ASCUS ANALYSIS AND THE INDUCTION OF PROTOPERITHECIA IN *SORDARIA BREVICOLLIS*

by

Douglas MacDonald

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This thesis was composed by myself and describes my own work except where otherwise stated either in the acknowledgements or in the text.

Some of the results here described have been previously published. This publication is bound at the back in Appendix II.

Acknowledgements

The results in Tables 5 and 10 involved the dissection of large numbers of asci. About half of these dissections were performed by Dr D.J. Bond whose assistance I gratefully acknowledge. I am also indebted to Miss S.L. Brown and Mr B. Doyle for occasional technical assistance with the biochemical assays described in Part 2. The organic phosphate assay techniques were kindly supplied by Dr I.W. Flynn. I am grateful to Dr N. Hastie for advice and assistance with the cAMP assays.

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ABSTRACT

The frequency of spindle overlap during ascus development in Sordaria brevicollis has been found by different workers to vary widely between crosses. This variation was confirmed and it was proposed that it can be accounted for largely by assuming that different types of asci have different rates of maturation and discharge. This assumption was confirmed by observation. In single point crosses the frequency of asci with a 2:2:2:2 distribution of wild type and mutant spores declined as the crosses matured. It was proposed that either spindle overlap asci or 2:2:2:2 asci per se matured and were discharged preferentially. Ascus dissection experiments failed to distinguish between these alternatives.

The production and distribution of perithecia and protoperithecia in Sordaria brevicollis is determined by environmental and genetic factors. It was found that the primary stimulus to protoperithecium production under laboratory conditions was an interaction between a growing culture and a physical barrier, such as a petri dish wall. Exhaustion of nutrients and the accumulation of organic substances in the medium did not play a major role in the induction of fruiting. Two genes, per-1 and per-2, which affect fruiting have been found. Per-1^- per 2^- cultures were completely devoid of protoperithecia. Per 1^- per 2^+ cultures have a characteristic phenotype with a fertile colony centre and a sterile margin. The concentration of some phosphorylated intermediates of carbohydrate metabolism dropped markedly in the sterile regions of cultures when their growth was impeded by a physical barrier. The nature of the interaction between a culture and a barrier was speculated on. No evidence was found to suggest a role for cyclic AMP in the regulation of these morphogenetic events.

Part 1

Variation in the frequencies of different ascus types during cross development in Sordaria brevicollis

Part 2

Protoperithecial production in Sordaria brevicollis

Part 1

Variation in the frequencies of different ascus types during cross development in *Sordaria brevicollis*

CHAPTER 1

Introduction

Ascus formation

Sordaria brevicollis (Olive & Fantini, 1961) is a heterothallic Ascomycete, the mating types being designated A and a. The mycelia of the two mating types carry both male and female reproductive structures and are morphologically indistinguishable. The life cycle is shown in Figure 1. There are no macroconidia and the microconidia germinate infrequently or not at all. The absence of asexual propagules makes the organism extremely non-prolific and contamination of cultures with *Sordaria* from an external source is virtually unknown. Their absence, however, removes one of the advantages of related fungi such as *Neurospora* in which the germination of conidia facilitates such procedures as the isolation of auxotrophic mutants.

A wild type culture grown in a 9cm petri dish differentiates protoperithecia, the female reproductive structures, after about 3.5 days growth. The protoperithecia are distributed evenly over the surface of the colony. They may be fertilized either by microconidiation, in which a suspension of microconidia in water is poured over them, or by confrontation of mycelia of opposite mating types.

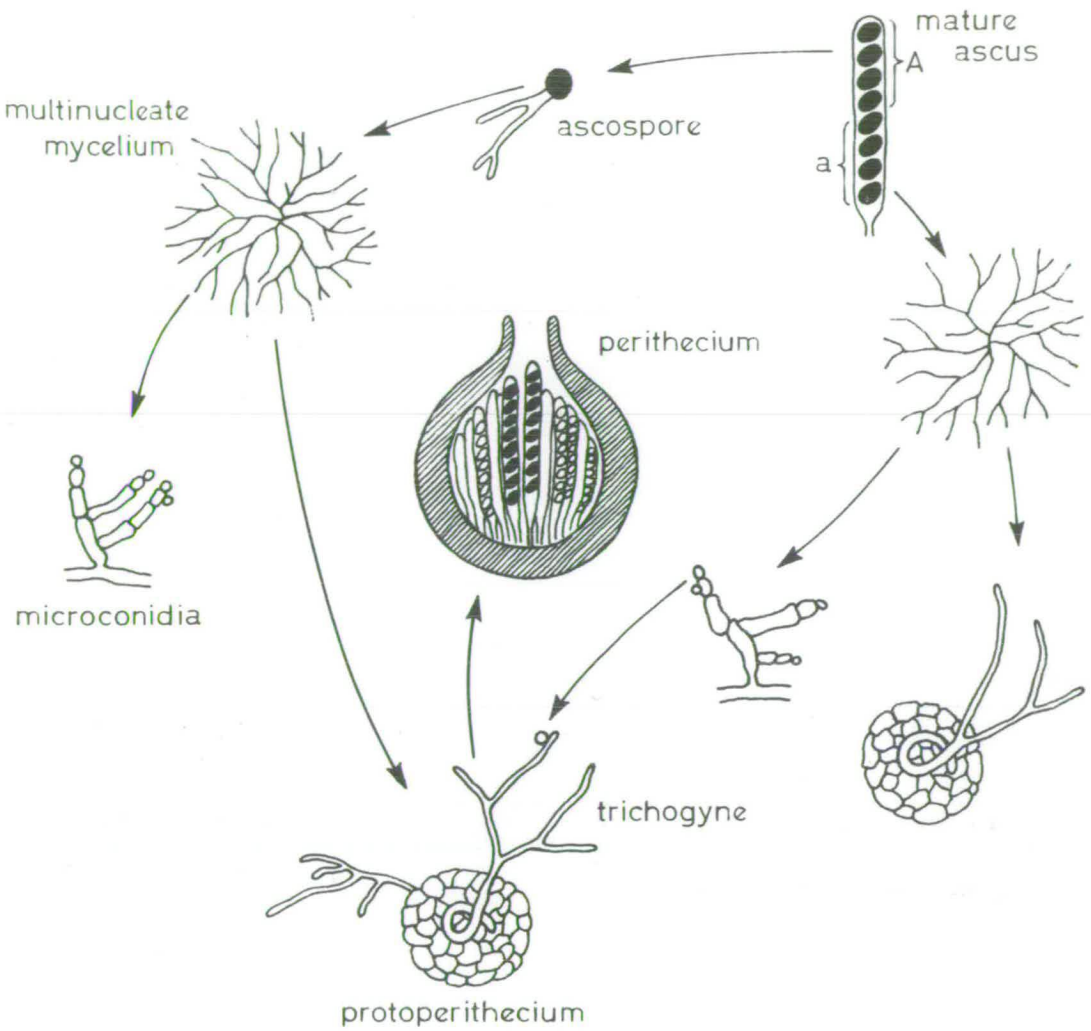
Ascus formation proceeds when a pair of nuclei, of opposite mating types, become associated and begin to divide synchronously. The products pass into ascogenous hyphae. At the same time the melanic perithecial wall develops and surrounds the numerous ascogenous hyphae.

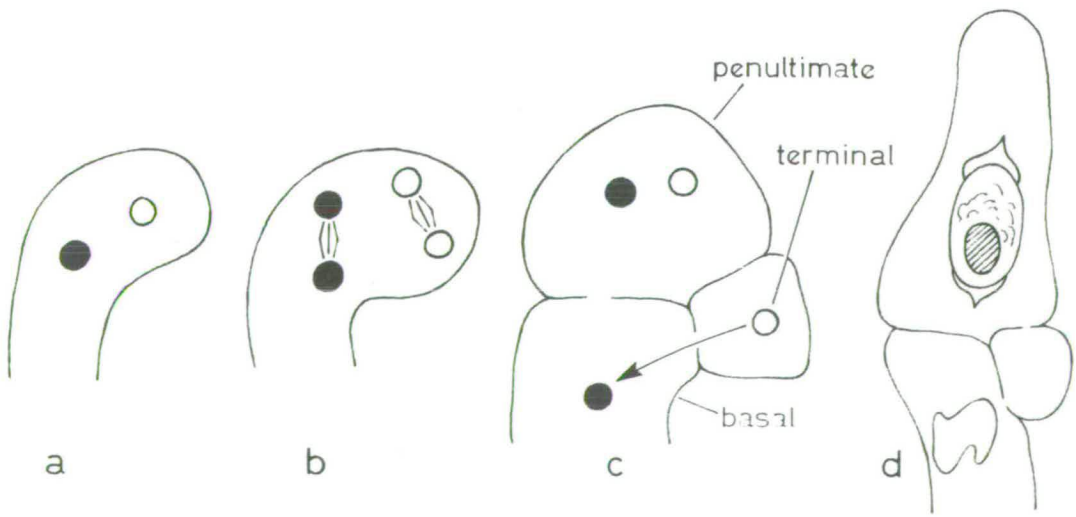
The further development of the asci in *S. brevicollis* has been the subject of cytological study by Mu'azu (1973). The pair of nuclei

Figure 1

Life cycle of *Sordaria brevicollis*

The lower part of the figure illustrates the early nuclear events occurring during ascus formation (From Mu'azu, 1973).


mature
ascus
A
a
ascospore
multinucleate
mycelium
perithecium
microconidia
trichogyne
protoperithecium


penultimate
terminal
basal
a
b
c
d

in the ascogenous hyphae divide and each daughter nucleus fuses with one of opposite mating type. One of the resulting diploid nuclei is contained in the ascus initial while the other is contained in the basal cell and probably breaks down eventually. The basal cell does not appear to form a secondary crozier (see Fig. 1).

The sequence of nuclear divisions in ascus formation is assumed to be similar to that described in Neurospora (Colson, 1934; Singleton, 1953). The first and second divisions of the nucleus in the ascus initial are normal meiosis. The third division is a normal mitosis forming an eight spored ascus. Mu'azu found all nuclei in an ascus dividing synchronously at each division.

At the first meiotic division the centromeres of ~~any~~ all bivalents separate. In the absence of biasing factors the four proximal spores of an ascus will contain copies of the centromere introduced into the bivalent by one parent while the distal spores will contain copies of that introduced by the other parent (Lindegren, 1933). A centromere-linked gene will most frequently segregate ~~unrecombined~~ with its centromere. A cross involving a pair of alleles will produce asci in which the distribution of the alleles is determined by the number and relationships of recombination events between the locus in question and its centromere. Sequencing asci can therefore be used to investigate the interaction of chromosomes during meiosis resulting in recombination and the associated events of gene conversion and post-meiotic segregation.

In S. brevicollis a large number of spore colour mutants are known and their use facilitates tetrad analysis.

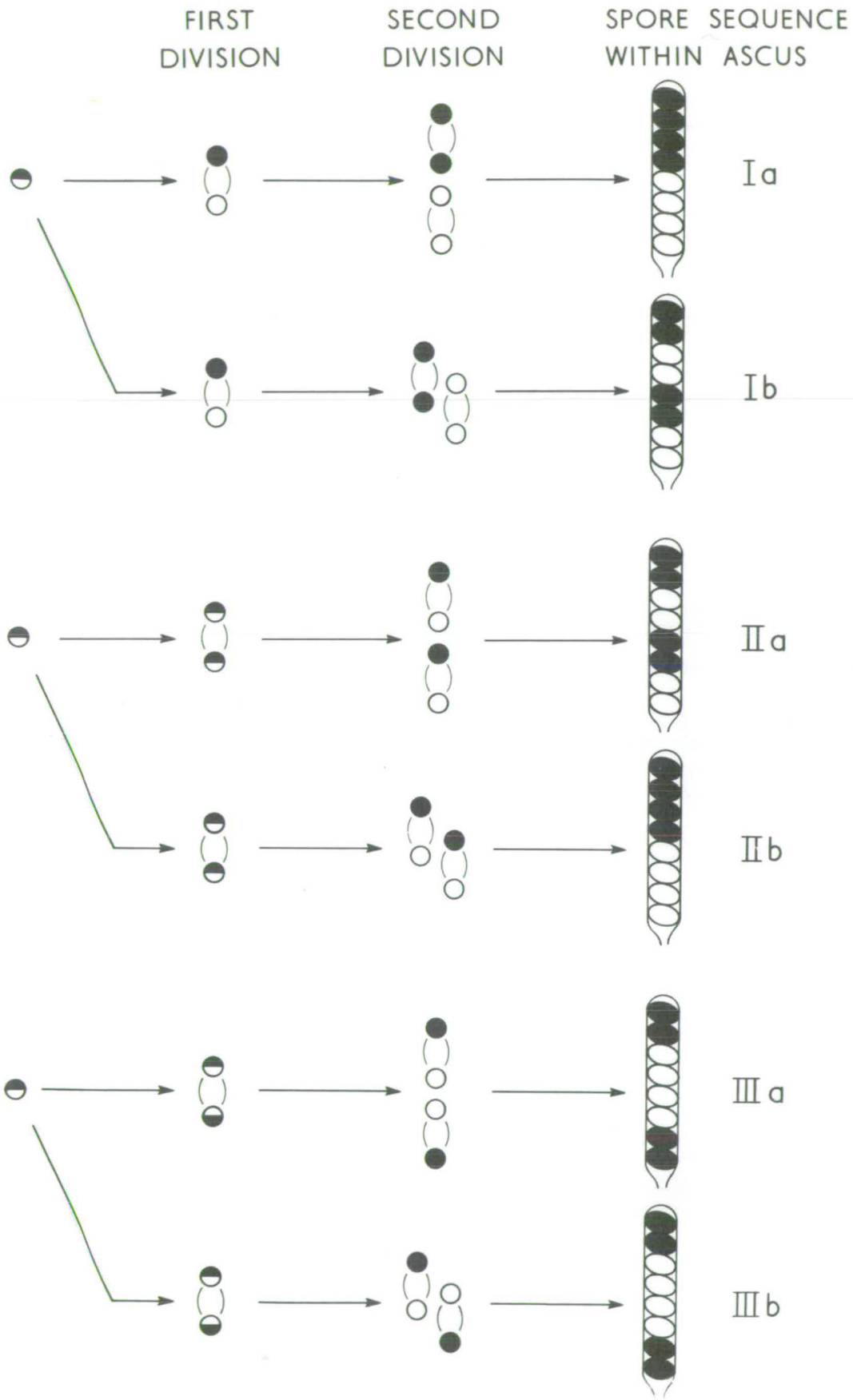
1st and 2nd Division Segregation

In single point crosses (wild type x single mutant) the majority of asci have one of three spore sequences, 4+:4m, 2+:2m:2+:2m or 2+:4m:2+. Their origins are shown in Figure 2. Ignoring for the moment the effects of spindle overlap, (Ib, IIb and IIIb in Fig. 2) it can be seen that the 4:4 arrangement arises from meiosis in which there is no recombination between the gene ~~in question~~ and its centromere, (Ia). The ~~nuclei are homogeneous~~ half-bivalents carry identical alleles after the first division of meiosis, thus 4:4 asci involve first division segregation of the wild type and mutant alleles. A single ~~recombination~~ chiasma between gene and centromere gives rise to a second division segregation sequence, either 2:2:2:2, (IIa), or 2:4:2, (IIIa). Symmetrical (2:4:2) and asymmetrical (2:2:2:2) sequences are equally probable, i.e. the inclusion of a chromatid in a recombination event does not affect the probability that it will ultimately be included in a terminal or a central spore pair. This is confirmed in S. brevicollis by the data of Chen (1965) for the spore colour mutant g_4. g_4 shows very loose linkage to the centromere of linkage group II (2:4:2 frequency = 31.2±1.4%) and has almost equal numbers of asymmetrical (328) and symmetrical (332) second division segregation sequences.

Certain infrequent meiotic events may give rise to spore sequences different from those described above. Gene conversion produces asci in which there are unequal numbers of wild type and mutant spores. Most commonly one of the alleles has been converted into the other in one or two spores giving 5:3 or 6:2 sequences. In post-meiotic segregation asci the number of spores may be 4:4 but the distribution is such that the alleles appear to have segregated at the post-meiotic

Figure 2

The origins of the three major types of asci and the consequences of partial second-division spindle overlap. Ia and Ib have no recombination between the spore colour mutant and its centromere. Recombination results, in the absence of spindle overlap, in asymmetrical (IIa) or symmetrical (IIIa) second division segregation sequences.


FIRST DIVISION
SECOND DIVISION
SPORE SEQUENCE WITHIN ASCUS
Ia
Ib
IIa
IIb
IIIa
IIIb

third division, e.g. 3:1:1:3. Gene conversion asci often also show post-meiotic segregation. In a 5:3 ascus at least one spore pair must contain one mutant and one wild type spore. Gene conversion and post-meiotic segregation are often associated with recombination events which occur very close to the segregating alleles. Post-meiotic segregations containing equal numbers of each spore type (i.e. aberrant 4:4's) cannot easily be detected as spore distribution may also be affected by spindle overlap.

Spindle overlap

Spindle overlap or nuclear passing gives rise to asci in which the spore sequence is changed by mechanical rearrangement of nuclei after the second meiotic division. Spindle overlap may be partial, in which a dividing nucleus interposes one daughter nucleus between the daughter nuclei of a second dividing nucleus, or complete, in which one pair of daughter nuclei is completely bracketed by another. Overlap may occur at the second or third divisions in ascus formation. The consequences of partial spindle overlap at the second division are illustrated in Figure 2. First division segregation asci are converted into 2:2:2:2s (Ia$\longrightarrow$Ib). Asymmetric second division asci are converted into 4:4s (IIa$\longrightarrow$IIb). Symmetric second division asci are unaltered. Complete second division overlap also causes transfers between classes. As the frequency of this event is less than 1% (Berg, 1966) its effect on ascus type frequencies is minimal.

Third division overlap may also be partial or complete. The products of some of the most common third division overlaps are shown in Figure 3.

Figure 3

The consequences of some of the most common partial third-division spindle overlap events.

3 : 1 : 1 : 3
2 : 1 : 1 : 1 : 1 : 2
2 : 2 : 1 : 1 : 1 : 1

Estimation of recombination and spindle overlap frequencies

In a random sample of asci the frequencies of different types of asci can be used to estimate the frequencies of recombination and spindle overlap. In single point crosses the frequency of recombination between a gene and its centromere is a function of the frequency with which the alleles involved segregate at the second division. As only half the spores in a second division segregation ~~asci~~ asci are recombinants the recombination frequency is calculated as half the frequency of such asci.

