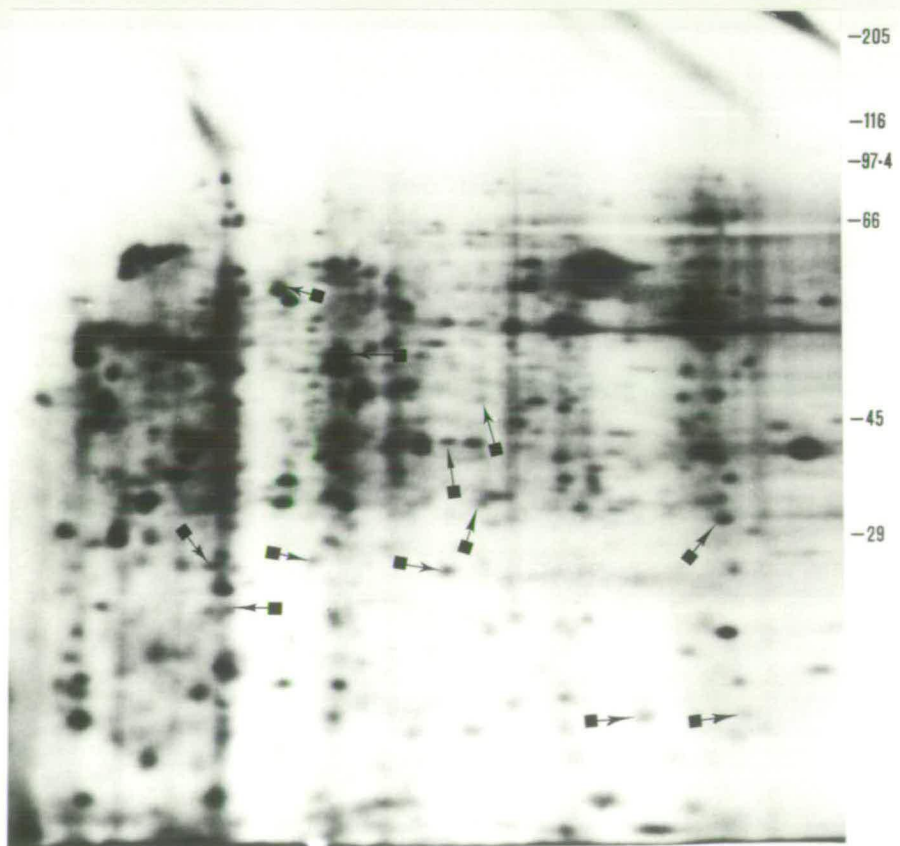
perithecial translation. Eleven more polypeptides were not observed until the perithecial stage.

It is interesting that the number of differences seen in the transition from the vegetative mycelium to protoperithecial production using this method is smaller than the number of differences observed in the *in vivo* labelling experiments. The significance of this in terms of different modes of control will be discussed later.

3.3.4. Construction of a Genomic Library.

Using the conditions described in the Materials and Methods, a library of fragments of *Sordaria brevicollis* genomic DNA was constructed.

Enrichment using Spi⁻ selection was chosen because of the high background of non-recombinant bacteriophage, as seen by plating on both selective and non-selective hosts at the same time. There was about 20 times as many plaques on WL87 (the non-selective host) as on WL95 in which Spi⁻ bacteriophage were selected.

Optimal ligation conditions were determined by combining different ratios (by weight) of EMBL3 arms and *S. brevicollis* genomic DNA fragments in pilot ligations. The final ligation reaction included 5µg of EMBL3 DNA and 1.25 µg of genomic DNA. This yielded a total of 4000 plaques on WL95.

The library was amplified as described, to enable a stock to be made for storage. The titre of this stock was 10^7 pfu ml^{-1}, so 10 µl is sufficient to provide a representative complement of library fragments.

The size of library required for a high probability (P = 0.99) of representation of any particular sequence is given by the formula:

$$N = \frac{\ln(1-P)}{\ln(1-x/y)}$$

(Clarke and Carbon, 1976),

where x is the insert size and y is the size of the genome. Assuming that the genomic fragments in the library is a random sample, and that each insert is 15 kilobases long, and that the *S. brevicollis* genome is similar to that of *Neurospora crassa* - 2.7 x 10^7 base pairs (Perkins, 1985), then the 'ideal' library would contain 8300 different fragments. Since this library contains 4000 different recombinant

bacteriophages, it is approximately half the size of an ideal library, and the probability that it contains any particular sequence would be 0.86 - so there is a chance of 14 per cent that a sequence would be missed.

3.3.5. Analysis of the Library.

The genomic library was first analysed for its capacity to represent the genome by screening with a cloned single-copy sequence - the *am* gene of *Neurospora crassa* and a cloned segment of repeated DNA - the tandem ribosomal sequence from *N. crassa*. Then, two approaches were made in the search for developmentally-regulated sequences. The library was screened for sequences differentially transcribed by using a 'non-specific' probe made from poly(A) RNA from *S. brevicollis*, and an experiment was performed to look for sequences equivalent to those isolated from dikaryon-specific mRNAs of *Schizophyllum commune*.

Specific sequences.

Before the library was screened for the presence of any specific sequence, a preliminary check was made to determine whether the sequence in question was present in the genomic DNA. For this, a specific probe was made from the cloned DNA, and used in hybridisation against digested *Sordaria brevicollis* genomic DNA bound to a nitrocellulose or Hybond N filter.

Neurospora crassa am sequence.

A 2.7kb fragment of *Neurospora crassa* genomic DNA containing the complete coding sequence of the glutamate dehydrogenase gene - a gift from Dr. J.H. Kinnaird - was used in the detection of an equivalent sequence in *S. brevicollis*.

Hybridisation conditions were as described in Materials and Methods for nitrocellulose filters. The result of the Southern analysis is shown in figure 17(i). The signal obtained in each track demonstrates the presence of a sequence equivalent to the cloned DNA probe, such that there is up to 2 kb showing homology (see *Eco*RI track).

On screening the library for a sequence equivalent to the *am* gene, however, there was no clone found, amongst 10,000 plaques, which hybridised to the *am* probe.

According to the calculation given in section 3.3.4, there is a chance of 14 per cent that a particular sequence will not be represented in a library of the size obtained in this work. This result adds proof to the argument that the library is not entirely satisfactory.

rDNA.