The notation used throughout is that of Whitehouse (1957) where:

p = frequency of 2nd division segregation

s = frequency of symmetric 2nd division segregation

r = frequency of asymmetric 2nd division segregation

x = frequency of partial spindle overlap at the 2nd division

In the absence of spindle overlap,

$$p = r + s$$

and $$r = s$$

$$p = 2s$$

1) Recombination frequency = $p/2 = s$

The maximum value of s is 0.33: Random spore analysis gives maximum recombination frequencies of 0.5. The disparity in these values results from the inability to recognize non-parental ditypes in single point crosses.

Spindle overlap is manifested as an excess of 2:2:2:2 over 2:4:2 sequences.

Excess asymmetric asci = r - s

This formula corresponds closely to the frequency of partial second division spindle overlap only for closely centromere linked genes. It does not take into account the loss of recombinant 2:2:2:2s through overlap converting them to 4:4s. As the frequency of recombinant 2:2:2:2s increases with gene - centromere distance excess asymmetric asci becomes an increasingly inaccurate measure of spindle overlap frequency.

The frequency of partial second division spindle overlap (x) is calculated using the formula:

2) $$x = \frac{r - s}{1 - 3s}$$

Whitehouse (1957) gives this formula as

$$x = \frac{2r - p}{2 - 3p}$$

Formula 2 is derived as follows

Total 2:2:2:2s = non-recombinant overlapped asci + $\frac{1}{2}$ recombinant non-overlapped asci.

$$r = (1-2s)x + \frac{1}{2} 2s (1-x)$$

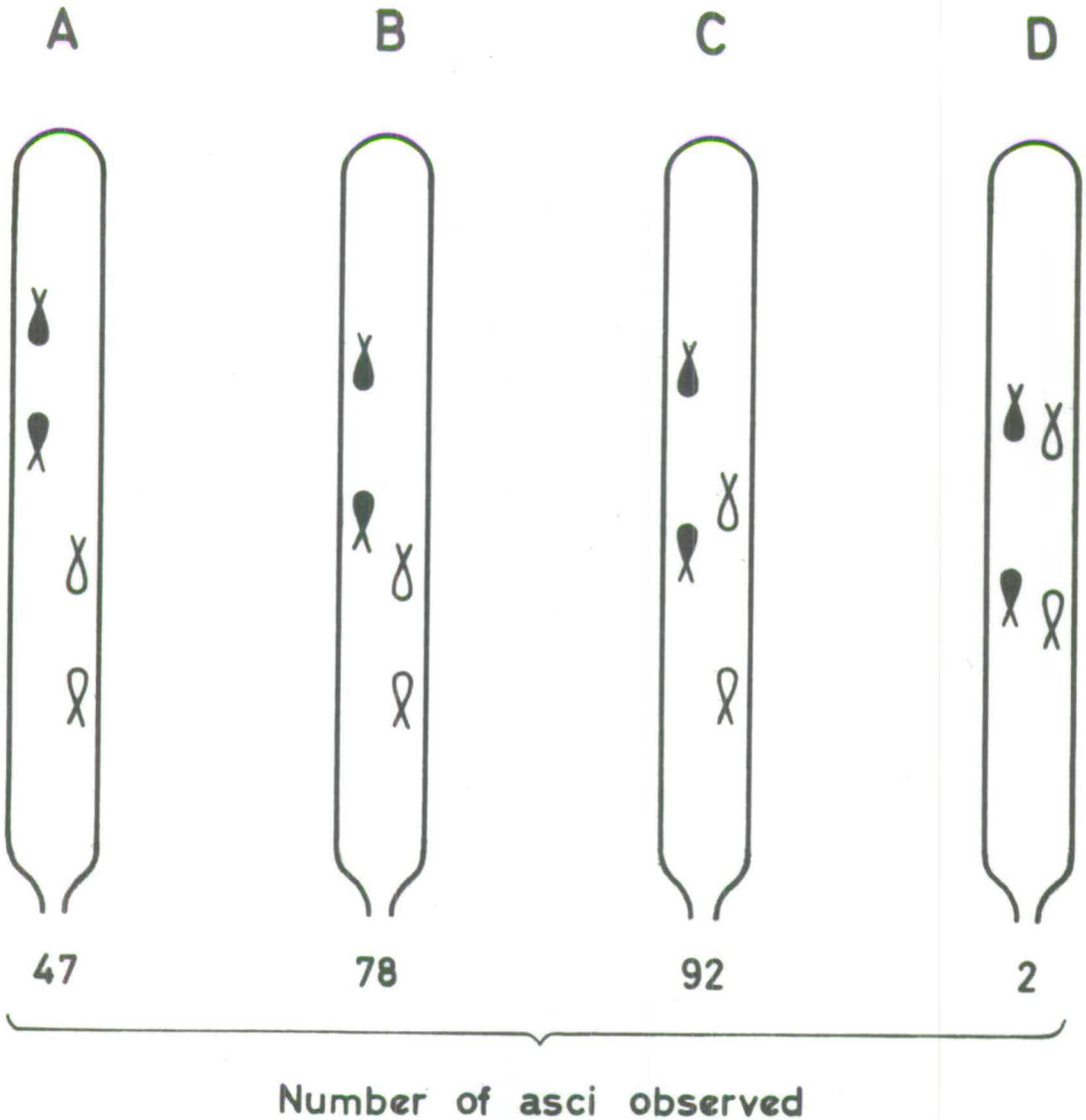
Solves to $x = \frac{r-s}{1-3s}$ (2)

Formulae 1 and 2 can be applied to any single point cross to give the frequencies of recombination and spindle overlap.

Mu'azu identified spindle overlap cytologically in S. brevicollis during interphase of the second division. He classified four types of nuclear arrangement, reproduced in Figure 4. Type C asci contain partial spindle overlap. Type B could result in third division over-

Figure 4

Four types of nuclear arrangement observed cytologically during interphase of the second division (from Mu'azu, 1973).


A
B
C
D
47
78
92
2
Number of asci observed

lap of central nuclei or in second division overlap. As third division overlap is independent of second division overlap (Berg, 1966) it is likely that type B asci lead to second division overlap with the same frequency as they lead to no second division overlap. Mu'azu does not comment on the discrepancy between the frequency of type C asci (42%) and the highest estimate of spindle overlap he obtained through tetrad analysis (19.4%).

Variation in spindle overlap frequency

Chen and Olive (1965) found variation from 0 to 78% in the frequency of excess asymmetric asci in 18 crosses involving 10 pairs of alleles. Their data, and the frequency of spindle overlap calculated using formula 2 appear in Table 1.

There are two sources of variation in the frequency of excess asymmetric asci. The first is variation in spindle overlap frequency (x), and the second is variation in gene - centromere distance (=s). Formula 2 compensates for variation in the value of s when calculating spindle overlap frequencies. As the gene - centromere distance increases the accuracy with which x can be estimated decreases. In a cross involving a centromere unlinked gene (e.g. g_4), equal numbers of the three major ascus classes are expected whatever the value of x.

Other workers have also observed a large amount of variability in spindle overlap frequency (Berg, 1966; Bond, 1969; Shaw, 1962; Mu'azu, 1973). Berg suggested that the range observed by Chen and Olive might be caused by differences in the genetic background of their isolates. She accounted for her smaller range (27% to 47%) on the grounds that her strains were more isogenic through backcrossing.

Table 1

Frequency of excess asymmetric asci and partial second division spindle overlap asci in single point crosses of 10 spore colour mutants. With the exception of the final column the data is that of Chen and Olive (1965).

$$\text{s.e. of \% excess asymmetric asci} = \pm\sqrt{\frac{\frac{f}{n}(1-\frac{f}{n})}{n}}$$

where f = [2:2:2:2] - [2:4:2] in a total of n

The frequency of spindle overlaps is calculated according to formula 2 (see p. 6) where

$$x = \frac{r-s}{1-3s}$$

The standard error of x is calculated according to the formula,

$$\text{variance of } x = \frac{a(b-c)^2 + b(a-c)^2 + c(a-b)^2}{(n-3c)^4}$$

where a = no of 4:4s; b = no of 2:2:2:2s and c = no of 2:4:2s.

The formula for the variance of x was kindly supplied by Dr E.C.R. Reeve.

Data from Chen & Olive (1966)							Frequency of partial second division spindle overlaps calculated by formula 2 (p. 6)
Cross no.	Cross	Locus	Spore sequence			% excess asymmetric asci	
			4:4	2:2:2:2	2:4:2		
1	$y_2 a x y_2^+ A$	y_2(I)	763	23	3	2.5±0.6	2.6±0.6
2	$y_2 A x y_2^+ a$	"	246	77	1	23.5±2.4	23.7±2.4
3	$b_1 a x b_1^+ A$	b_1(II)	186	395	15	63.8±2.0	69.0±2.1
4	$b_1 A x b_1^+ a$	"	129	588	17	77.8±1.5	83.6±1.6
5	$g_3 a x g_3^+ A$	g_3(II)	485	12	10	0.4±0.3	0.4±1.0
6	$g_3 A x g_3^+ a$	"	428	165	10	25.7±1.8	27.1±1.9
7	$g_4 a x g_4^+ A$	g_4(II)	49	61	62	-0.6	7.1±70.9
8	$g_4 A x g_4^+ a$	"	404	328	332	-0.4	-5.9±41.2
9	$g_2 a x g_2^+ A$	g_2(III)	156	201	11	51.6±2.6	56.7±2.8
10	$g_2 A x g_2^+ a$	"	473	47	14	6.2±1.0	6.7±1.5
11	$g_5 a x g_5^+ A$	g_5(III)	1,015	493	250	13.8±0.8	24.1±2.0
12	$g_5 A x g_5^+ a$	"	800	211	145	5.7±0.7	9.2±2.3
13	$y_4 a x y_4^+ A$	y_4(V)	177	87	0	32.9±2.9	32.9±2.9
14	$y_4 A x y_4^+ a$	"	412	236	0	36.4±1.9	36.4±1.9
15	$h_3 a x h_3^+ A$	h_3(III)	506	72	31	6.7±1.0	7.9±1.8
16	$h_3 A x h_3^+ a$	"	896	173	52	10.8±0.9	12.5±1.4
17	$g_7 a x g_7^+ A$	g_7(III)	1,432	401	42	19.1±0.9	20.5±1.0
18	$g_7 A x g_7^+ a$	"	342	226	8	37.8±2.0	39.5±2.1

She also found that the difference in overlap frequency between reciprocal crosses on the same plate was less than that between repeat crosses. This observation suggests that environmental rather than genetic factors were largely responsible for the variation.

Mu'azu (1973) examined the effect of temperature on spindle overlap frequency in a cross of a y_4 allele, which is inseparable from the centromere of linkage group V, with wild type. The frequency rose from 8.6% at 15°C, through 12.6% at 20°C to 19.4% at 25°C and fell to 15.1% at 37°C. One very marked result of the experiment was a four fold increase in partial third division overlaps at 37°C. Mu'azu suggested that the increase in third division spindle overlap at higher temperatures might result from an increase in the rate of ascus elongation relative to the rate of nuclear division. Bistis (1956) reported an increase in spindle overlap with decreasing temperature in Ascobolus stercorarius.

Differential maturation of asci

A difficulty of tetrad analysis in Ascomycetes is achieving synchronization of crosses. A cross proceeds from fertilization through ascus formation, maturation and final dehiscence. The time-scale of these processes may vary depending on the genetic background of the cross and on environmental conditions. In addition the total number of mature asci produced by each perithecium may vary considerably.

Fertilization is the only point which can be accurately fixed in time, after that it is difficult to say exactly what stage of maturity a cross is at. It seemed possible that the large amount of variability observed in spindle overlap frequencies was not so much a consequence of real variability between crosses as of variability within a cross

as a result of sampling it at only one time point.

Lamb (1966, 1967) investigated polarized segregation, and differential maturation and bursting of asci in Ascomycetes. In Neurospora crassa he found a decline in the frequency of second division segregation of the 'asco' spore colour mutant from 30% to 15% as the cross matured. In Sordaria fimicola he found no decline in second division segregation frequency but found a decline with time of 4:4 asci with wild type spores proximal. To account for these observations he suggested that the rate of maturation of asci was affected directly by the distribution of wild type and mutant spores, rather than by the meiotic history of the ascus which is indicated by spore distribution. Lamb proposed two mechanisms by which spore distribution might affect the rate of ascus maturation. The first is that the translocation of nutrients up the ascus may be affected by spore distribution. As such transport is probably mediated by active transport mechanisms impaired metabolism in the vicinity of mutant bearing nuclei could bring about slower maturation of asci with mutant spores proximal. His second hypothesis is that the nature of the apical spore pair may affect the dehiscence of the apical pore.

In order to test the hypothesis that the apparent frequency of spindle overlap in S. brevicollis varied with cross maturity samples of asci from single point crosses of spore colour mutants were scored on successive days.

Materials and Methods

Strains

The following strains of Sordaria brevicollis were used in this work.

1) S229 is a yellow spore colour mutant. It is an allele of the y_4 locus and is very closely linked to the centromere of linkage group V. It arose spontaneously and was isolated by the author.

2) S187 is also a yellow spore colour mutant, the phenotype being indistinguishable from that of S229. It is an allele of the y_9 locus and is approximately 15 map units from the centromere of linkage group II. It was isolated following U.V. treatment by Bond (1969).

3) c79 is a buff spore colour mutant, also located on linkage group II. It is a member of the b_1 locus and is approximately four units from the centromere. It was kindly supplied by Professor L.S. Olive.

4) DAS 6 is a grey spore colour mutant. It was kindly supplied by Dr H.K.L. Whitehouse and is probably unlinked to the centromere of linkage group II at the g_4 locus.

5) mo-1 is a morphological mutant isolated and mapped by M.H.V. Cooray. It is located approximately mid-way between the b_1 locus and the centromere. The phenotype of this strain is characterized by slower growth than wild type and by an altered, 'fluffy', morphology at the colony margin.

Crossing techniques

Crosses were carried out at 25°C in 9cm petri dishes. 20mls of corn meal agar was the standard crossing medium and plates were

routinely used within 48 hours of pouring. Male and female parents were grown separately for four days. About 4mls of sterile distilled water ~~was~~ were then poured over the culture designated as the male parent. The surface of the mycelium was agitated with a glass rod and the resulting suspension of microconidia poured over the female parent which was shaken to ensure wetting of the entire mycelium. Excess water was poured off. Perithecia became visible after a further two days of incubation.

For rapid testing of the mating type or spore colour of an isolate crosses were performed by confrontation. Up to four inocula were placed simultaneously on one plate. Perithecia were formed after about four days of growth along the line where the ~~mycelial fronts~~ hyphae of strains of opposite mating type met. This method had the advantages of speed and economy.

Scoring techniques

Perithecia were harvested by scraping the surface of a culture with a tungsten needle, and crushed in a drop of 10% (w/v) sucrose solution on a slide. The sucrose solution prevents osmotic bursting of asci (Lamb, 1966). Clumps of asci liberated by crushing were transferred to a clean slide and teased apart with tungsten needles, again in 10% sucrose solution. The resulting groups of asci were flattened under a coverslip and scored at a magnification of x400.

Asci were classified according to the arrangement of spores of different phenotypes. Every ascus on a prepared slide was examined. Those in which all eight spores could not be seen were discounted. Examples of this type of ascus were those in which one or more spores were missing or obscured by debris on the slide. In this way

scoring biases were minimised. Asci in which the spores had not developed their mature colour but in which the phenotypes were nevertheless distinguishable were scored as partially mature. The number of completely immature asci was also recorded. In these the wild type and mutant spores were indistinguishable.

Spore isolation and ascus dissection

Fresh isolates were obtained by spreading discharged spores on 4% agar and picking off individual spores into tubes containing Vogel's (1956) N medium. 0.7% sodium acetate was added to the medium to stimulate germination when wild type isolates were required. Spore colour mutants germinated without this supplement.

Asci were dissected on 4% agar with tungsten needles under a magnification of x80. Spores were picked into tubes of Vogel's medium, numbered so as to retain the same order as in the ascus.

The formulae for all media are given in Appendix I.

CHAPTER 2

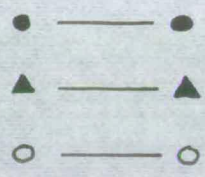
Analysis of single point crosses

Results

Figure 5 shows the frequency of 2:2:2:2 asci in three crosses sampled on successive days. Each cross involved a different spore colour mutant. The data from which the graphs were extracted appears in Table 2 (A-C). A marked decline in 2:2:2:2 frequency was apparent in all crosses. It is proposed that this phenomenon is largely responsible for the reported observations of variation in spindle overlap frequencies. A plot of spindle overlap frequency with time was obtained by applying formula 2 to the data (Figure 6). The decline in spindle overlap frequency appeared to be very similar for the three crosses. There may be some temporal displacement due to difficulties of synchronization. The result of the cross involving c79 for example appears to be displaced by about twenty four hours to the right. This could have been the result of some environmental factor causing slower development of this cross or it could be a property of the c79 genotype. ~~In any case~~ It is clear that a sample of asci from the three crosses taken on any particular day may show a wide variation in spindle overlap frequencies, e.g. from 57% to 33% on day 7 and from 44% to 21% on day 9. The range of spindle overlap values was 66% to 21%. Of eighteen crosses performed by Chen (1965) seven gave values significantly outwith this range. One of these was higher (83.6±1.6%). Five of the remainder ~~lie~~ lay between 2% and 13% and one (Cross 5, Table 1) had no significant excess of 2:2:2:2s.

Figure 5

Frequency of 2:2:2:2 asci between 4 and 10 days after fertilization in three single point crosses.



S229 x wild type

c79 x wild type

S187 x wild type

Data taken from Table 2 (A-C)

$$\text{s.e.} = \pm \sqrt{\frac{\frac{f}{n}\left(1-\frac{f}{n}\right)}{n}}$$

where f is the number of 2:2:2:2 asci in a sample of n.