A clone, pMF2, containing the coding sequences for the 17S, 5.8S and 28S ribosomal RNAs of *Neurospora crassa* – a gift from Dr. R.L. Metzenberg – was used as with the *am* clone, to detect the rDNA sequence, first in the *Sordaria brevicollis* genome, and then in the genomic library. the result of the Southern analysis is shown in figure 17(ii).

There is a band of DNA at around 8 kb in all but the *Sal* track, indicating that there is a tandem repeat of this size in the *S. brevicollis* genome. The signal at the top of the *Sal* track indicates that there is no *Sal* site within the repeat sequence. The 'ladder' of bands in the *Eco*RI track is possibly due to a partial digestion of DNA.

On screening the library, about 150 plaques of a plate lysate of 6000 gave a positive signal, so about 2.5% of the cloned fragments contain ribosomal sequences. Since the number of rDNA genes in *N. crassa* is about 200, representing approximately 8% of the genome (Krumlauf and Marzluf, 1980), there appears to be no undue bias for the cloning of ribosomal, and therefore perhaps other repeated, DNA sequences, in the making of the library.

Figure 17

Analysis of the Genomic DNA of *Sordaria brevicollis.*

Genomic Digests Probed with:

(i)   *Neurospora crassa am* Gene.

(ii)   *Neurospora crassa* rDNA clone.

The tracks are as follows:

S. brevicollis genomic DNA digested with:

*Bam*HI   (B)

*Eco*RI   (E)

*Sal*I   (S)

*Hind*III   (H)

Markers are BRL 1kb ladder, sizes in kb.
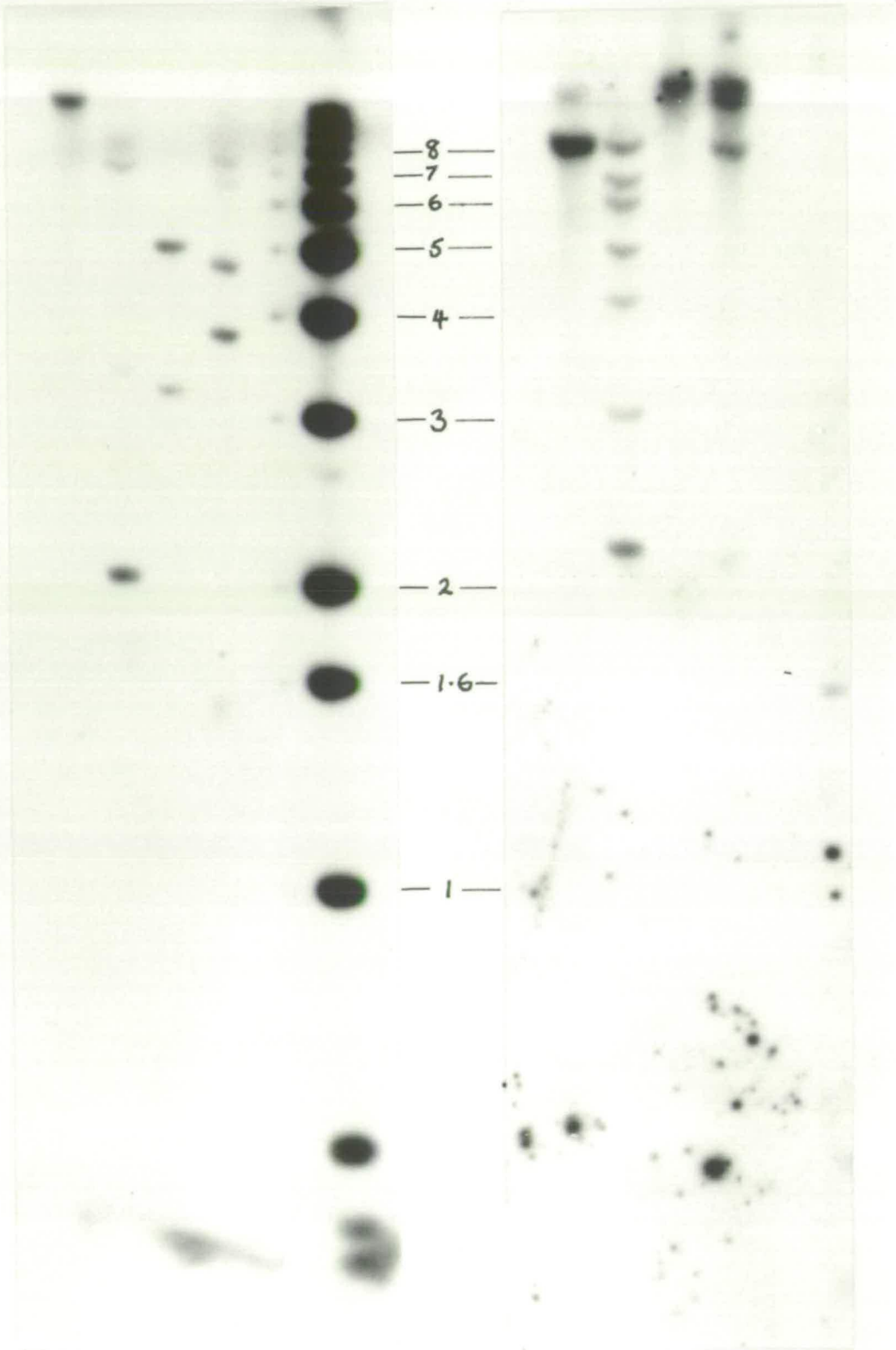
pSC clones.

A set of clones containing sequences that are reported to be developmentally regulated in the Basidiomycete *Schizophyllum commune* were obtained – a gift from Prof. J.G.H. Wessels – and used to determine the presence of equivalent sequences in *Sordaria brevicollis.*

On hybridisation to Southern filters, each gave a signal, although the patterns had some similarity to each other. Since the probes were prepared from cDNA clones, it was possible that the pattern on the Southerns was due to the poly d(A) 'tails' hybridising to d(AT)-rich regions of the *S. brevicollis* genome. To test this possibility and to overcome this putative artefact, poly d(A) was included in the pre-hybridisation and hybridisation mixes of a second experiment, to block out poly d(A) in the probe from hybridising to poly d(T) sequences in the genomic DNA, and to compete with poly d(A) in the filter-bound DNA for hybridisation with poly d(T) in the probe.