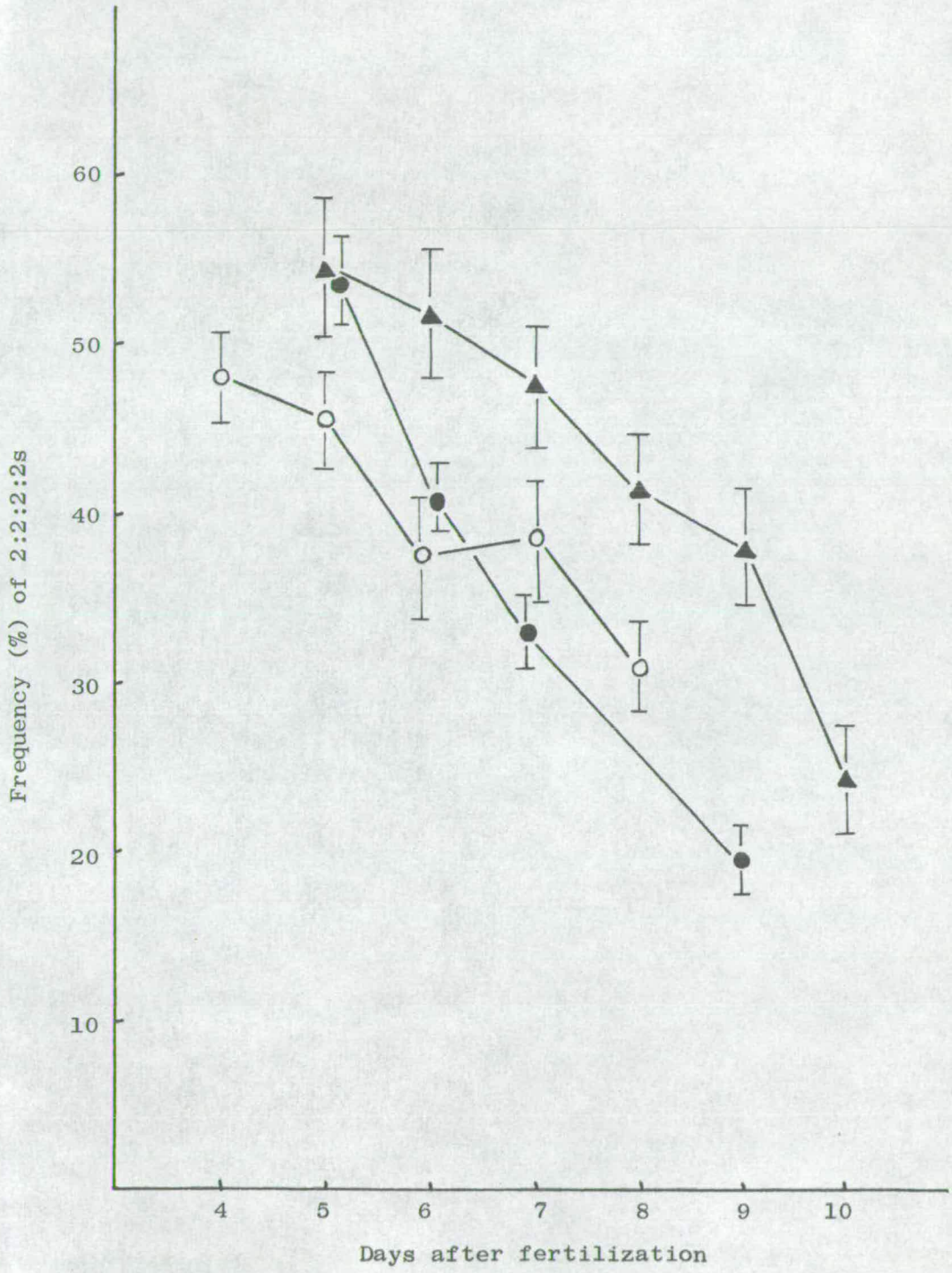

60
50
40
30
20
10
Frequency (%) of 2:2:2:2s
4
5
6
7
8
9
10
Days after fertilization

Table 2(A-C)

Numbers of different classes of asci from three single point crosses.

mat. = asci in which all spores had developed their mature colour

imm. = asci in which the mature spore colours were only partially developed though differences between phenotypes could be distinguished.

Immature asci were those in which the different spore colours could not be distinguished.

Aberrant asci were those with unequal numbers of wild type and mutant spores e.g. 5:3 and 6:2

Sequence of spores	Days after fertilization							
	5		6		7		9	
	mat.	imm.	mat.	imm.	mat.	imm.	mat.	imm.
4:4	222	15	290	22	345	11	444	18
2:2:2:2	288	4	215	10	183	2	114	7
2:4:2	6	0	0	0	2	0	1	0
3:1:1:3	10	0	7	0	13	1	15	2
2:1:1:1:1:2	5	1	5	0	7	0	7	0
2:2:1:1:1:1	0	0	0	0	0	0	0	0
2:3:1:1:1	0	0	0	0	0	0	0	0
3:2:1:2	1	0	3	0	2	0	2	0
2:3:2:1	0	0	0	0	0	0	0	0
2:2:1:2:1	0	0	0	0	1	0	0	0
Other 3rd div. overlap sequence	0	0	0	0	0	0	0	0
Aberrant	3	1	4	6	2	0	1	0
Immature	-	200	-	137	-	110	-	149
Total	535	221	524	173	555	124	584	176

Table 2A

Numbers of different types of asci from a cross of a yellow spore colour mutant (S229) with wild type.

Isolate nos. M21.1(S229A) x M20.4(a).

Sequence of spores	Days after fertilization											
	5		6		7		8		9		10	
	mat.	imm.	mat.	imm.	mat.	imm.	mat.	imm.	mat.	imm.	mat.	imm.
	25	32	22	14	38	11	54	48	48	3	85	11
	28		35	15	29	15	54		59	5	54	21
	47	72	51	31	44	10	49	19	44	1	26	4
	34		39	17	50	6	51		40	1	26	3
	3	5	7	4	4	2	6	6	6	1	4	1
	2		5	1	8	11	5		3	1	2	0
3:1:1:3	0	5	1	1	11	3	7	1	9	0	7	0
2:1:1:1:1:2	1	2	1	1	4	3	10	0	6	0	3	0
2:2:1:1:1:1	3	0	6	0	5	0	1	0	5	0	1	0
2:3:1:1:1	2	0	2	0	1	0	1	0	1	0	1	0
3:2:1:2	2	1	1	0	3	0	1	0	0	0	4	0
2:3:2:1	0	0	1	0	0	0	0	0	0	0	1	0
2:2:1:2:1	0	0	1	0	0	0	0	0	0	0	0	0
Other 3rd div. overlap sequence	3	0	0	0	1	0	2	0	0	0	0	0
Aberrant	2	0	2	0	0	0	0	0	0	0	0	0
Immature	-	167	-	119	-	68	-	47	-	37	-	39
Total	149	284	174	203	198	129	241	121	221	49	214	79

Table 2B

Numbers of different types of asci from a cross of a buff spore colour mutant (c79) with wild type. Isolate nos. B88.2 (c79A) x M11.32(a).

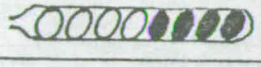
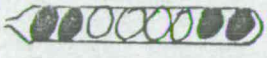
Sequence of spores	Days after fertilization									
	4		5		6		7		8	
	mat.	imm.	mat.	imm.	mat.	imm.	mat.	imm.	mat.	imm.
	53	4	50	2	57	0	34	2	84	1
	44	8	41	2	55	1	34	0	67	1
	64	9	74	1	46	1	34	0	41	1
	85	9	73	2	51	0	34	0	52	0
	18	2	29	6	14	0	15	2	25	0
	28	6	27	2	15	1	11	2	21	1
3:1:1:3	3	0	5	0	3	0	5	1	4	0
2:1:1:1:2	5	0	7	1	9	0	1	0	6	0
2:2:1:1:1:1	2	0	4	0	0	0	0	0	0	0
2:3:1:1:1	0	0	0	0	1	0	0	0	0	0
3:2:1:2	2	0	5	0	2	0	1	0	0	0
2:3:2:1	0	0	0	0	1	0	0	0	0	0
2:2:1:2:1	0	0	0	0	0	0	0	0	0	0
Other 3rd div. overlap sequences	0	0	0	0	0	0	0	0	0	0
Aberrant	6	1	7	0	6	0	6	0	1	0
Immature	-	730	-	191	-	51	-	20	-	120
Total	310	769	322	207	260	54	175	27	301	124

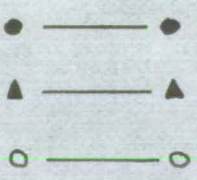
Table 2C

Numbers of different types of asci from a cross of a yellow spore colour mutant (S187) with wild type.

Isolate nos. M35.1(mo⁻S187A) x D1.7(a)

Figure 6

Frequency of asci containing partial second division spindle overlaps between 4 and 10 days after fertilization in three single point crosses.



S229 x wild type

c79 x wild type

S187 x wild type

Data taken from Table 2 (A-C)

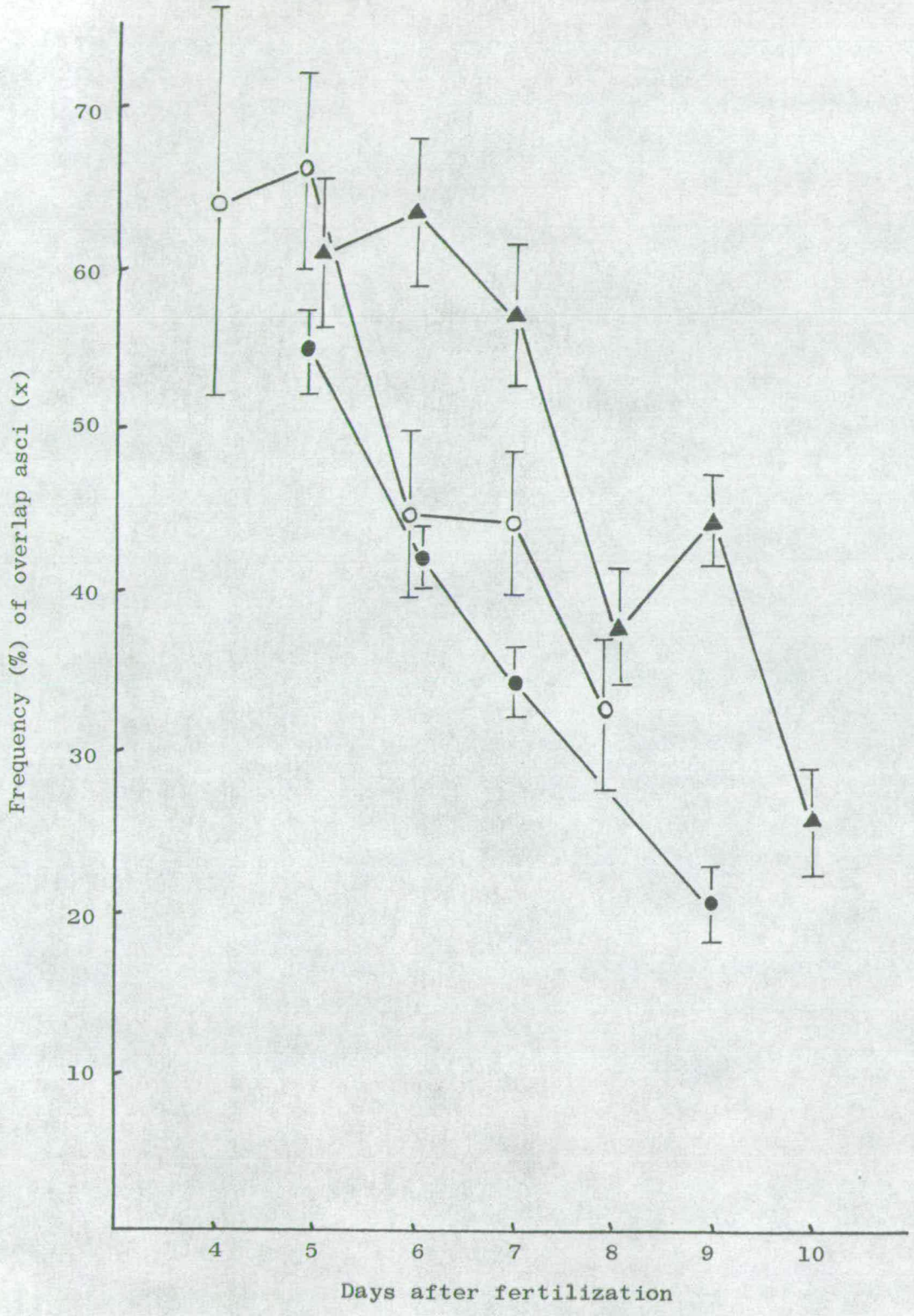
The frequency of spindle overlap is calculated according to formula 2 (see p. 6) where;

$$x = \frac{r-s}{1-3s}$$

The standard error of x is calculated according to the formula,

$$\text{variance of } x = \frac{a(b-c)^2+b(a-c)^2+c(a-b)^2}{(n-3c)^4}$$

where a = no. of 4:4s; b = no. of 2:2:2:2s; c = no. of 2:4:2s in a total of n.


70
60
50
40
30
20
10
Frequency (%) of overlap asci (x)
4
5
6
7
8
9
10
Days after fertilization

The results of the single point crosses imply that asci with a 2:2:2:2 distribution of wild type and spore colour mutant spores mature and/or discharge earlier than those with 4:4 distributions. As Lamb (1966), suggested this may be a consequence of some inherent property of 2:2:2:2s per se. Alternatively it may be that asci with partial second division spindle overlaps mature and/or discharge early. This will manifest itself as a decline in 2:2:2:2 frequency in crosses involving centromere linked genes. Means of distinguishing these possibilities will be discussed.

Preferential maturation and discharge of asci

Both preferential maturation and preferential discharge were probably responsible for the decline of 2:2:2:2 asci with time. It could be supposed that some property of 2:2:2:2s made them complete the developmental process from ascus initial to discharged ascospores more quickly than 4:4s. Alternatively the first ascus initials formed after fertilization could be more likely to develop into 2:2:2:2s than those formed later. Within any class of asci the developmental process was by no means synchronized. Even perithecia in which the majority of asci had been discharged still contained some immature asci. Discharged spores were usually first seen about forty-eight hours after the first mature asci could be scored. Preferential maturation and discharge will be referred to simply as preferential discharge.

The hypothesis of preferential discharge of spindle overlap asci

Suppose that asci formed early in a cross are more likely to contain spindle overlaps than those formed later. This might be the

result of a shorter mean ascus length at nuclear division in early asci. Whatever the mechanism of the effect the preferential discharge of asci with partial second division spindle overlaps will result in a decline in 2:2:2:2 frequency with time. This only applies to 2:2:2:2 distributions of centromere linked alleles. In the extreme case of S229, which is inseparable from its centromere, all 2:2:2:2s contain spindle overlaps. As centromere distance increases so will the number of 2:2:2:2s which are recombinant rather than overlap. Spindle overlap asci will also be increasingly found in the 4:4 and 2:4:2 classes through overlap of recombinational asci. When the centromere and gene are not linked the three classes of asci will be expected to be equal in size and the proportion of overlap and non-overlap asci will not vary between the classes. In this case the preferential discharge of 2:2:2:2s will vanish. Figure 7 illustrates this effect in a theoretical situation, based on the observed decline of 2:2:2:2 frequency in the S229 x wild type cross. For ease of computation the decline is taken to be linear from r = 60% to r = 20% in six days. In the cross of S229:

$$s = 0 \qquad r = x$$

Using the values of x at different times, graphs can be constructed showing the expected decline in 2:2:2:2 frequency (r) for different values of s. Values of r were calculated from formula 2, rearranged so that,

$$r = x + s - 3xs \qquad (2A)$$

Inserting the values of s found from the crosses of c79 and S187, the predicted and observed declines can be compared (Figure 8).

Figure 7

Theoretical effect of increasing centromere distance(s) on the decline in 2:2:2:2 frequency with time. It is assumed that the frequency of asci containing partial second division spindle overlaps (x) declines linearly from 60% to 20% over a six day period.