Under these conditions, and with the lowest degree of stringency (57°C in 2 x SSC) there was no signal now detected in the filters, suggesting that no *S. brevicollis* sequences corresponding to the *Schizophyllum commune* developmentally regulated genes existed.

mRNA screening.

To detect sequences in the library of genomic *Sordaria brevicollis* DNA which were differentially transcribed during sexual development, the method of plaque filter hybridisation was adopted. Replica filters from a plate lysate were hybridised to end-labelled mRNA from the different developmental states of the fungus. Plaque transfers were made on Hybond N nylon filters, in order that they could be re-used if necessary. Hybridisation conditions were as for Hybond N in Materials and Methods. End-labelled mRNA from vegetative mycelium hybridised to filter-bound plaques showed around 450 positive signals per 6000 plaques, so about 0.75% of all fragments were represented in the mRNA from vegetative mycelium.

However, no signal was now detected using mRNA from either 4 day-old (protoperithecial) mycelium, nor from 3 day-old perithecia. It is unlikely that a

signal would be undetectable, considering the results of the *in vitro* translation experiments, in which a considerable amount of translation was evident from the 2D gels. A more likely explanation is that some other part of the screening process has failed. This is despite a number of attempts to repeat the experiment. For example, the DNA might not have been bound to the filter, or the RNA probe may have been lost during the Sephadex chromatography, or degraded at some other stage.

The screening was repeated using radiolabelled cDNA rather than mRNA, but again no signal was obtained. The reason is likely to be that there was insufficient DNA bound to the filter. Unfortunately time did not allow further investigation of the failure, but future work would certainly focus on the screening for developmentally regulated sequences.

CHAPTER 4
DISCUSSION

The Ascomycete fungi have been widely used in research for many years. In particular, the filamentous Ascomycetes have played an important role in the investigation of cellular aspects of sexual reproduction. Genetic analysis of meiosis and spore formation is well documented (for review, see Raju, 1988), and hyphal and mycelial growth patterns have been extensively studied (see Prosser, 1983).

The characteristic features of the sexual cycle of filamentous Ascomycetes are well known, although the mechanisms involved in the initiation of sexual development are not yet fully understood (see Moore-Landecker, 1988; Bond and Broxholme, 1988).

Sordaria brevicollis has found a niche amongst other filamentous Ascomycetes used in research for a number of reasons. In addition to the ease of its manipulation and maintenance in the laboratory, there are numerous spore colour mutations which have provided a useful means of demonstrating the processes of genetic recombination, and hence aid linkage analysis (see Whitehouse, 1982 for review). Complementation between alleles of spore colour mutation loci has enabled *S. brevicollis* to be used in the analysis of origins of aneuploidy and in the testing of environmental agents for aneuploid induction (Fulton and Bond, 1983; 1984).

The physiological features of *S. brevicollis* when grown on cornmeal agar have made it a useful organism in the investigation of the mechanisms of the sexual response to environmental stimuli. The characteristic "edge effect", in which fruiting structures (protoperithecia) are initiated only after mycelial growth is disturbed, has been studied a great deal, although the mechanism underlying this phenomenon is not yet fully understood (Bond and Broxholme, 1988).

The clear edge effect observed when *S. brevicollis* is grown on cornmeal agar has been exploited in this work as a basis for the separation of the sexual phase of the fungus from the vegetative, mycelial phase, since pure

mycelium can be obtained that is free from sexual structures. The synchrony of protoperithecial initiation has enabled them to be harvested in the knowledge that, at least in the early stages following an initiation event, the majority of protoperithecia are at the same morphogenetic stage.

That *S. brevicollis* is a heterothallic fungus has been of use in the preparation of perithecia. Since protoperithecia will remain as such until a cross to the opposite mating-type is made, the age of perithecia in a cross can be predicted from the time of fertilisation.

The edge effect in *Sordaria brevicollis*, the switch from vegetative colonisation of the substrate to sexual morphogenesis as a result of either reaching the edge of the containing vessel, or as a response to a change in the substrate composition that causes a disturbance in the growth rate, or on confrontation with another microorganism, can reasonably be expected to occur in nature, especially when the colonisation of a substratum by a number of individual fungi is considered. Thus the use of the edge effect provides a system for the study of a developmental switch that possibly represents a phenomenon that would be a fundamental part of the life cycle in the natural habitat of this organism. During the course of this work, conditions were found in which a few of the protoperithecia in a culture would increase in size, resulting in the formation of structures that resembled perithecia, although frequently a neck was absent, and only rarely would spores be formed. This phenomenon occured only in older cultures, at least seven days after inoculation, and it appeared to be enhanced when the culture had been kept in dark conditions. When spores were formed within these perithecium-like structures, they were contained within an ascus, as would spores of a normal cross, but all eight spores of a single ascus were found to be of one mating-type, that of the parent culture. Experiments are now in progress to determine the origin of these perithecia, and by heterokaryon construction it should be established whether the spores arise from one nucleus or two. It is possible that this phenomenon represents the capacity of *Sordaria brevicollis* to resort to a homothallic life cycle, and if so, it may have implications for the evolution of heterothallism or of homothallism in fungi. In comparing all fungi, there is a variety of different types of sexuality, as reviewed by Burnett (1975). The different levels of complexity seen in the Basidiomycetes, for example, would no doubt prove a very daunting prospect for the analysis of the

mechanisms involved at the gene level. It is possible, therefore, that the analysis of a relatively simple system, such as the biallelic heterothallism normally seen in *S. brevicollis* and other Ascomycetes would be a more accessible means of analysing the mating-type process.

A second phenomenon that had not previously been recognised in the development of *S. brevicollis* was the appearance, under certain conditions, of spherical bodies similar to protoperithecia yet larger and lacking both a trichogyne and an ascogonium. These structures only appeared when a culture was grown on cornmeal agar in continuous light. Their appearance was not dependent on the mycelial front having previously reached the edge of the Petri dish, as is the case with protoperithecia.

The identification of the physiological role, if any, of these structures, has not yet been established. It may be that they are analogous to the sclerotia described for *Aspergillus nidulans* and *Sclerotinia sclerotiorum*, which are involved in survival during adverse conditions (see Coley-Smith and Cooke, 1971).