The theoretical declines are calculated from formula 2A (see page 15) where

$$r = x + s - 3xs$$

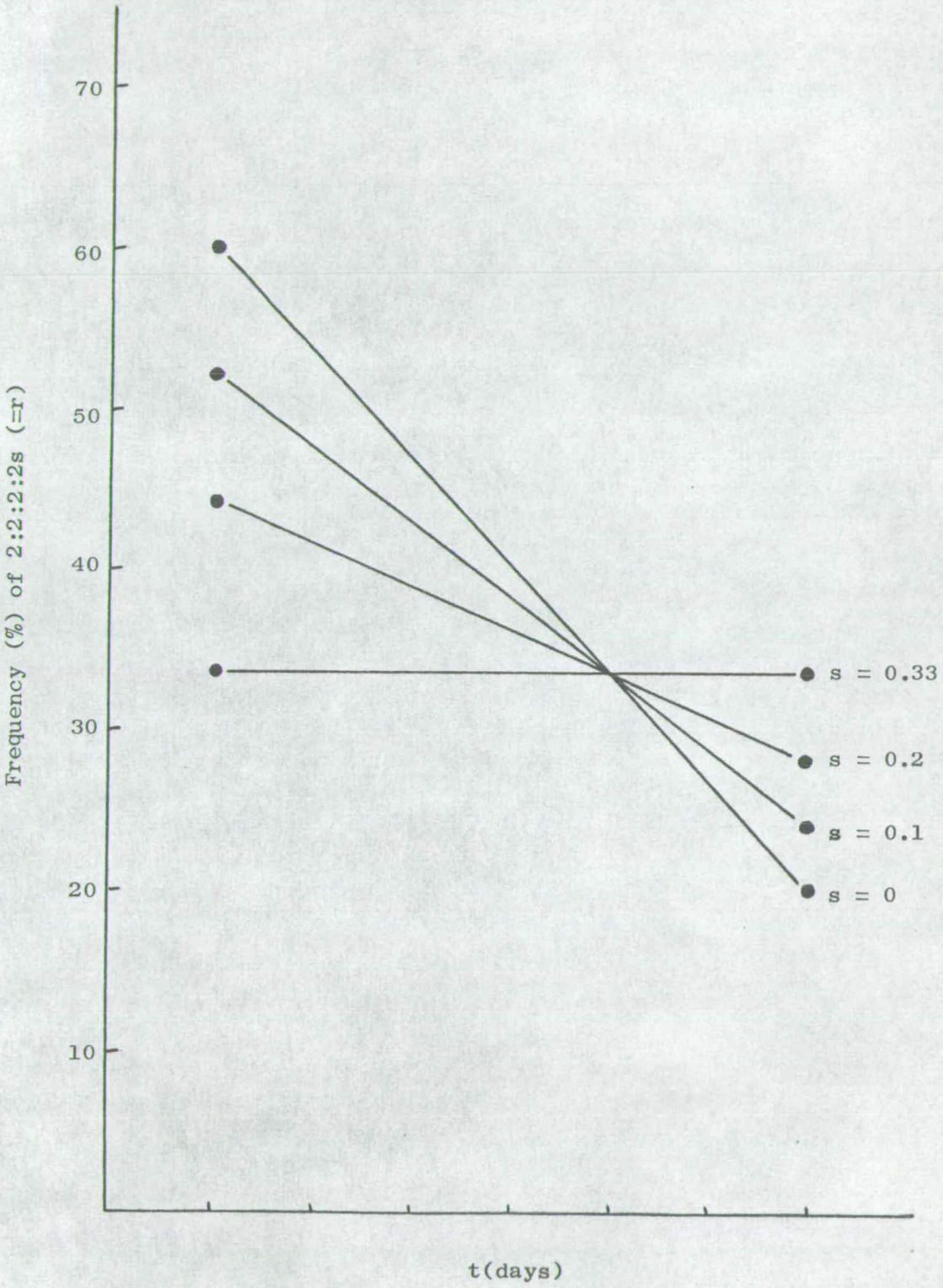

70
60
50
40
30
20
10
Frequency (%) of 2:2:2:2s (=r)
s = 0.33
s = 0.2
s = 0.1
s = 0
t(days)

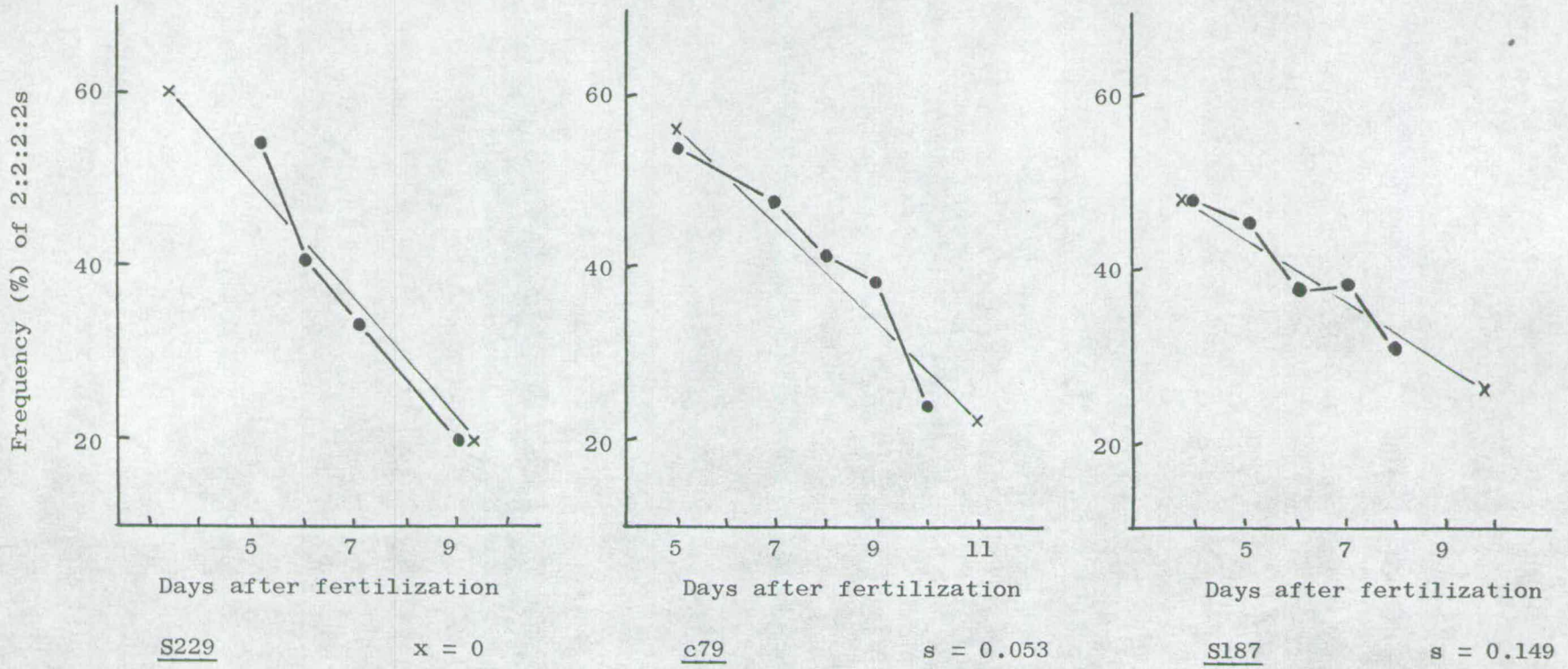
Figure 8

Predicted and observed decline in 2:2:2:2 frequency with time for three single point crosses.

The predicted decline is calculated from formula 2A (see page 15) where

$$r = x + s - 3xs$$

assuming that the frequency of partial second division spindle overlap (x) declines linearly from 60% to 20% over a six day period.


Frequency (%) of 2:2:2:2s
60
40
20
5
7
9
Days after fertilization
S229
x = 0
60
40
20
5
7
9
11
Days after fertilization
c79
s = 0.053
60
40
20
5
7
9
Days after fertilization
S187
s = 0.149

A second prediction of the hypothesis of preferential discharge of spindle overlap asci is that the frequency of 2:4:2 asci will not change with time. As the size of the 2:4:2 class is neither added to nor subtracted from by spindle overlap the proportion of 2:4:2s containing overlaps will be the same as the frequency of overlaps in the cross as a whole. The decline in 2:4:2s as a result of preferential discharge of overlap asci will be proportional to the decline in the total ascus pool. The ratio of 2:4:2s to total asci is therefore unaltered by changes in x.

$$s = (s-xs) + xs = s$$

s is independent of x

The observed frequencies of 2:4:2s are shown against time in Table 3. There was no significant change in 2:4:2 frequency in any of the three crosses.

Cross	Days after fertilization							Hetero-geneity χ^2	P	Total	Frequency (%)
	4	5	6	7	8	9	10				
S229 x +	-	6/523	0/524	2/555	-	1/584	-	-	-	9/2186	0.41 ±0.12
c79 x +	-	7/149	14/174	13/198	12/241	10/221	7/214	4.9	0.30	63/1197	5.26 ±0.65
S187 x +	46/310	56/322	30/260	26/175	46/301	-	-	2.9	0.50	204/1368	14.91 ±0.96

Table 3

Numbers and frequency of 2:4:2 asci in single point crosses between 4 and 10 days after fertilization. Data extracted from Table 2(A-C). The figures in the columns headed 'days after fertilization" represent $\frac{f}{n}$ where f is the number of 2:4:2s in a total sample of n asci.

$$\text{s.e.} = \pm \sqrt{\frac{\frac{f}{n}\left(1 - \frac{f}{n}\right)}{n}}$$

A third expectation arising from preferential discharge of spindle overlap asci is that the proportion of recombinant asci in the 2:2:2:2 class will increase with time. This proportion can be predicted for any given values of x and s.

$$\frac{\text{recombinant 2:2:2:2s}}{\text{Total 2:2:2:2s}} = \frac{(s-xs)}{x(1-2s) + (s-xs)}$$

$$= \frac{s - xs}{x + s - 3xs} \qquad (3)$$

Inserting values of x and s into equation 3, for example s = 0.15 and x declining from 0.6 to 0.2 with time, the predicted proportion of recombinant/total 2:2:2:2s increases from 12.5%, (x = 0.6), to 46.2%, (x = 0.2).

The hypothesis that spindle overlap asci are preferentially discharged leads to the predictions discussed above. The data from the single point crosses of S229, c79 and S187 do not appear to contradict any of the predictions. Two experiments have been performed which further test the predictions.

Cross involving centromere-unlinked marker

The isolate DAS 6 was used to test the prediction that the decline in 2:2:2:2 frequency will approach zero as the ~~gene — centromere distance~~ frequency of recombination approaches 33.3%. DAS 6 is a grey spore colour mutant, probably an allele of g_4 which has a recombination frequency of 31.2 ± 1.4% (Chen, 1965). In this work the recombination frequency was 34.8 ± 1.7%. A cross of this isolate x wild type was sampled on four successive days. None of the samples had a frequency of 2:2:2:2 asci significantly different from 33.3% (Table 4).

Day	Spore sequence			Total	Frequency of 2:2:2:2s (%)
	4:4	2:2:2:2	2:4:2		
4	31	24	34	89	27.0 ± 4.7
5	65	73	74	212	34.4 ± 3.3
6	90	69	78	237	29.1 ± 3.0
7	79	86	93	258	33.3 ± 2.9

Table 4

Numbers of asci in the three major ascus classes from a cross of a grey spore colour mutant (DAS 6) with wild type

$$s.e. = \pm \sqrt{\frac{\frac{f}{n}\left(1 - \frac{f}{n}\right)}{n}}$$

where f = no. of 2:2:2:2s in a total of n

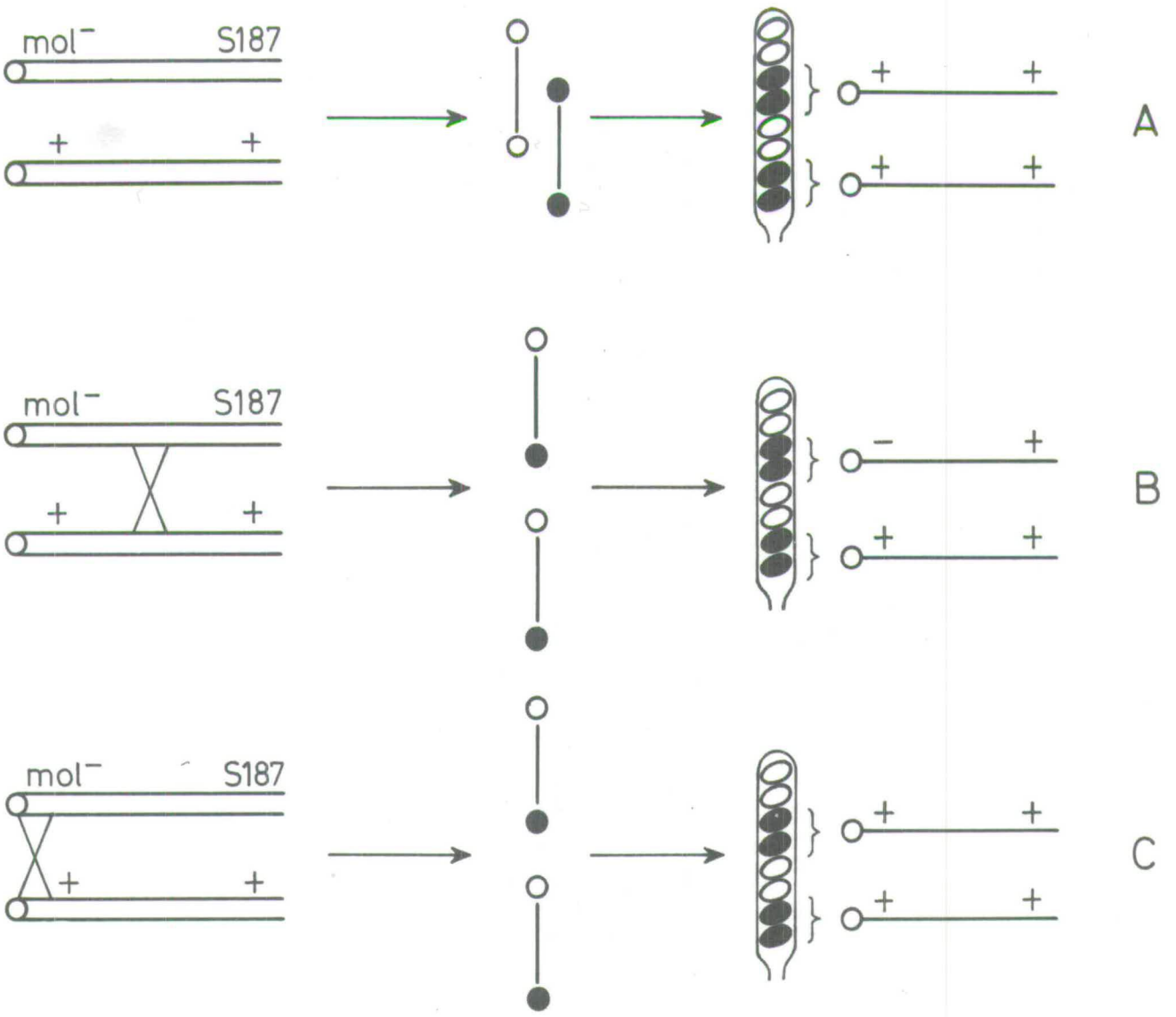
Proportion of recombinant 2:2:2:2s

The prediction that the proportion of recombinants in the 2:2:2:2 class would increase with time was tested by dissecting asci from a cross of mo-1$^-$ S187 x wild type. The morphology and mating type of black spores in 2:2:2:2 asci from this cross was ascertained. Almost all 2:2:2:2s can be classified as one of three types illustrated in Figure 9. Spindle overlap asci, (type A) will retain the parental configuration of S187 and mo-1. The black spores will therefore be of wild type morphology. Non-overlap 2:2:2:2s (types B and C) will contain a recombination between S187 and its centromere. The majority of these recombination events will occur distal to mo-1 and one of the black spore pairs will be mo-1$^-$ (type B). The ~~centromere distances~~ recombination frequencies of mo-1 and S187 with their centromere are 2 and 15% respectively. 2/15 of recombinants between S187 and the centromere will therefore have the recombination on the proximal side of mo-1 (type C) and will be

Figure 9

The three most common origins of 2:2:2:2 asci in a cross of ♂ mo-1 S187 x ♀ + +.

For explanation see page 18.


mol⁻
S187
+
+
A
mol⁻
S187
+
+
−
+
+
+
B
mol⁻
S187
+
+
+
+
+
+
C

indistinguishable from type A in terms of the segregation of mo-1 and S187.

The proportion of recombinant 2:2:2:2s is,

$$\frac{\text{Type B + Type C}}{\text{Type A + Type B + Type C}} \qquad (4)$$

As Types A and C are indistinguishable the proportion may be calculated as,

$$\frac{\text{Type B} + \frac{2}{15}\text{ Type B}}{\text{Total}} \qquad (4A)$$

2:2:2:2s from a cross of mo-1 S187 x wild type were dissected 4 and 8 days after fertilization. This is one of the single point crosses discussed previously. The values of r and s on days 4 and 8 are;

$$r_4 = \frac{100}{310} \quad ; \quad r_8 = \frac{155}{301}$$

$$s_4 = \frac{45}{310} \quad ; \quad s_8 = \frac{46}{301}$$

Introducing the value of x found by formula 2 into formula 3 the expected frequency of recombinant 2:2:2:2s can be calculated.

(3) $$\frac{s - xs}{x + s - 3xs}$$

Substitute (2) $\frac{r - s}{1 - 3s}$ for x

(3A) $$\frac{\text{Recombinant 2:2:2:2s}}{\text{Total 2:2:2:2s}} = \frac{s(1-r-2s)}{r(1-3s)}$$

Applying formula 3A to the data the expected proportion of recombinant 2:2:2:2s increases from 10.6% on day 4 to 31.4% on day 8. The experimental results appear in Table 5. The predicted enrichment of

the recombinant fraction of the 2:2:2:2 class did not occur. This experiment was repeated with asci from a different cross. The result was very similar and is shown in Table 5A. In the repeat experiment each ascus dissected was taken from a different perithecium. This ensured a representative sample of 2:2:2:2s and avoided any bias which might have resulted from drawing the entire sample from only a few perithecia. These results suggested that 2:2:2:2 asci per se were preferentially discharged rather than some group of asci within the 2:2:2:2 class, e.g. spindle overlap asci.

Days after fertilization	2:2:2:2 asci		Observed frequency of recombinants (%)	Expected frequency of recombinants (%)
	Non-recombinant	Recombinant		
4	76	20	20.8±4.1	10.6%
8	81	23	22.1±4.1	31.4%

Table 5

Number and frequency of recombinant 2:2:2:2s from a cross of a yellow spore colour mutant (S187) with wild type. [Isolate nos. M35-1 (mo⁻S187a) x D1.7(A)]

Days after fertilization	Spore sequence			Expected frequency of recombinant 2:2:2:2s	Observed recombinant 2:2:2:2s	
	4:4	2:2:2:2	2:4:2		no	frequency (%)
5	74	63	24	21%	25/96	26.0±4.5
9	110	55	28	38%	21/92	22.8±4.4

Table 5a

Number and frequency of recombinant 2:2:2:2s from a cross of a yellow spore colour mutant (S187) with wild type. [Isolate nos. M26.1 (mo⁻S187a) x M23.8(A)]

The expected frequencies of recombinant 2:2:2:2s are calculated using formula 3A (see page 19). Where

$$\frac{\text{Recombinant 2:2:2:2s}}{\text{Total 2:2:2:2s}} = \frac{s(1-r-2s)}{r(1-3s)}$$

The hypothesis of preferential discharge of 2:2:2:2 asci per se

If it is supposed that 2:2:2:2s are preferentially discharged because of some inherent property of this spore sequence it must be considered whether the observations that apparently supported the hypothesis of preferential discharge of spindle overlap asci can be explained. These observations were that the frequency of 2:4:2s did not vary with time and that the observed decline in 2:2:2:2 frequency decreased predictably with increasing gene - centromere distance.

If all asci which were not 2:2:2:2s matured and discharged at the same rate than the frequency of 2:4:2s would be expected to increase as the 2:2:2:2 frequency decreased. As this was not the case it must be assumed that 2:4:2s and 4:4s behave differently in their rates of discharge. In Figures 10, 11 and 12 a graphic model is devised to illustrate the fluctuations in the various ascus classes in a theoretical situation.