Whatever these sclerotium-like bodies are, and whatever their function in the physiology of *Sordaria brevicollis*, a number of interesting questions arise concerning the mechanisms for their initiation, and their construction (compared, for example, with that of protoperithecia). The role of light in their initiation could be investigated with a view to the identification of a mechanism for the adoption of alternative developmental pathways. As yet, there has not been described a part of the life cycle of *S. brevicollis* outside the sexual phase, which would allow the fungus to withstand adverse conditions such as dehydration. It is believed that the sclerotia observed in some other Ascomycetes perform such a function, and thereby would enable an individual colony to survive without the need to enter the sexual cycle, which would depend, in *S. brevicollis*, upon encounter with a strain of the opposite mating-type. Whether or not the switch to this developmental pathway can be attributed solely to the effect of constant light, or at least to long periods of it, remains to be determined, but it is an interesting possibility.

To be useful in the experimental analysis of morphogenetic changes in the life cycle of an organism, the different stages must be accessible in such a way that they can be separated from other stages. Thus, in *S. brevicollis*,

mycelium must be obtainable that is free of sexual structures, protoperithecia should be developed only so far and not be at the post-fertilisation proliferation stage. Similarly, protoperithecia would ideally be free of undifferentiated mycelium, and perithecia would be free of unfertilised protoperithecia as well as mycelium.

The physiological characteristics of *S. brevicollis* when grown on cornmeal agar satisfy all of these requirements. Cultures remain as vegetative mycelium until after the colony has reached the wall of the Petri dish. The use of a cellulose membrane placed over the surface of the agar medium allows a rapid and efficient method for harvesting of the mycelium, since hyphae have not yet ramified into the substrate and therefore the mycelium can be lifted off by scraping with a microscope slide.

After edge contact, protoperithecia are produced all over the surface of the culture. If an inoculum is placed directly onto the agar surface, protoperithecia are present throughout the culture, above and below the surface. However, with the use of a cellulose membrane, placed as described above, the protoperithecia tend to be situated above the surface of the mycelium. This not only aids the monitoring of protoperithecial production, but also allows efficient harvesting by scraping gently across the surface of the culture with a cover-slip. While this provides ample material for 'cold' protein analysis and *in vivo* labelling of proteins, the yield of messenger RNA from protoperithecia is very low, and their purification for RNA extraction is neither useful nor convenient. For this reason, 4 day-old mycelium was used for the extraction of mRNA from the protoperithecial stage of sexual development. The assumption was made that mycelium of this age would contain the messenger RNAs involved in the initiation and early development of protoperithecia, since they were being produced in abundance by this time, and therefore would be suitable for the analysis of this stage.

Of course, there are changes in the metabolism of the culture when undergoing the transition from vegetative growth to sexual reproduction besides those directly concerned with the morphogenesis itself. For example, there would undoubtedly be some degree of re-utilisation of resources so that mycelial constituents would perhaps be catabolised to provide an energy source for the development of protoperithecia, as described by Zonneveld,

1980. There would be seen, in comparisons between the two mycelial types, changes not directly, if at all related to the production of protoperithecia. Another example would be the changes involved in the attainment of a phase of quiescence, as in *Podospora anserina* (Boucherie and Bernet, 1980; reviewed in Bond and Broxholme, 1988), in which novel proteins may be synthesised as part of the process, and not easily distinguishable from those directly involved in the morphogenesis. Such potential artefacts will be considered later in the light of the results obtained.

The heterothallic nature of *S. brevicollis* means that the perithecial stage – that is, the stage in which the fruitbody undergoes the transition from the structure specialised for the reception of the male gamete, to the maturation of the ascocarp involved in the production and dispersal of the ascospores – does not occur until the culture encounters cells of the opposite mating-type. This can be easily controlled in the laboratory by choosing the time at which the cross is made. Since the time of crossing is known, the development of the perithecia can be assumed to be approximately synchronous in the whole culture, and thus perithecia can be described as being of a known age. In this way, perithecia have been compared at 1, 2, 3 and 4 days after fertilisation. Further characterisation of the stages of perithecial maturation has been made by scanning electron microscopy of the external morphology and internal structure of the fruitbodies (Broxholme, Read and Bond, manuscript in preparation).

Harvesting of perithecia is possible using the same method as that for protoperithecia, but for large-scale purification, a new method has been devised. The use of a muslin disc placed over the surface of the agar in place of the cellulose provides numerous benefits, as described in the Results (section 3.1). The appearance of the perithecia above the surface of the muslin may be a similar effect to that typically observed in a standard technique with *Neurospora crassa* in which a 'wick' of filter paper in the crossing medium enhanced the fertility, and the perithecia in this case were preferentially situated on the parts of the wick that were out of the medium itself. Opinions may vary as to the reasons behind this phenomenon. It may be that there is a reduced supply of nutrients in the wick, therefore favouring sexual development (see Introduction, section 1.2.1.), or the preference for perithecia to be supported by a firm base. Both of these would be plausible

and not mutually exclusive explanations for the phenomenon. In any case, the positioning of the perithecia above the muslin provides simple harvesting of the perithecia, in addition to the added benefits described earlier (Results, section 3.1.).

4.1. Approach to the Project.

After an initial characterisation of the sexual process in *Sordaria brevicollis*, in physiological terms, had been completed in order that definitive preparations of the individual morphogenetic stages could be made, a progressive investigation into molecular changes coincidental with the morphogenesis was undertaken.

Three methods were adopted; the analysis of proteins of known identity which are believed to be involved with the morphogenetic process, the analysis of proteins of unknown identity, by investigation of differences in the patterns of gene expression in different stages, and the analysis of changes in the patterns of transcription during the morphogenesis.

4.1.1. Proteins of known identity: Phenoloxidases.

Many workers have reported correlations between phenoloxidase activity and sexual development. Often these observations have been concerned with the phenoloxidase activity in the whole culture, while others have noted a localisation of activity to the fruitbody itself.

Hirsch (1954) observed the coincidental appearance of tyrosinase activity in a colony of *Neurospora crassa* with melanin formation and sexual development. Tyrosinase activity was assayed by the oxidation of DOPA to dopachrome by a soluble extract of the mycelium. MacDonald (1976) attempted to compare tyrosinase activity in *S. brevicollis* with that in *N. crassa*, by repeating the experiments of Hirsch (1954). Although the *N. crassa* control showed a similar level of activity as that in the original results, no tyrosinase activity could be detected in any stage of the life cycle of *S. brevicollis*, with any of the culture conditions used.