Figure 10 is based on the observations from the single point crosses and shows the frequencies of different ascus classes against time. Figure 11 is a graph of the mean number of mature asci contained in each perithecium against time. It is purely conjectural. Combining Figures 10 and 11 gives Figure 12, a graph of the mean number of asci in the three major classes per perithecium against time. The behaviour of each class of asci is similar but the peaks of mature ascus numbers are separated temporally. 2:2:2:2s have some inherent advantage over 4:4s which enable them to mature on average one day earlier. 2:4:2s have a disadvantage to 2:2:2:2s equal to their advantage over 4:4s. It is stressed that only Figure 10 is

Figure 10

Stylized variation in the frequencies of the three major classes of asci, based on observations of single point crosses.

o———o 2:2:2:2 asci show a linear decline in frequency

x———x 2:4:2 asci show no variation

●———● 4:4 asci show a linear increase in frequency

Figure 11

Conjectural variation in the mean number of asci per perithecium throughout the period of ascus maturation and discharge.

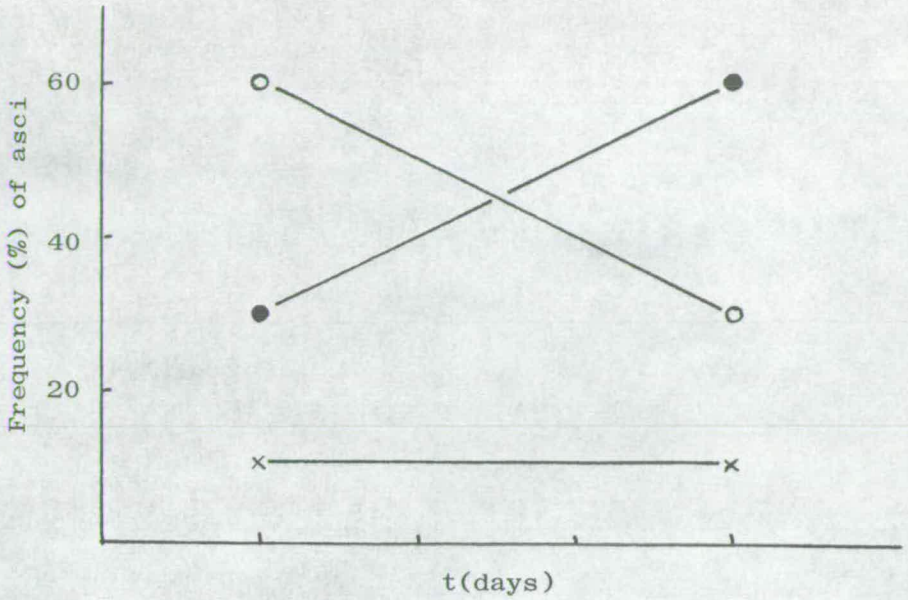


Figure 10

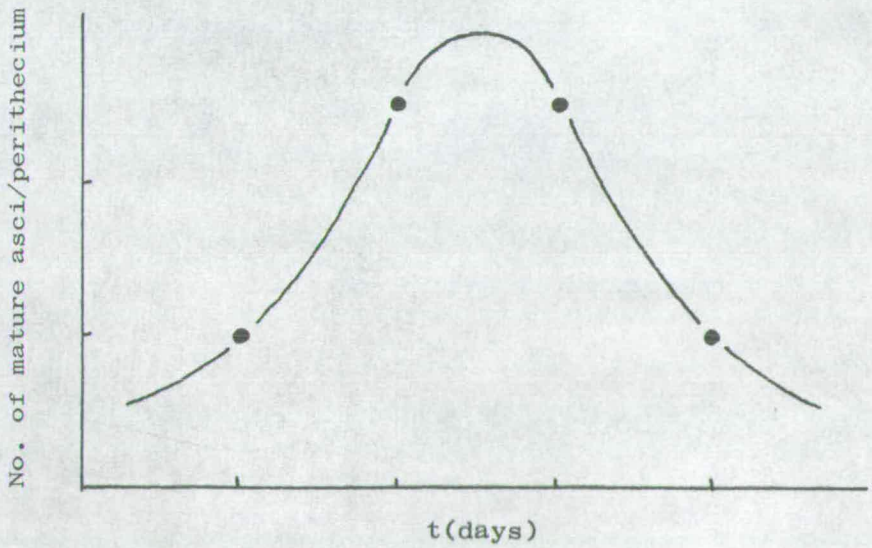


Figure 11

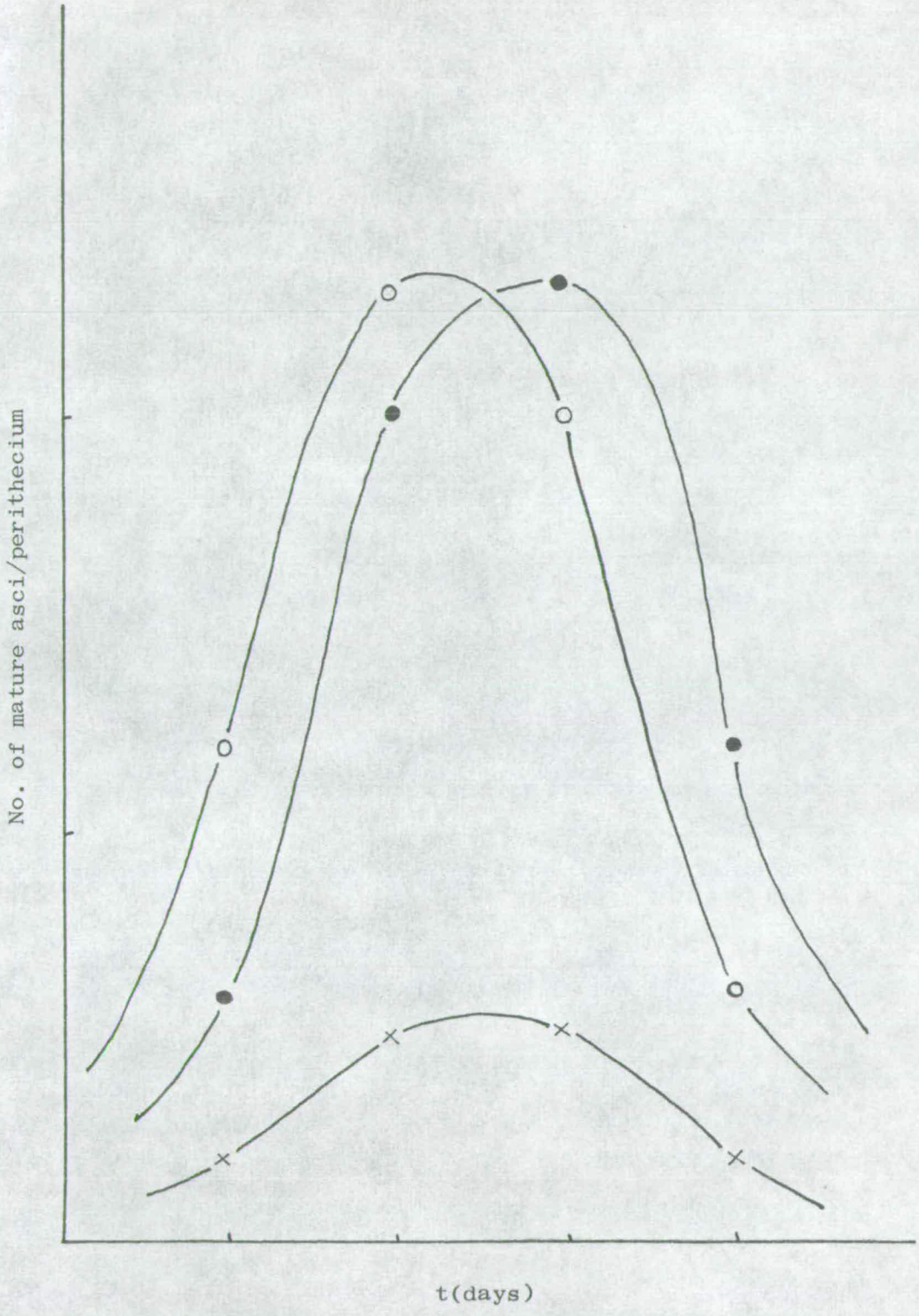
Figure 12

Mean number of mature asci of the three major ascus classes per perithecium throughout ascus maturation and discharge. This graph is obtained by combining figures 10 and 11.

o——— o 2:2:2:2s

x——— x 2:4:2s

●——— ● 4:4s


No. of mature asci/perithecium
t(days)

based on observation. The model devised in Figures 11 and 12 could probably have been derived with unequal frequencies of 2:2:2:2s and 4:4s (i.e. spindle overlap frequency ≠ 50%) and asymmetric distributions of ascus numbers with time, without being inconsistent with Figure 10.

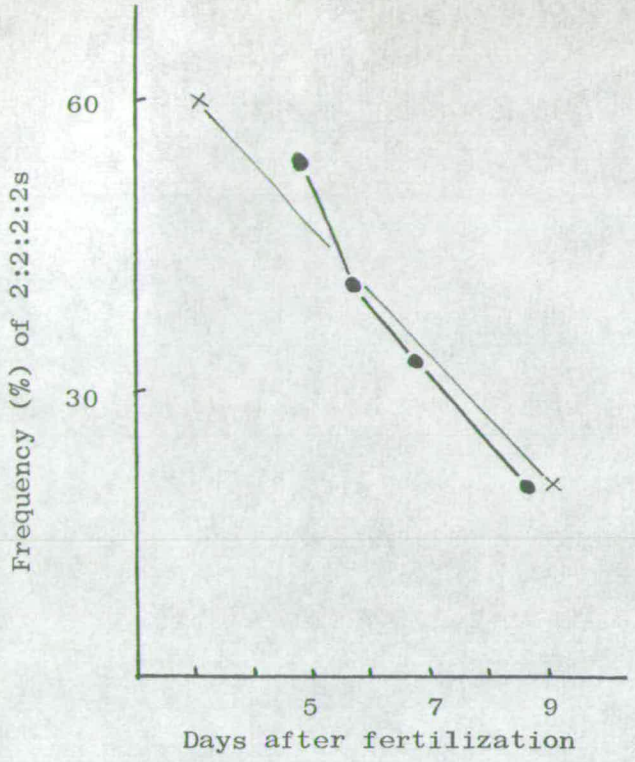
The cross of S229 x wild type shows a drop in 2:2:2:2 frequency approximately corresponding to a linear decline from 60% to 20% over a six day period. Taking these values and assuming a linear decline in 2:2:2:2 frequency with no variation in 2:4:2 frequency it is possible to predict the frequencies of different ascus types in the other single point crosses.

	Day 1	Day 6
4:4	0.40(1-s)	0.80(1-s)
2:2:2:2	0.60(1-s)	0.20(1-s)
2:4:2	s	s

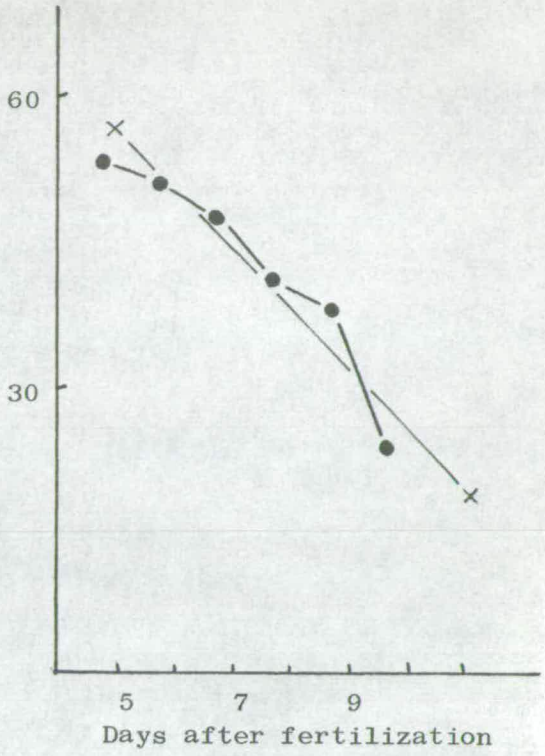
By inserting the value of s found in the crosses of S187 and c79 the predicted and observed declines in 2:2:2:2 frequency can be compared, (Figure 13). The only inconsistency between model and observations appears to be the failure of the DAS 6 cross to show any significant decline in 2:2:2:2 frequency. An assumption underlying the hypothesis of preferential discharge of 2:2:2:2s per se is that there should be a difference between the parental spore phenotypes. The distribution of these different spores affects the rate of ascus maturation in some way. What the critical difference may be is unknown but Lamb (1967) (see p. 9) suggests that it may be the extent to which the active transport of nutrients is impaired in the vicinity of mutant bearing nuclei. The difference between the two spore types could be expressed as the distance between the peaks of the graphs of

Figure 13

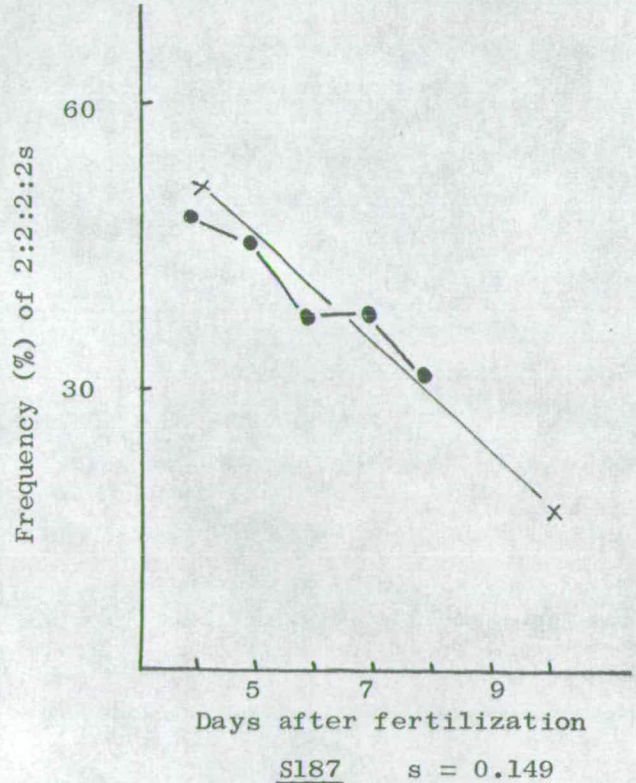
Predicted and observed decline in 2:2:2:2 frequency for four single point crosses. The predicted decline is calculated on the assumption that the frequency of 2:2:2:2s in the absence of recombination, decline from 60% to 20% over a six day period through the preferential discharge of 2:2:2:2s per se (see page 22).



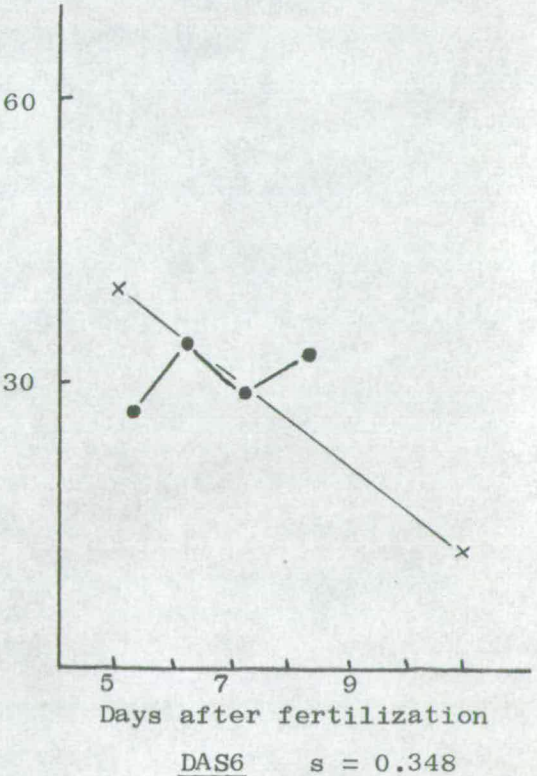
S229 s = 0



c79 s = 0.053



S187 s = 0.149



DAS6 s = 0.348

2:2:2:2 and 4:4 frequency in Figure 12. The result of the DAS 6 x wild type cross could therefore be accounted for by assuming that there is only a very small difference between wild type and DAS 6 spores.

Lamb observed differential maturation between reciprocal sequences within classes of asci, e.g. 4+:4m and 4m:4+. No such effect has been found in this work.

Conclusions

In three out of four single point crosses there was a decline in the frequency of 2:2:2:2 asci as the cross ~~developed~~ matured. Of the two hypotheses proposed to account for the decline one, that spindle overlap asci are preferentially discharged, leads to a number of testable predictions. The only unfulfilled prediction is that the proportion of recombinant 2:2:2:2s will increase with time.

The second hypothesis, that 2:2:2:2s per se are preferentially discharged leads only to one direct prediction. That is that the proportion of recombinant and overlap 2:2:2:2s will not change as the cross develops. A model of cross development has been devised which fits the observed variations in ascus type frequencies. It is likely that almost any observation could be shown to be consistent with some model of cross development.

As an experiment has been performed which appears to exclude the hypothesis of preferential discharge of spindle overlap asci the results of the single point crosses are taken to favour the alternative hypothesis that 2:2:2:2s per se are preferentially discharged.

CHAPTER 3

Preferential discharge of asci from other than single point crosses

The hypothesis that asci containing a 2:2:2:2 arrangement of wild type and colour mutant spores are preferentially discharged because some inherent property of this arrangement permits faster maturation of such asci was tested by two experiments. The first involved a cross of two spore colour mutants and the second a cross which did not involve any spore colour mutants.

Two point cross

(II, 4% from centromere) (II, 15% from centromere)

A cross was made between c79 (buff) and S187 (yellow). Parental ditypes from this cross contained only buff, and yellow spores. Tetratype asci contained one spore pair of each of four phenotypes, buff, yellow, wild type and double mutant, (white). Non-parental ditypes contained two wild type and two double mutant spore pairs. If the phenotype and arrangement of the spores within the ascus was the critical factor in determining the rate of maturation and discharge it was thought possible that preferential discharge would be shown by groups of asci which did not necessarily contain spindle overlaps. For example preferential discharge of tetratypes could be taken as evidence that the presence of a wild type spore pair enhanced the maturation rate. Failure to find any preferential discharge within the parental ditypes could suggest that the difference between buff and yellow spores was insufficient to give any arrangement of the spores an increased maturation rate. Such results would indicate that the preferential discharge of 2:2:2:2s in single point crosses was a consequence of their spore sequence rather than of spindle overlap.

The results of the two point cross are shown in Table 6. The asci were classified by the order of the spores they contained. For example y:b:y:b represents an ascus with a 2:2:2:2 arrangement of buff and yellow spores, with a yellow spore pair at the distal end of the ascus. This is a parental ditype, either with a partial second division overlap or a recombination between buff and the centromere. b:-:+:y represents a tetratype ascus containing, from the apex, a pair of buff spores, a double mutant pair, a wild type pair and a yellow pair. This ascus contains a 2:2:2:2 arrangement of the yellow (S187) allele and a 4:4 arrangement of the buff (c79) allele. It arises from a recombination between c79 and S187 but probably does not contain a spindle overlap as this would be unlikely to give a 4:4 arrangement of c79.

The raw data in Table 6 contains a large amount of information. That which is relevant to the discussion which follows has been extracted and is presented in Tables 7-9. The following questions were asked about the two point cross.

1) Is there preferential discharge of tetratype asci?

The frequency of tetratype asci is shown in Table 8. The expected frequency of tetratypes can be calculated from the single point crosses and is approximately twice the difference between the centromere distances of S187 (14.9%) and c79 (5.4%).

$$\text{Expected tetratypes} = 2(14.9\% - 5.4\%)$$

$$= 19\%$$

Table 6

Numbers of different types of asci from a cross of a yellow spore mutant (S187) with a buff spore colour mutant (c79). [Isolate nos. M26.4 (mo⁻S187a) x B88.2 (C79A)].

The numbers in the column headed 'sequence of buff allele' represent;

1 - a 4:4 sequence

2 - a 2:2:2:2 sequence

3 - a 2:4:2 sequence

Extracts from this table are also presented in Tables 7, 8 and 9. and in Figures 14 & 15.

Ascus type	Sequence of buff allele	Sequence of spores	Days after fertilization					
			5	6	7	8	9	10
Parental Ditypes	1	b:b:y:y	74	87	110	118	133	51
	1	y:y:b:b	62	80	108	139	127	76
	2	b:y:b:y	96	61	105	68	83	34
	2	y:b:y:b	118	81	87	66	86	25
	3	b:y:y:b	14	8	12	9	15	4
	3	y:b:b:y	9	9	7	8	11	5
Tetratypes	2	+:b:y:-	3	4	3	6	9	5
	3	+:b:-:y	1	2	1	1	0	0
	1	+:y:-:b	2	0	7	9	7	5
	1	+:y:b:-	2	2	7	13	12	4
	3	+:-:b:y	0	4	0	3	0	1
	2	+:-:y:b	5	7	5	7	4	7
	3	b:+:y:-	1	2	2	1	1	0
	2	b:+:-:y	6	6	8	6	5	4
	3	b:y:+:-	0	0	0	0	2	1
	2	b:y:-:+	6	5	9	6	9	3
	1	b:-:y:+	5	7	6	7	6	9
	1	b:-:+:y	0	5	12	5	10	4
	2	y:b:+:-	2	3	8	6	3	2
	3	y:b:-:+	1	1	1	0	1	0
	1	y:+:b:-	0	1	4	4	9	11
	1	y:+:-:b	2	2	5	6	10	4
	2	y:-:+:b	4	8	12	7	2	7
	3	y:-:b:+	0	2	1	1	10	1
	1	-:b:y:+	2	3	6	19	1	9
	1	-:b:+:y	2	3	3	2	7	4
	3	-:+:y:b	2	1	2	1	2	0
	2	-:+:b:y	5	10	5	12	12	2
	3	-:y:+:b	0	2	1	0	0	0
	2	-:y:b:+	1	11	16	6	1	4
Non-Parental Ditypes	1	+:+:-:-	0	0	0	0	0	0
	1	-:-:+:+	0	0	0	0	0	0
	2	+:-:+:-	0	0	0	0	0	0
	2	-:+:-:+	1	0	1	0	0	0
	3	+:-:-:+	0	0	1	1	1	0
	3	-:+:+:-	1	1	0	1	1	0
Others			3	0	3	0	2	1
Total			430	421	558	538	581	283
Immature			230	156	180	134	81	27

		Days after fertilization											
		5		6		7		8		9		10	
		no.	%	no.	%	no.	%	no.	%	no.	%	no.	%
Distribution of buff and yellow spores in Parental Ditypes	4:4	136	36.5	167	50.8	218	50.8	257	63.0	260	57.1	127	65.1
	2:2:2:2	214	57.4	145	44.1	192	44.8	134	32.8	169	37.1	59	30.3
	2:4:2	23	6.2	17	5.2	19	4.4	17	4.2	26	5.7	9	4.6
	Total	373		329		429		408		455		195	
Distribution of buff alleles in Tetratypes	4:4	15	28.8	23	25.3	50	40.3	65	50.8	71	58.2	50	57.5
	2:2:2:2	32	61.5	54	59.3	66	53.2	56	43.8	45	36.9	34	39.1
	2:4:2	5	9.6	14	15.4	8	6.5	7	5.6	6	4.9	3	3.4
	Total	52		91		124		128		122		87	

Table 7

No. and frequency of asci with different spore distributions in parental ditypes and tetratypes from a cross of a yellow spore colour mutant (S187) with a buff spore colour mutant (c79). [Isolate nos. M26.4 (mo^{-}S187a) x B88.2 (c79A)]. Data extracted from Table 6.

Days after fertilization	Undischarged Tetratypes (%)	Discharged Tetratypes No	Discharged Tetratypes %
5	12.1 ± 1.6	-	
6	21.6 ± 2.0	$\frac{40}{183}$	21.9 ± 3.1
7	22.2 ± 1.8	$\frac{41}{176}$	23.3 ± 3.2
8	23.8 ± 1.8	$\frac{54}{201}$	26.9 ± 3.1
9	21.0 ± 1.7	$\frac{29}{121}$	24.0 ± 3.9
10	30.7 ± 2.7	$\frac{47}{189}$	24.9 ± 3.1

Table 8

Frequency of tetratypes from a cross of a yellow spore colour mutant (S187) with a buff spore colour mutant (c79). [Isolate nos. M26.4 (mo⁻S187a) x B88.2 (c79A)]. Undischarged tetratypes are those prepared from perithecial harvests. Discharged asci were counted on petri dish lids. The lid was renewed each day to collect only those asci discharged during a 24 hour period. Data on undischarged tetratypes extracted from Table 6

$$s.e. = \pm \sqrt{\frac{\frac{f}{n}\left(1 - \frac{f}{n}\right)}{n}}$$

Between six and nine days after fertilization the tetratype frequency varied little around 22%. It is proposed that tetratype asci were not preferentially discharged. The low and high values on days five and ten can be accounted for by supposing that on day five many tetratype asci are misclassified. Before becoming black, wild type spores must first pass through yellow colour and a large number of immature tetratypes in the earliest stage of the cross might be taken as parental ditypes. On the last day of scoring, when most of the asci have been discharged many of the remainder have yellow spores which darken and become difficult to distinguish from wild type. It is thought likely that such asci were responsible for the apparent increase in tetratype frequency on day ten.

The frequency of tetratypes amongst discharged asci on the petri dish lid was scored at the same time as that amongst undischarged asci. The results, shown in Table 8 do not provide any evidence for preferential discharge of tetratypes.

2) Is there preferential discharge of 2:2:2:2s in parental ditype asci?

Figure 14 illustrates the decline in 2:2:2:2 frequency with time in parental ditypes. The decline was similar to that found in single point crosses. Whereas this result would be expected if spindle overlap asci were preferentially discharged it is not an a priori expectation of preferential discharge dependent on the arrangement of spores with different phenotypes. It could be postulated, however, that the difference between buff and yellow spores is sufficient for their sequence to affect the rate of ascus maturation.

3) Is there preferential discharge of 2:2:2:2s for the buff locus in tetratypes?

If spindle overlap asci were preferentially discharged a decline in the frequency of tetratype asci with a 2:2:2:2 arrangement of the buff locus would be expected. On the other hand there would be no such expectation if the sequence of spores of different phenotypes were the important factor. Figure 15 shows that such a decline occurred and was similar in magnitude to that in single point crosses and in the parental ditypes.

4) Does the position of the wild type spores in a tetratype ascus affect the rate of maturation and discharge?

Table 9 shows the frequency within tetratypes of asci with wild type spores in each of the four possible positions. There was no

Figure 14

Frequency in parental ditypes of asci with a 2:2:2:2 distribution of buff and yellow spores from a cross of two spore colour mutants (c79 x S187).

Data taken from Table 6.

$$se = \pm \sqrt{\frac{f/n(1-f/n)}{n}}$$

where f is the number of 2:2:2:2 asci in a total of n.

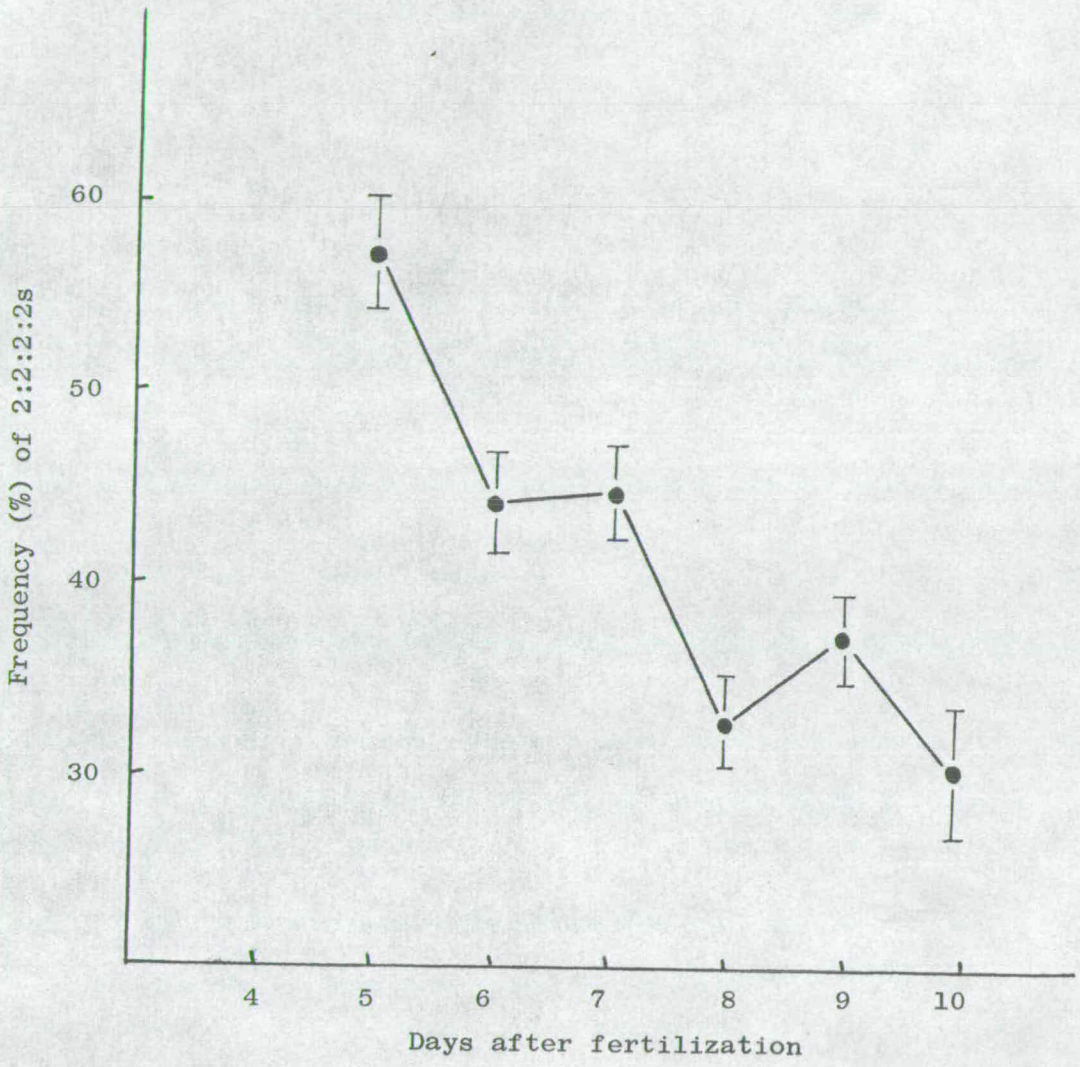

60
50
40
30
Frequency (%) of 2:2:2:2s
4
5
6
7
8
9
10
Days after fertilization

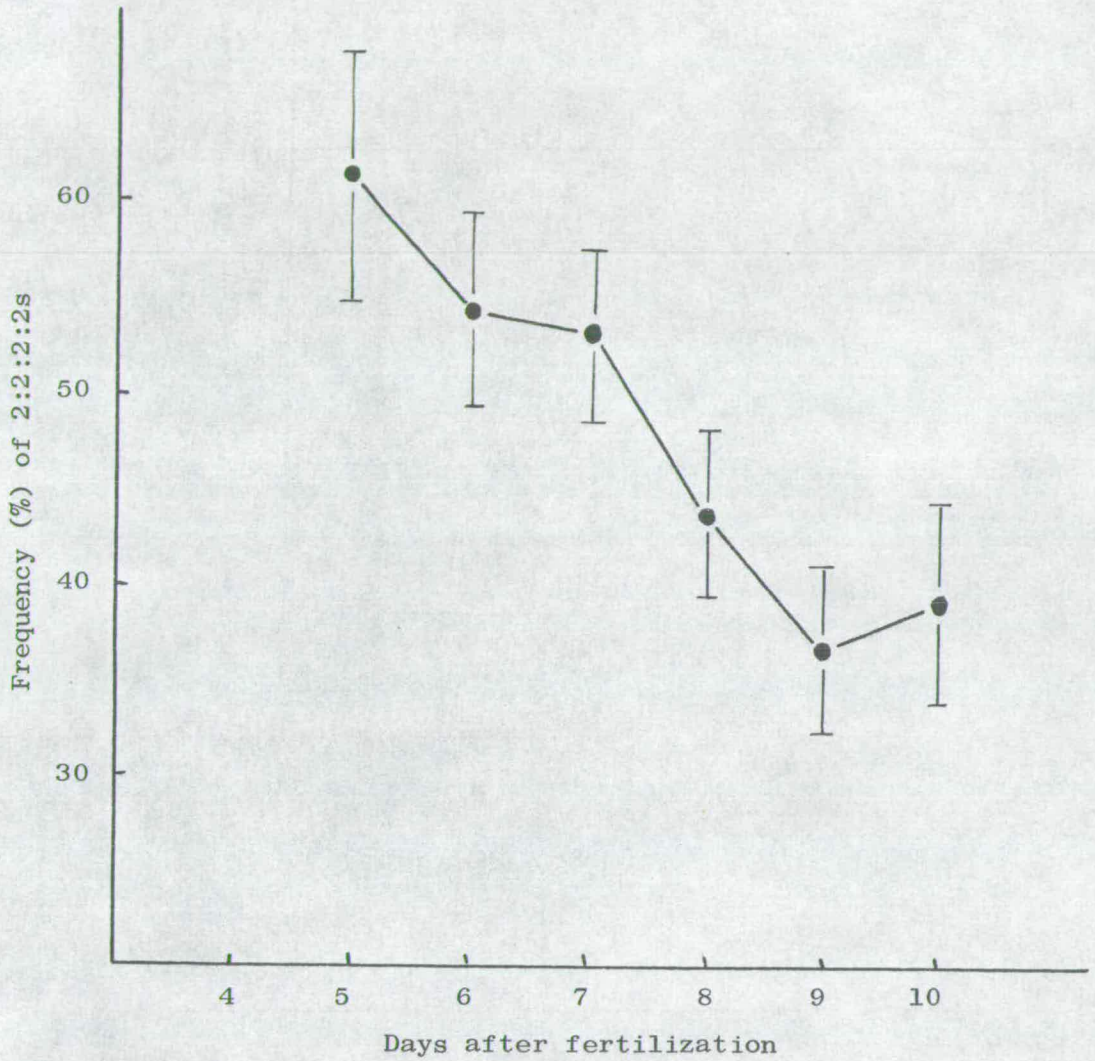
Figure 15

Frequency in tetratypes of asci with a 2:2:2:2 distribution of buff (c79) alleles from a cross of two spore colour mutants (c79 x S187).

Data taken from Table 6.

$$s.e. = \pm \sqrt{\frac{f/n(1-f/n)}{n}}$$

where f is the number of 2:2:2:2 asci in a total of n.


Frequency (%) of 2:2:2:2s
60
50
40
30
4
5
6
7
8
9
10
Days after fertilization

apparent tendency for the frequency of any position to decline or increase with time.

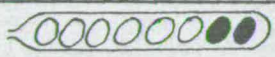
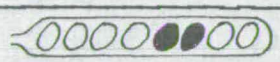
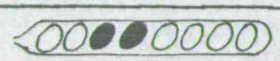
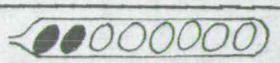
Position of wild type (black) spores in tetratypes	Days after fertilization											
	5		6		7		8		9		10	
	no	%	no	%	no	%	no	%	no	%	no	%
	13	25.0	19	20.9	23	18.6	39	30.5	32	26.2	22	25.3
	12	23.1	30	33.0	33	26.6	40	31.3	32	26.2	19	21.8
	18	34.6	25	27.5	37	29.8	25	19.5	33	27.0	21	24.1
	9	17.3	17	18.7	31	25.0	24	18.8	25	20.5	25	28.7
Total	52		91		124		128		122		87	

Table 9

No and frequency of tetratype asci with black spores in each of the four possible positions in a cross of a yellow spore colour mutant (S187) with a buff spore colour mutant (c79). [Isolate nos M26.4 (mo^-S187a) x B88.2 (c79A)].
Data extracted from Table 6.

Heterogeneity $\chi^2_{15} = 15.9 \quad p > .50$

The answers to these four questions indicated that the presence and position of wild type spores in asci from the two point cross did not affect the rate of ascus maturation and discharge. There was, however, preferential discharge of asci having a 2:2:2:2 distribution of the buff allele c79. While these results do not exclude the hypothesis that preferential discharge is a consequence of the distribution of spores they are entirely consistent with the hypothesis that spindle overlap asci are preferentially discharged.