In this Project, phenoloxidase activity has been demonstrated to occur in *S. brevicollis*, but not in the same manner as that described in *N. crassa*. Phenoloxidase activity was clearly seen by the use of *in situ* staining with

phenoloxidase indicators. With each of the indicators used, activity was only seen in the fruitbody itself, not in the whole culture, and there was little evidence for activity in a soluble extract of the fungus – a result reminiscent of that of MacDonald (1976).

Thus there is a clear difference between the phenoloxidase activity in *N. crassa* and *S. brevicollis.* While in both there is an increase observed during the transition to the sexual phase, there is a distinct localisation of activity within the fruitbody of *S. brevicollis,* and little or none elsewhere. Hirsch (1954) did not report a localisation of activity in the protoperithecia of *N. crassa,* but it cannot be assumed that this is not the case. The major difference, therefore, is the lack of a soluble form of phenoloxidase activity associated with the sexual development of *S. brevicollis.*

The demonstration by Hermann *et al.* (1983) of a localisation of phenoloxidase to the cleistothecial primordia of *Aspergillus nidulans* and their enveloping hulle cells is similar to that described here. The hypothesis to explain the pattern seen in *A. nidulans* was that the (laccase) is synthesised in the hulle cells, then transported in a soluble form to the developing primordia, where it is used to cross-link hyphal walls during their aggregation. The cross-linking function of phenoloxidases was suggested by Bu'Lock (1967).

The possibility that phenoloxidase activity is responsible for, or at least correlated with, hyphal aggregation and cohesion is further supported by the results reported here. Activity was predominantly in the parts where hyphal aggregation was active. While there was a generally even staining of the protoperithecia, there was a clear increase of activity in the neck regions of the perithecia. In particular, there was very intense staining at the apex of the neck, where according to Read and Beckett (1985) aggregation and cohesion of periphyses would be occuring in the differentiation of the neck peridium.

There was also phenoloxidase activity observed when the sclerotium-like bodies produced during continuous light conditions were investigated. The localisation differed from the pattern seen in protoperithecia in that there was a more intense staining in the centre of the sclerotia, rather than an even staining throughout. This observation complements that of Read (manuscript in preparation) that there is a loose arrangement of hyphae in the periphery of the sclerotium, while the centre is generally more tightly packed with hyphal

aggregates.

All the observations described in the results (section 3.2) support the suggestion by Bu'Lock (1967) of a role for phenoloxidases in hyphal aggregation and cohesion. It appears that phenoloxidase activity is not restricted to sexual morphogenesis, as seen by the activity within the sclerotia, which do not take part in the sexual cycle. Rather, it is specific to the aggregation, and more specifically, cohesion of hyphae during the formation of multicellular structures.

It was mentioned earlier (Section 1.5.3.) that it is likely that there are numerous roles for phenoloxidases in fungi. It would be unlikely that the mechanism implicated by my results, that of hyphal cohesion, is directly relevant to all the different processes described elsewhere. It is more probable that there are many functions of phenoloxidases, of which that involved in the formation of multicellular structures might represent just one, which may or may not be related to those in other systems.

4.1.2. Proteins of unknown identity.

There are several systems in which changes in gene expression have been investigated during a differentiation process, although few of these involve sexual differentiation, or even more generally the transition to a multicellular state.

In the analysis of sexual development, the use of PAGE has led to initial observations that help to form the basis for further work. Nasrallah and Srb (1973) reported a major protein component of the perithecia of *Neurospora*, which was absent from extracts of mycelium. Further work (Nasrallah and Srb, 1977) describes comparisons between the perithecial proteins of different species of *Neurospora* and other filamentous Ascomycetes. The results were inconclusive – immunological cross-reactivity of the putative major perithecial protein with protein extracts of the perithecia of other fungi did not always show a correlation with the presence of a major band in a polyacrylamide gel. Perhaps by using the more sensitive technique of 2D-PAGE to identify the protein band, and the combined use of monoclonal antibodies with Western blotting would provide a more complete analysis of this phenomenon.

Similar work to that by Nasrallah and Srb (1973) has been reported for the morphogenesis of sclerotia in some fungi (Russo *et al*, 1982; Insell *et al*, 1985; Newsted and Huner, 1987). A similar result was found; that of the presence of a major polypeptide species in the sclerotia, which appears to dominate the protein profile.

Although it provides an adequate method for an initial analysis, the use of cold protein extracts makes it impossible to describe the different protein profiles in terms of differential rates of synthesis. The technique of pulse-labelling with radioactive (^{35}S) methionine has allowed a more accurate description of the rates of synthesis of individual polypeptides to be made. A considerable amount of interest has focussed on the sexual development of the yeast *Saccharomyces cerevisiae*. Analysis of the proteins synthesised by yeast during sporulation showed a rather different situation from that reported for perithecial morphogenesis in *Neurospora*. A major protein species specifically associated with the sexual phase was not detected as such, although there were a number of differences demonstrated by each group of workers which still supported the presence of phase-specific proteins. Trew *et al* (1979) detected nine polypeptide species specific to a/α diploid cells in log phase, and eleven that were present in sporulating, but absent from vegetative cells, however these were also observed in a/a cells under certain conditions. Wright and Dawes (1979) described 18 changes in the cellular polypeptides during meiosis and sporulation, among a background of some 400 species. Further to this, Wright *et al*. (1981) demonstrated the temporal pattern of such changes, and also the possibility that phosphorylation of proteins may play an important part in the control of the differential gene expression.

DeVries and Wessels (1984) found 30 polypeptides synthesised by a fruiting dikaryon of *Schizophyllum commune* which were absent from the parent monokaryons. Also, seven polypeptides were found to be secreted into the medium specifically by the dikaryon in surface-grown cultures but not in liquid medium. Two of these were shown to be associated specifically with the hyphal wall of the dikaryon, irrespective of the culture conditions.

The present work describes the analysis of changes in the protein profiles during the sexual morphogenesis of *Sordaria brevicollis* in two ways. First, the

detection of differences in the cold protein profiles of the different developmental states, and later using a pulse-labelling technique to identify differences in the patterns of protein synthesis during the morphogenesis.

As an initial experiment (result not shown), an attempt was made to repeat the findings by Nasrallah and Srb (1973). No repeatable difference was found in the SDS-PAGE profiles stained with coomassie blue. However, with the use of 2D-PAGE and the more sensitive method of silver staining, a number of differences were observed, although none appeared to be of the magnitude of that expected for a major perithecial protein. It is assumed that the actual number of differences between the protein profiles is more than that observed, since there is a high level of background staining, possibly due to the many proteins which are only evident on reduction of the silver stain.

While the method of silver staining is very sensitive, the precise nature of the stain is not fully understood. It might be, therefore, that the degree of staining, or indeed colouration using this method, is not necessarily dependent on the quantity of protein present within a particular spot, and certainly there is no basis for a quantitative comparison of different spots on the gel. Therefore, the stained gel may give a somewhat distorted view of the polypeptides present.

As mentioned above, the detection of cold proteins in polyacrylamide gels does not necessarily represent a true picture of the patterns of protein synthesis during the developmental process. A more useful method in this respect is the use of radiolabelled amino-acids for pulse-labelling of polypeptides before detection of newly-synthesised protein by fluorography.

First, a method for the efficient uptake and incorporation of the ^{35}S-methionine was required. Other workers using pulse-labelling of filamentous fungi have used mycelium suspended in liquid medium to which the ^{35}S-methionine has been added. Mycelium thus has direct contact with the solution containing the label, and therefore uptake should not be hindered. For *S. brevicollis* grown on cellulose membrane, mycelium was easily labelled either by flooding a culture with a solution of ^{35}S-methionine in sterile dH$_2$O, or by inverting a segment of the membrane onto an appropriate volume of the same solution in a Petri dish. While labelling of mycelial protein in this way rarely presented problems, specific labelling of protoperithecia or perithecia

was comparatively difficult. This is perhaps because protoperithecia and perithecia are not necessarily able to take up label as efficiently through their surfaces as mycelium, so there would be a tendency for more label to be taken up by the underlying mycelium than by the fruitbodies themselves.

A method was therefore devised for the simple and efficient labelling of any stage in the sexual development of *S. brevicollis*. The material was harvested as described in Materials and Methods, and incubated with the ^{35}S-methionine in a test-tube before protein preparation was performed in the same tube. The convenience of the whole labelling and extraction process being done in the same vessel meant that there was minimal loss of sample during the transfer of material from one tube to another. The ability to prepare many samples without the need for cleaning of tubes etc. considerably reduced the time involved for a large experiment.

In vivo labelling analysis was performed for several stages in the sexual cycle. Undifferentiated, vegetative mycelium was used as a control to identify proteins that were specifically expressed during the sexual phase. Protoperithecia were taken from a 6 day-old unfertilised culture. A 6 day-old culture was used because of the high yield of protoperithecia available by the normal harvesting method. At this time, protoperithecia were still being initiated while other stages in protoperithecial development were also represented in the harvested material. At this stage the various maturities of the protoperithecia were of little consequence, since the resulting gel represented proteins that were synthesised during both the initiation and development of the protoperithecia. Perithecia were known to be reasonably well synchronised, since the time of fertilisation was known by the cross being made at a specific time. Thus, perithecia of a certain 'age' were known to be at no later a stage of maturation, although the possibility of some protoperithecia being fertilised at a later time than others could not be ruled out. Since there are clear differences between the protein profiles of different ages, however, it was assumed that this assignment of age to the perithecia was valid – there were major differences seen between perithecia fertilised within 24 hours of each other.

Fourteen polypeptides were identified which appeared in protoperithecia but not in vegetative mycelium, and 17 others appeared only after fertilisation.

As shown in Table 2, there are numerous different patterns of expression with respect to the morphogenetic stage in which individual spots first appear, and also to the subsequent pattern of expression in later stages. This would suggest that there is no one single mechanism involved in the induction of such proteins. It is more likely, and indeed would be expected, that there is a complex sequence, or network, of interactions resulting in the differential patterns of expression. The results of the *in vivo* labelling experiments provide a convenient way of describing the types of change occuring in 'net gene expression', that is, the synthesis of the phase-specific polypeptides. It is evident that not all such proteins are synthesised with the same temporal pattern, and similarly, there are clear differences between the relative levels of synthesis of individual protein species at different time-points during the developmental program.

4.1.3. Analysis of changes in RNA population.

Although the analysis of polypeptides synthesised *in vivo* gives useful information about such control phenomena, it gives no immediate indication of the level at which the control is taking place. *In vitro* translation is one method which would show differences between the translatable mRNA species present at the different stages. Thus, control mechanisms that would normally act at the translational level *in vivo* would not be expected to contribute to changes in the patterns of protein synthesis seen in these gels.

In vitro translation also provides a basis for the use of mRNA to identify differences in transcription during the developmental sequence, and then to isolate the genomic DNA sequences undergoing differential expression. This was part of the intended program of work in this project.

The results of *in vitro* translations using mRNA from vegetative mycelium, protoperithecial mycelium, and perithecia, shown in figures (15 – 16), give the indication that there are many polypeptide (and hence mRNA) species which are common to all stages in the sexual development. However, many of these have different intensities relative to their counterparts in the other stages. Indeed, compared with the *in vivo* gels, the *in vitro* translations were more difficult to compare, partly because of the variation in intensity of the 'landmark' protein species.

There is evidence from these gels that there are qualitative differences between the mRNA populations of the different stages, and therefore some degree of trancriptional control is involved.

In comparing the *in vitro* translation results, it can be seen that, in general, the gels of the different developmental stages are very similar in the patterns of polypeptides. The differences indicated (figures 15 – 16) demonstrate that there are clearly identifiable changes in the diversity of poly(A) RNA species during the morphogenetic process. While many species evidently cease to be synthesised on the transition from vegetative to protoperithecial mycelium, as can be seen by the reduced number of spots in figure 16 *iii*, there are some species that only appear after this transition. Thus the major changes appear to be in the switching off of gene expression. This kind of pattern might be expected in the transition of the mycelium from a phase of vegetative growth to one of quiescence, where growth is reduced – the metabolic status of the mycelium would be altered in that certain biochemical pathways would be closed off, either for lack of substrate or because of the reduced need for a product. The apparent lack of 'new' species in the protoperithecial mycelium may be accounted for by the contamination of the protoperithecia with large quantities of vegetative material, such that only major changes in the fruiting structures would be represented in the observed patterns, less prominent changes being masked by the background pattern.