Cross not segregating for spore colour markers

Asci from a cross of two wild type spore colour isolates were

dissected and the sequence of the mating type alleles scored. This experiment was devised to test the hypothesis that 2:2:2:2 asci were preferentially discharged when the two spore phenotypes involved differed in such a way as to give this sequence an increased rate of maturation. Possible mechanisms for this effect have been discussed (see page 9). It was thought that the absence of any difference in spore colour might mean that 2:2:2:2 asci in this cross would not show more rapid maturation and discharge than other sequences. On the other hand if spindle overlap asci were preferentially discharged the absence of a spore colour difference would not be expected to have any effect on the decline in 2:2:2:2 frequency.

The results appear in Table 10. There is a clear decline in the frequency of 2:2:2:2 sequences between five and eight days after fertilization, from 48% to 32%, ($0.05 \rangle p \rangle 0.01$).

Days after fertilization	Distribution of mating type alleles					
	4:4	2:2:2:2	2:4:2	Total	Ambiguous	Unscoreable
5	49	47	2	98	5	17
8	62	31	2	95	2	8

Table 10

Numbers of asci with specified distributions of the mating type alleles (A/a) from a cross of two wild type isolates sampled 5 and 8 days after fertilization. [Isolate nos. M36.5a and M36.4A].

Unscoreable asci were those in which two or more spores failed to germinate. Ambiguous asci were those which could not be put into one of the three major ascus classes either directly or by invoking one partial third division spindle overlap event.

The statistical significance of the difference between 2:2:2:2 frequency on days 5 and 8 was tested by a contingency X^2 test

$$\chi^2_1 = 4.09 \qquad 0.05 \rangle p \rangle 0.01$$

Conclusion

The experiments presented in this chapter clearly show that the proportions of different types of asci in *Sordaria brevicollis* change as a cross matures. It is proposed that this is the major cause of the fluctuations in spindle overlap frequency which have been noted by different workers. Two hypotheses have been proposed to account for this phenomenon. Of these, one is very difficult to disprove, namely that the distribution of phenotypically different spores in an ascus affects the rate of maturation and discharge of asci. The preferential discharge of asci with a 2:2:2:2 distribution of mating type alleles but no other apparent phenotypic difference is unexpected and hard to account for on this hypothesis. So too is the preferential discharge of tetratype asci with a 2:2:2:2 distribution of the buff allele in the two point cross.

The second hypothesis, that spindle overlap asci mature and are discharged earlier than non-overlap asci, leads to a number of direct predictions. With one exception the experimental observations fit the prediction extremely well. The exceptional result is the failure of the proportion of recombinant 2:2:2:2s and non-recombinant 4:4s to increase with time. No explanation has been proposed or tested which would explain this result without excluding the possibility that overlap asci are preferentially discharged.

Part 2

Protoperithecial production in *Sordaria brevicollis*

CHAPTER 1

Introduction

One of the central problems of biology is understanding the control of morphogenetic processes. The physical and chemical reactions occurring within an organism are organized both spatially and temporally in such a way as to produce a specific and predictable phenotype. Environmental fluctuation can be tolerated and adapted to without loss of integrity. Genetic information within a cell is continually interacting with external sources of information, which may be other cells in the same organism or may be environmental sources, independent of the organism.

The differentiation of identical cells requires different morphogenetic responses to different external information. The study of the control of morphogenesis in a particular organism involves investigation of the stimuli which induce differential responses and of the way in which such external stimuli interact with the internal, genetic information of the organism. Clearly different organisms show different levels of complexity in these interactions. For example the induction of the lac operon in *E. coli* is stimulated in the presence of exogenous lactose in a way which involves relatively few chemical components with specific functions which are well researched and understood. On the other hand the processes involved in embryogenesis of higher organisms lead to the differentiation of numerous cell types and their organisation into tissues and organs with specific functions. This involves the communication of information about the position of cells within the organism and the induction of different-

iation within a rigorously controlled spatial and temporal framework. Although while the basic processes involved may be similar in many ways to those involved in the induction of the lac operon many features of embryogenesis are not understood.

Morphogenesis in Sordaria brevicollis is relatively simple. Hyphal elongation and branching are the most obvious events occurring during the vegetative phase of the life cycle. The differentiation of hyphae to form incipient female sexual reproductive bodies, the protoperithecia, is the other main morphogenetic process. It is with this event that this part of the thesis is principally concerned.

In the course of routine crosses of S. brevicollis it was noticed that the distribution of fruiting bodies on the mycelial surface was not random. Symmetrical patterns of perithecial distribution, centred on the inoculum were the rule rather than the exception. Different parts of the mycelium were to some extent autonomous and differed in the extent to which they differentiated fruiting structures. This investigation was initiated in an attempt to discover what stimulated a culture to produce protoperithecia, how this stimulus interacted with the genetic content of the organism and why different parts of cultures reacted to the stimulus in different ways.

Cultures in circular petri dishes are radially symmetrical and any radius contains all the information required to describe the entire culture. It could be said, therefore, that what is being investigated is differentiation in a one-dimensional system. Growth along any radius is controlled by internal and external factors in such a way that any point on the radius has a specific probability of differentiating. It was hoped that such a system, involving relatively

simple differentiation might be sufficiently amenable to investigation to yield results of general application to morphogenetic studies.

In the account that follows the production of protoperithecia in a wild type culture is described. The effect of various environmental changes on such a culture was then investigated. Two mutant strains with altered protoperithecial production were obtained and an attempt was made to find biochemical changes in the mycelium correlated with fruit body formation.

Materials and Methods

The materials and methods which apply generally to this part of the thesis are given here. Details of biochemical assay techniques are given where appropriate in the relevant sections of the text. The formulae for all media are given in Appendix 1.

Strains

The strains of Sordaria brevicollis used were all isolated from stocks held by Dr D.J. Bond. Their complete history was not documented but they had been subjected to a high degree of inbreeding and many had received some form of mutagenic treatment in their recent past. Strains mentioned in the text were all wild type at all known loci except where otherwise stated.

Pairs of alleles at the perithecial-1 (per 1) and perithecial-2 (per 2) loci were isolated from a cross which had not been subjected to mutagenic treatment. Both parents in the cross, however, came from stocks which had been treated with either UV or NTG in their recent past (Bond and MacDonald, manuscript submitted for publication).

Definition of crossing phenotype

Crossing phenotype was the term used to describe the appearance of a fertilized culture in terms of the number and distribution of perithecia. It also came to include the number and distribution of protoperithecia and the time of onset of protoperithecial production.

Standard growth and crossing technique

Except where otherwise stated cultures were grown and crossed in 9cm plastic petri dishes on 20mls of corn meal agar (CMA) medium

at 25°C. Crosses were fertilized four days after inoculation, with microconidia of opposite mating type. Microconidial suspensions were obtained by pouring about 4mls of sterile distilled water over a four day old culture and agitating the surface of the mycelium with a glass rod. The suspension was filtered through two layers of sterile muslin to remove protoperithecia and fragments of mycelium. About 2mls of the suspension was poured onto the culture to be fertilized and the petri dish shaken to ensure even coverage of the mycelium. Excess water was poured off. It has been shown, (Bond, unpublished results), that the concentration of microconidia in a typical suspension of this sort can be increased or decreased by a factor of ten without any noticeable change in the resulting crossing phenotype.

Fresh isolates of strains were obtained by spreading ascospores on 4% CMA (CMA + 2.3% Difco Agar) to which 0.7% sodium acetate was added to stimulate spore germination. After overnight incubation at room temperature germinated spores were picked off under a dissecting microscope into tubes containing Vogel's (1956) N medium.

Defined media

Experiments to investigate the effect on crossing phenotypes of alterations in the nutrients available were carried out on Westergaard and Mitchell's (1947) medium from which KNO_3 had been omitted. This was solidified with 1.5% Difco agar and other supplements were added as mentioned in the text. The crossing phenotypes of cultures grown on CMA with alterations in the sugar supply were determined on the normal CMA medium from which glucose and sucrose had been omitted and supplements added according to the text.

Perithecial and Protoperithecial production in wild type cultures

Under the standard conditions described in Materials and Methods the diameter of a culture of Sordaria brevicollis increased linearly at a rate of about 2mm per hour. Between inoculation and the onset of the linear growth phase there was typically a lag of approximately 12 hours, (see Fig. 16). The mycelium from an inoculum placed in the centre of a 9cm petri dish therefore reached the dish wall in about 2.5 days. Cultures were routinely fertilized on the fourth day after inoculation and perithecia were clearly visible after a further two days. The perithecia were distributed over the entire mycelial surface, though their density was not necessarily equal on all parts of the culture. Plate 1 shows a typical wild type culture four days after fertilization.

The asci of S. brevicollis contain four pairs of spores, each pair derived from a single mitotic division. The members of a pair can be considered to be genetically identical in most instances. Any phenotypic differences within a spore pair can thus be attributed to environmental rather than genetic factors.

Although differences in the number and distribution of perithecia were often observed between isolates grown under standard conditions very few such differences were apparent between the members of a spore pair. Plate 2 shows crosses of cultures grown from the eight spores derived from one ascus, and illustrates the similarity between members of a spore pair. It was concluded from many such asci that any variability which may have been present in the standard growth and crossing condition did not have significant effects on the development of fruiting structures in different isolates.

Figure 16 Increase in colony diameter with time of three wild type isolates on CMA in 9cm petri dishes.

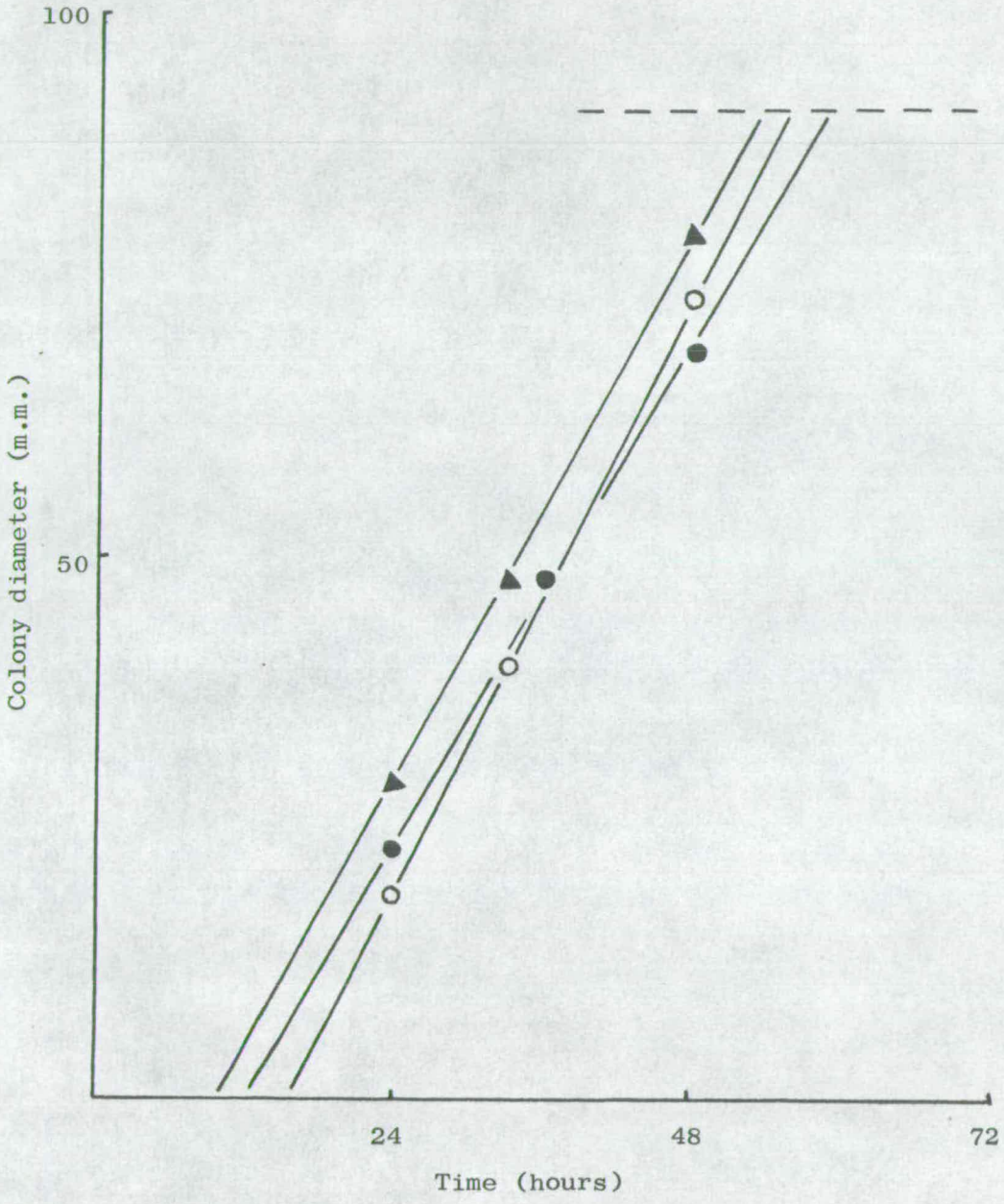
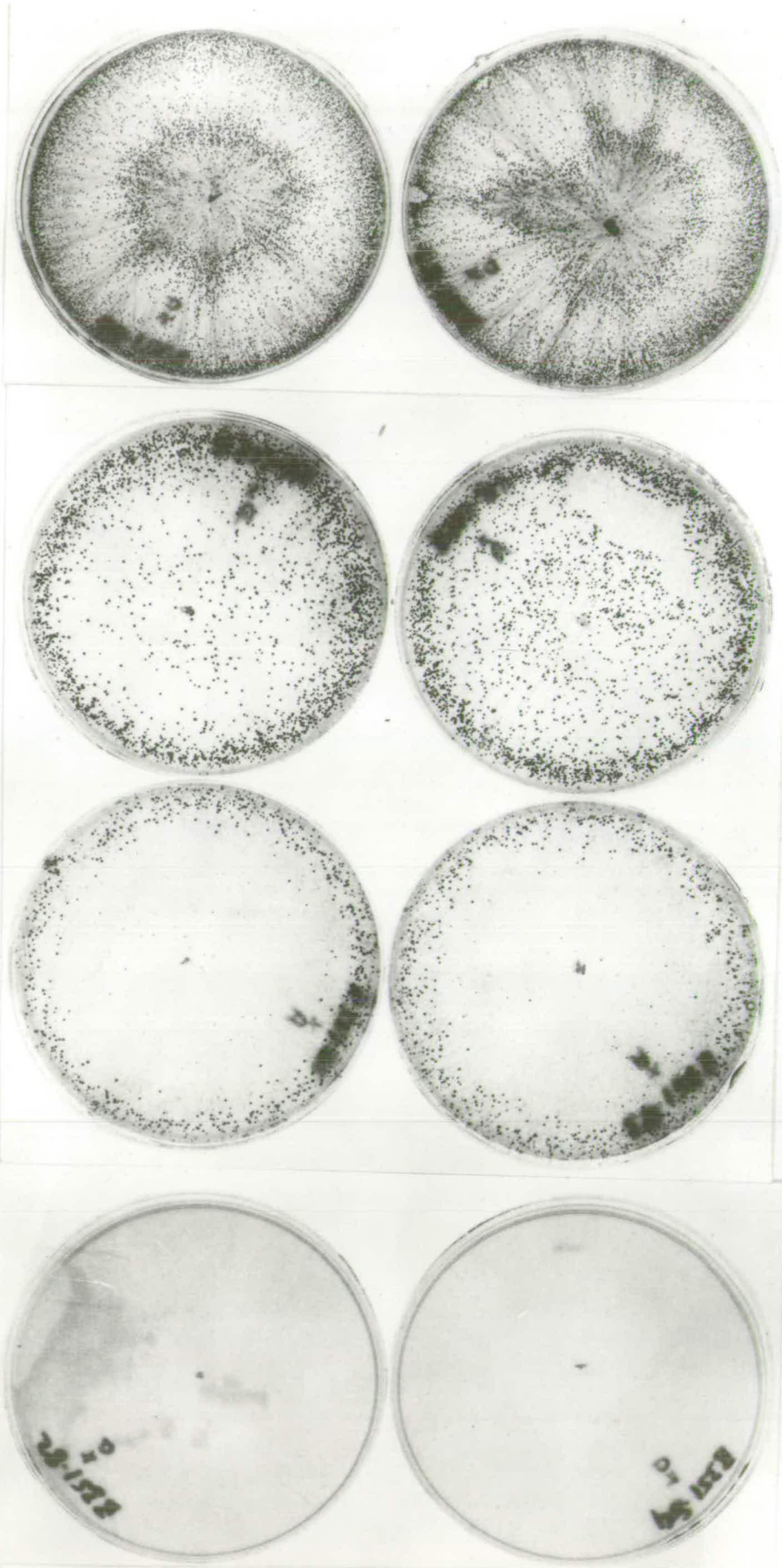

100
50
Colony diameter (m.m.)
24
48
72
Time (hours)

Plate 1 Crossing phenotype of a typical wild type isolate on CMA in a 9cm petri dish

Z1SJ.dx
+ 6

Plate 2 Crosses of cultures derived from the eight spores of a single ascus illustrating the similarity between members of a spore pair, i.e. the products of a single mitosis. The top pair is female sterile, the genetic basis of this phenotype is explained later in the text.

All crosses on CMA in 9cm dishes.



A consequence of this conclusion was that the differences in perithecial numbers and distribution between isolates derived from different mitoses must have been the result of genetic differences. The strains used in this work came from inbred laboratory stocks and it would not have been surprising had they been relatively homogeneous. Some of the more distinctive crossing phenotypes were backcrossed in an attempt to classify them genotypically. It was found that, with two exceptions which will be discussed later, the distinctive phenotype of a culture almost invariably failed to appear in its offspring. It was concluded that polymorphism was present at a large number of loci involved in the production and distribution of fruiting structures. In their relatively recent past most strains used had been subjected to some variety of mutagenic treatment. It is probable that this was largely responsible for the variability observed. In Aspergillus nidulans it has been estimated that there are between 300 and 800 loci involved in conidiophore production (Martinelli and Clutterbuck, 1971). It is possible that a similar number of loci may determine crossing phenotype in S. brevicollis.