In the comparison between protoperithecial mycelium and perithecia, it is clear that most of the changes now occur in the appearance of new species, rather than the reduction in expression seen before. While these changes appear to occur coincidentally with the proliferation of the fruitbodies, it cannot be assumed that they are all directly related to the morphogenetic process. However, since the RNA was prepared from purified perithecia, it is more likely that these changes are morphogenetically regulated, than that they are due to metabolic changes in the underlying mycelium.

The numbers of mRNA species that show changes in the morphogenetic sequence may well represent only a minimum estimate of the total changes occuring. For example, there may be changes that are not detectable by this method of analysis, perhaps because of the location within the fruitbody, or within the cell, which may have an effect on the extractability of a particular

species of RNA. Also, *in vitro* translation as a method of analysis has a limitation in that detection of polypeptides synthesised in the cell-free system depends to a certain extent upon their methionine content. Thus, it may be assumed that the number of species involved may be extrapolated to an unknown, larger figure, which might more easily be detected by using a different system of analysis. For example, Timberlake (1980) used a technique of mRNA - cDNA hybridisation to determine the complexity of poly(A) RNA, and hence to analyse differential transcription of conidiating *Aspergillus nidulans*. During this morphogenetic transition, it was estimated that between 700 - 1100 new species of poly(A) RNAs were synthesised, constituting some 20% of the total poly(A) RNA.

The changes observed in the *in vitro* translation experiments do not reflect the changes seen by *in vivo* pulse-labelling, and this result is not unexpected. In the former, detection of species depends upon the efficient preparation of high quality poly(A) RNA of which the ease of extraction may depend upon the location within the cell or tissue, and this might not reflect the extractability of the equivalent protein product in the *in vivo* experiments. Many of the polypeptide differences observed by *in vivo* labelling were of high molecular weight, often over 100 kDa; polypeptides of this size would rarely be detected in *in vitro* translations, owing to the requirement of very high quality, intact mRNA. Also, if post-translational modification (such as glycosylation) of a protein were responsible for its high molecular weight, this would not occur in the cell-free system. Thus, the prominent polypeptides with high molecular weights such as P13 and P14 (figures 11*i* - 13*vi*) would not necessarily be observed as such.

In addition, since protoperithecia were purified for the *in vivo* labelling experiments, relatively minor differences would be observed easier than in the protoperithecial mycelium from which mRNA was prepared. This would explain the relative numbers of new polypeptides detected in the protoperithecial stage by the two methods of analysis.

4.2. Molecular biology of sexual development.

The analysis of molecular events in the sexual morphogenesis of *Sordaria brevicollis* has shown that there are several clearly identifiable changes in the nett gene expression, as visualised by the comparison of proteins synthesised

in vivo from different stages in the morphogenetic process. Such changes have also been shown by comparing the translation products of poly(A) RNA isolated from different stages. However, owing at least partly to the difficulty in preparing high quality RNA from certain stages, the *in vivo* patterns of new polypeptides are not fully represented in the *in vitro* translation analysis. The intended approach to the isolation of the genes involved in the morphogenetic process was to be by the identification of genomic sequences that specifically hybridised to labelled RNA from the sexual phase. This detection method would not involve the limitations conferred by *in vitro* translation. RNA species would be represented by radioactive labelling irrespective of the quality of the preparation, and there would be a reduced chance of a particular species not being represented during the screening program. Furthermore, should problems arise because of developmentally regulated sequences being in the same fragment of genomic DNA as those expressed in mycelium, there are methods available to overcome this. One such method would be to pre-hybridise the genomic sequences with an excess of unlabelled RNA from the undifferentiated state, prior to hybridisation with the labelled RNA from the sexual stage. This would have the effect of blotting out sequences transcribed in the mycelium, leaving other sequences available for hybridisation. This method of 'hybridisation-competition' is similar to that used by Mangiarotti *et al.* (1981) in isolating sequences specifically expressed in differentiating cells of *Dictyostelium discoideum.* Another method would be to adopt the 'cascade hybridisation' technique of Timberlake (1980) whereby labelled cDNA would be prepared from the mRNA of the sexual material, and from this, phase-specific sequences are purified by removal of those which hybridise to RNA from undifferentiated mycelium.

Work on the morphogenetic development of the slime mould, *Dictyostelium discoideum* illustrates some features which might be appropriate in the investigation of the *Sordaria* system. Firtel (1972) showed, by RNA-DNA hybridisation kinetics, that between 36 and 38% of the single-copy portion of the genome is differentially transcribed through the developmental sequence. Williams and Lloyd (1979) identified cDNAs whose complementary RNAs changed in abundance during the morphogenesis. This was shown by hybridising radioactively labelled RNA to filter-bound cDNA; the strength of the resulting signal was taken to be proportional to the abundance of the specific RNA corresponding to the cloned cDNA. They found that only a small fraction

of RNA species increased significantly during the morphogenesis. Rowekamp and Firtel (1980) isolated developmentally regulated genes from *D. discoideum* by screening cDNA libraries with end-labelled RNA from different developmental stages. Mangiarotti *et al.* (1981) selected cloned fragments of genomic DNA containing developmentally regulated sequences by hybridising with an excess of RNA from the undifferentiated state simultaneously with labelled mRNA from aggregating cells. An interesting observation made here was that several clones appeared to encode a set of discrete mRNA species which accumulated in the same temporal pattern. Later it was estimated that there are between 2000 and 3000 species of RNA induced during aggregation. Transcriptional control was demonstrated in that RNA sequences present at all stages were transcribed from the nuclei at all stages. But sequences specific to aggregation and post-aggregation stages were transcribed only at these stages.

Molecular analysis of sexual development in filamentous fungi has been begun with investigations into the sexual cycle of the Basidiomycete, *Schizophyllum commune* (Dons *et al.* 1984; Mulder and Wessels, 1986). Wessels' group (see Wessels, 1987) have found that the mRNAs corresponding to their cDNA clones are indeed under the control of both the mating-type genes. Wessels *et al.* (1987) have since shown that the expression of these genes, which can occur in conditions inhibitory to fruiting, are probably necessary for, but not sufficient for, the fruiting process.

Developmental regulation of phase-specific gene products by means of transcriptional control is a well-studied concept. This is perhaps not surprising on account of it being more accessible to investigation than other modes. The extraction and comparison of messenger RNA from different developmental stages is a quite powerful tool by which a limited number of conclusions can be drawn.

There is still much more information required before a mechanism for an integrated program of transcriptional, or other control can be elucidated. By identifying the elements that show changes during the cycle we can monitor these changes under varying conditions, perhaps ultimately describing the circumstances in which differentiation occurs. However, we will still have little evidence for the nature or identity of the elements that perform the controlling

function, nor of the mechanism by which it acts or of the function of the gene product under control.

After the cloning of developmentally regulated genes, further investigation into the mechanisms of transcriptional control would involve comparisons of the regions of the genome concerned with the initiation of transcription, namely the 'operator' and 'promoter' regions lying upstream of the coding sequence. There is evidence that genes transcribed under a common mechanism show some similarity in their 5' upstream regions, suggesting a mechanism involving the recognition of a specific sequence or sequence pattern in this region. There has been considerable progress in the analysis of the quinic acid utilisation genes of *Neurospora crassa*. Structural genes for enzymes involved in the process are found in a group known as the *qa* cluster, which also contains the regulatory genes *qa-1F* and *qa-1S* (Giles *et al.*,1985). The activator protein encoded by *qa-1F* has an affinity for numerous sites in the gene cluster, each characterised by a 16 base-pair sequence of partial dyad symmetry (Baum and Giles, 1986; Baum *et al.*, 1987). One of these sites lies between the *qa-1F* and *qa-1S* regulatory genes, thus implying some degree of autoregulation. In addition, the presence of multiple binding sites at variable positions upstream of the *qa* structural genes appears to allow greater transcriptional control by the *qa-1F* protein.

If the clustering of genes with related function is a general rule, it may be that the approach taken in this project, that of screening for genomic fragments containing developmentally regulated genes, would allow such clusters to be found instantly. Such a situation has been found by Timberlake's group in the investigation of genes expressed specifically during conidiation in *Aspergillus nidulans*. It is interesting that there is a non-random distribution of these phase-specific genes in the genome, such that there is a cluster of sequences of which the transcripts accumulate specifically in conidia (Zimmermann *et al.*,1980; Timberlake and Barnard, 1981; Orr and Timberlake, 1982). Miller *et al.* (1987) investigated the effects of altered chromosomal position on the expression of a gene from the *spoC1* gene cluster. The gene became deregulated when at non-homologous chromosomal sites – the level of expression in vegetative cells was elevated, while that in conidia was variable. In addition, the insertion of the non-regulated gene, *argB*, into the region repressed expression of the gene in vegetative hyphae, while

expression in conidia was variable. As well as this evidence for a *cis* acting control mechanism, there was evidence for a *trans* acting element in the regulation of the *spoC1* genes.

If the mechanism of developmental regulation of genes were to be similar in nature to that of such systems as the *qa* cluster of *Neurospora crassa* or the *SpoC1* cluster of *Aspergillus nidulans*, then the possibilities for further investigation of the *Sordaria* system described here is all the more promising.

Clustering of genes with related functions also suggests a role for chromatin structure in gene control. That the conserved binding site for the *qa-F1*-encoded activator protein includes an inverted repeat sequence would be consistent with a role for the conformation of the DNA in this region, perhaps in recognition by the binding protein.

It is interesting that there is evidence that the target of the *qa-1F*-encoded repressor protein appears to be the activator protein itself rather than the DNA (Giles *et al*, 1987). This would imply that there may be more than one level of control in other systems also. It would not be surprising, therefore, if a complex sequence of events such as in multicellular differentiation were to turn out to involve a very complicated mechanism of gene control.

There could be no doubt that the individual steps involved are required in more than one pathway. For example, the construction of protoperithecia, perithecia and sclerotia in *Sordaria brevicollis* would all involve similar mechanisms for cell adhesion, though the end result in each case is different, and the initiating factors for the three pathways are also dissimilar.

Therefore, we are confronted with what promise to be a complicated story, beginning with the concept of alternative morphogenetic pathways, possibly involving similar mechanisms in the morphogenesis itself, and terminating with the appearance of discrete multicellular structures, of different external morphology, internal anatomy and function.

I. Abbreviations used in this Thesis

Ac	Acetate
ADBP	4-amino-dibromophenol
ATP	Adenosine Triphosphate
BME	β-mercaptoethanol
CMA	Cornmeal agar (see appendix 2)
CsCl	Caesium chloride
DA+	Dissecting agar + acetate (see appendix 2)
DMA	Dimethyl aniline
DOPA	Dihydroxyphenylalanine
DTE	Dithioerythritol
DTT	Dithiothreitol
dH_2O	distilled water
EDTA	Ethylene diamine tetraacetic acid
EGTA	ethylene glycol-bis(β-amino ethyl ether)-N,N,N',N'-tetraacetic acid
EtBr	Ethidium bromide
HOAc	Acetic acid
KOAc	Potassium acetate
L	see appendix 2
min.	minutes
m.o.i.	multiplicity of infection; the ratio of phage particles per cell in a liquid lysate.
NaOAc	Sodium acetate
NH_4OAc	Ammonium acetate
pfu	plaque-forming units
PSB	Phage suspension buffer
rpm	Revolutions per minute

RT	Room temperature
SDS	Sodium dodecyl sulphate
STET	see appendix 2
T	T broth; as L broth without yeast extract
TCA	Trichloroacetic acid
TMB	Tetra-methyl benzidine
TMN	see appendix 2

II. Media and Buffers

CMA	Per litre: 17g Cornmeal agar (Difco), 2g glucose, 3g sucrose, 1g yeast extract
DA+	CMA + 23g per litre Difco agar + 0.7% Sodium acetate
L broth	1% Tryptone, 0.5% Sodium chloride, 0.5% Yeast extract
L agar	L broth + 1.5% Bacto-agar
L top agar/ agarose	L broth + 0.7% Bacto-agar/agarose
PSB	Phage suspension buffer; TMN + 0.05% gelatin
STET	8% sucrose, 0.5% Triton X-100, 50mM EDTA pH 8.0, 50mM Tris-Cl pH8.0
T broth	1% Tryptone, 0.5% Sodium chloride
TMN	10mM Tris-Cl pH 7.5, 0.1mM NaCl, 10mM $MgCl_2$
Vogels agar	Vogel's (1956) salt solution + 2% glucose + 1.5% Difco agar

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