Crossing phenotype was initially defined as the distribution of perithecia in a culture fertilized after four days of growth. Perithecia arose from fertilized protoperithecia and their distribution presumably reflected an underlying distribution of protoperithecia. Protoperithecia were formed during the growth of the organism and the stage of growth at which they appear was another aspect of the crossing phenotype. Information about the distribution of protoperithecia and the development of crosses with time was obtained by counting fruiting structures in a series of microscope fields along the radii

of cultures over a series of time intervals. One problem encountered with this technique was that of partially buried protoperithecia. As all the protoperithecia were not in one focal plane accurate counting was difficult and laborious. To overcome this difficulty cultures were grown on a layer of dialysis membrane placed on top of the normal agar medium. This technique effectively limited growth and fruiting to a single plane.

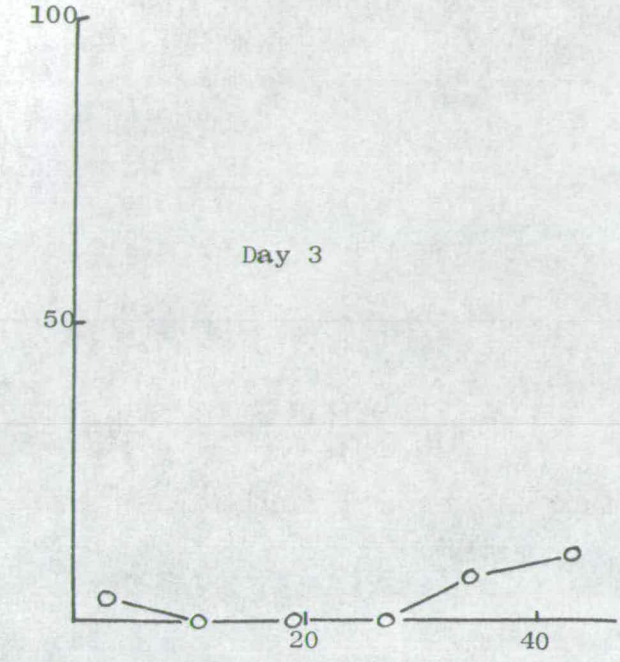
Graphs derived from counts of protoperithecia and perithecia, with and without dialysis membrane are shown in Figures 17 and 18.

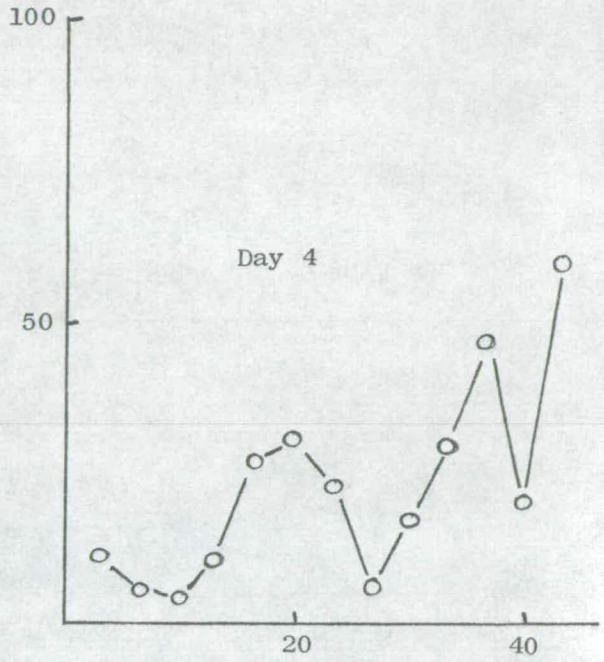
Three main conclusions were drawn from the graphs. The first was that the distribution of perithecia is a reasonably good indicator of the distribution of protoperithecia. There was no apparent effect of the position of a protoperithecium on the probability of its being fertilized. Second, the graphs showed that protoperithecia were produced at all parts of a culture more or less simultaneously. Under standard conditions they first appeared on the third day after inoculation. This contrasts with observations on organisms such as Aspergillus niger (Yanagita and Kogane, 1962) in which conidiophores are continuously produced at a fixed distance behind the advancing colony margin. Cultures of A. niger show a range of developmental states from immature conidiophores in the outer regions of the colony to aged conidiophores in the centre.

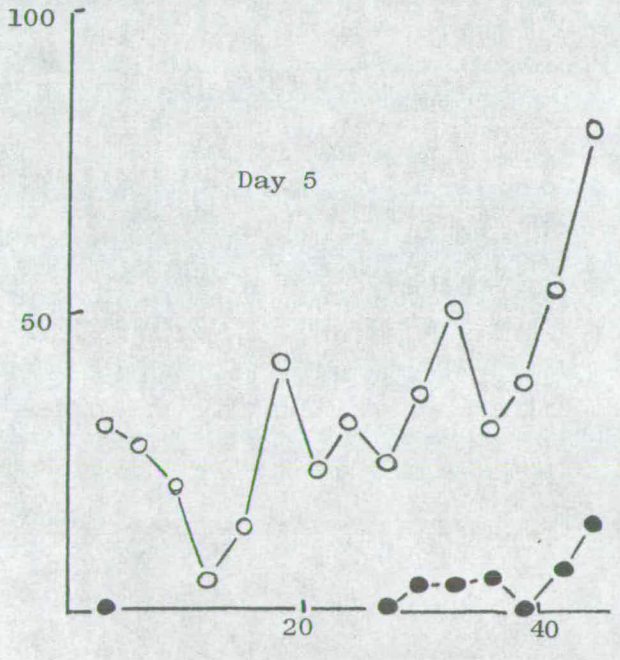
The third conclusion drawn from the graphs was that crosses performed on dialysis membrane were more fertile than those without membrane i.e. there were greater numbers of fruiting structures present. The time of first appearance of protoperithecia was approximately the same with or without membrane, though their distribution was not

Figure 17 Number and distribution of fruiting structures in a wild type culture on CMA

Numbers of protoperithecia (o — o) and perithecia (● — ●) along radii of a wild type culture between 3 and 6 days after inoculation. Distance from the inoculum (mm) is plotted on the abscissa. Number of fruiting structures per microscope field ($\approx 6mm^2$) is plotted on the ordinate. Numbers were determined each day in successive microscope fields along one randomly chosen radius.


100
50
Day 3
20
40


100
50
Day 4
20
40


100
50
Day 5
20
40

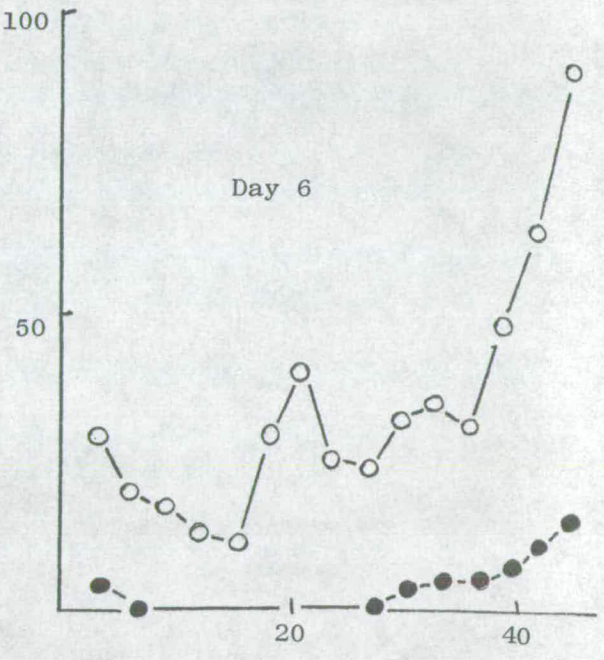
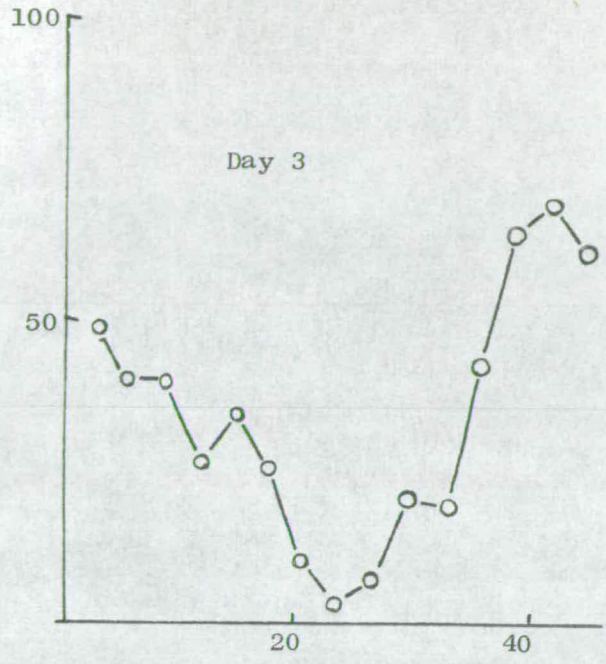
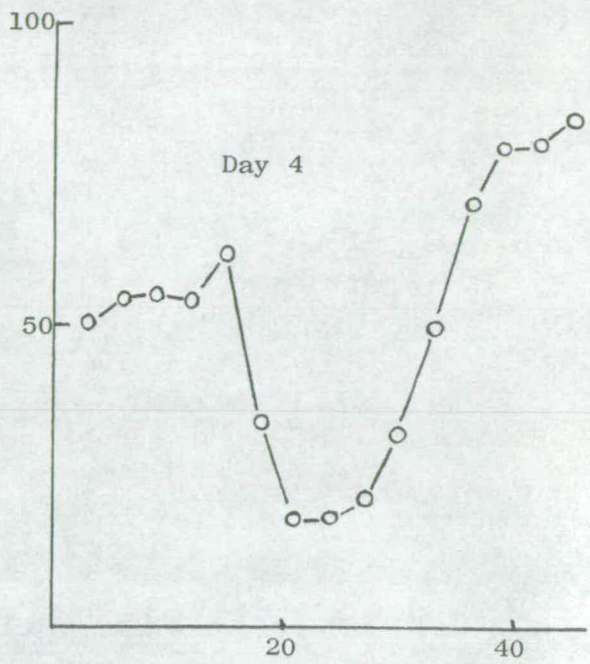
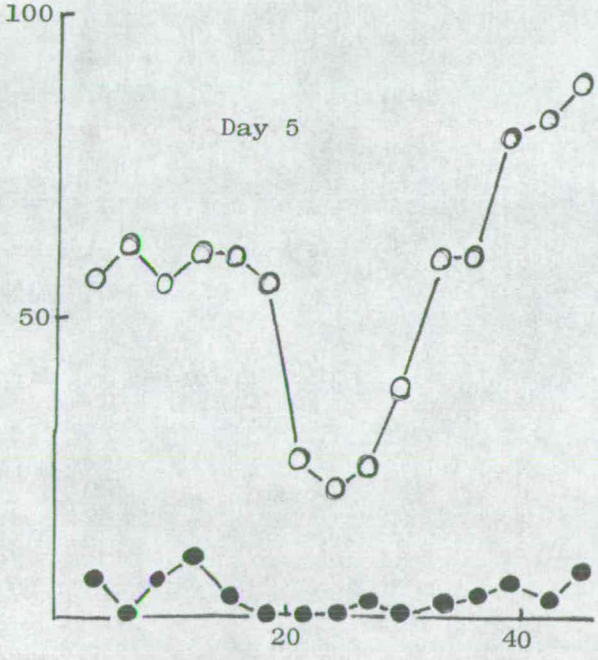

100
50
Day 6
20
40

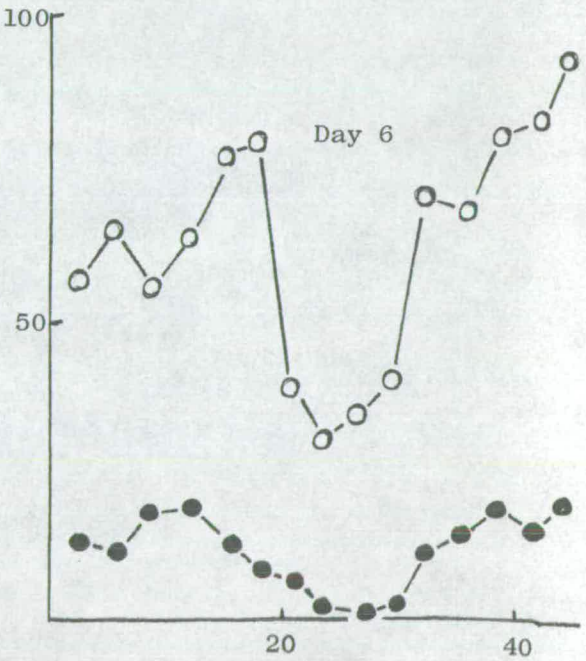
Figure 18 Number and distribution of fruiting structures in a wild type culture on CMA with dialysis membrane

Numbers of protoperithecia (o —— o) and perithecia (• —— •) along radii of a wild type culture between 3 and 6 days after inoculation. Distance from the inoculum (mm) is plotted on the abscissa. Number of fruiting structures per microscope field ($\approx 6mm^2$) is plotted on the ordinate. Numbers were determined each day in successive microscope fields along one randomly chosen radius.


100
50
Day 3
20
40


100
50
Day 4
20
40


100
50
Day 5
20
40


100
50
Day 6
20
40

necessarily identical.

Perhaps the most interesting phenomenon observed in the course of the analysis of wild type crossing phenotypes was of their development with time. Within a twenty-four hour period all parts of the mycelium produced protoperithecia in large numbers. Subsequently there was comparatively little protoperithecial production. Evidently whatever stimulus induced vegetatively growing mycelium to produce protoperithecia it acted at a specific time and had a simultaneous action on the whole colony. Although an entire culture differentiated at the same time there was evidence to show that different regions were to some extent discrete. The formation of patterns of perithecial distribution suggested that not all parts of a colony had the same response to the stimulus.

Factors affecting protoperithecial production

The onset of the production of fruiting bodies in fungi is known to be affected by a variety of factors. The availability of nutrient is often critical in this respect. Also the relative proportions and sources of nutrients may be important. Physical factors such as light and mechanical factors such as the presence of a barrier to normal growth affect reproductive morphogenesis in some species of fungi. These are all external controlling factors, the onset and extent of fruiting being dependent on the environment in which the organism is growing. The onset of fruiting may also be controlled by internal factors. For example fruiting may be initiated by the accumulation of substances produced metabolically within the mycelium and excreted into the medium. The existence of a 'fruit inducing substance' has been demonstrated in extracts of Coprinus macrorhizus (Uno and Ishikawa, 1971). Extracts of other fungi have been shown to stimulate fruiting in Schizophyllum commune (Leonard and Dick, 1968) and Venturia inaequalis (Wilson, 1927). All differentiation is, of course, ultimately under the control of internal factors, the genes. It is with the interaction between genes, other internal factors and external factors that morphogenetic studies are concerned.

Effects of nutrient availability

Introduction

In Neurospora crassa, Westergaard and Mitchell (1947) showed that the amounts and proportions of carbon and nitrogen sources in the growth medium were important determinants in the production of perithecia. The maximum numbers of perithecia were formed on medium

containing 1.8% (w/v) of glucose and 0.1% (w/v) of potassium nitrate (KNO_3). They tested a number of different sources of carbon and nitrogen. Optimum numbers of perithecia were obtained on carbon sources containing glucose, e.g. sucrose, maltose and starch. The best source of nitrogen was KNO_3. Similar results were obtained by Hirsch (1953) who also showed that protoperithecia failed to develop into perithecia until the nitrate ions in the medium were exhausted.

In penicillia and aspergilli the carbon/nitrogen balance also appears to be a general condition controlling fruiting. In some species of penicillia perithecial formation is optimum with polysaccharides as carbon sources (Basu and Bhattacharyya, 1962). Low concentrations of nitrate ions inhibit perithecium formation in Venturia inaequalis (Ross, 1959).

It has been proposed (Klebs, 1900) that sexual reproduction in fungi is favoured by growth on relatively poor nutritional conditions. This principle certainly holds true for other morphogenetic pathways, such as the production of secondary metabolites in some fungi which increases enormously after the termination of the initial growth phase of a culture. If these considerations applied to Sordaria brevicollis it might be expected that the onset of protoperithecial production, and ultimate crossing phenotype, could be affected by alterations in the constitution of the medium, particularly the carbon/nitrogen balance. Experiments were conducted to test this possibility.

Results

Wild type cultures of S. brevicollis were grown and crossed on media containing different sources and amounts of carbon and nitrogen.

The fertility of a cross under different conditions was graded into one of four categories by inspection of the numbers of protoperithecia and perithecia produced. Small variations in the pattern of protoperithecial distribution were ignored. Any distinctive crossing phenotypes, or obvious correlations between protoperithecial distribution and, for example, increasing concentration of a particular carbon source were looked for. The second parameter investigated under different growth conditions was the time of first appearance of protoperithecia. This could be measured as absolute time, i.e. time after inoculation, or related to the rate of growth of a particular culture. In the majority of cases absolute and relative time were effectively the same as most variations in the composition of the medium made little difference to the radial growth rate. In practice two measurements of the time of first appearance were made, one of these being the time elapsed after inoculation. The second measurement was of the colony diameter at the time of first appearance, or in the case of cultures which had reached the plate wall before producing protoperithecia, the time elapsed between reaching the wall and protoperithecial production.

The results of the investigation into the effects of altered nutrient supply on crossing phenotypes are shown in Tables 11 - 13. They show that the amount and source of carbon and nitrogen supplies affect the number of protoperithecia and perithecia produced. For example the addition of 5 or 10% sucrose to minimal medium gives highly fertile crosses whereas lower concentrations have relatively few perithecia. Glucose, on the other hand produces most fertile crosses at a concentration of 0.5 to 2% and few or no perithecia at

Table 11 The effects of providing KNO_3 and ammonium tartrate as nitrogen sources on growth and fruiting. Cultures were grown on Westergaard and Mitchell's (1947) medium supplemented with various concentrations of the two salts.

Figures in the columns headed 'growth' represent the increase in diameter (mm) of the colony during 24 hours while growth was linear. The number of fruiting structures was classified by eye into one of four categories (—, +, ++, or +++).

All cultures were regularly inspected for the appearance of protoperithecia. It was found that every fertile culture had no protoperithecia earlier than 16 hours after it reached the edge but that these were present within a further 12 hours.

% Ammonium tartrate w/v		% KNO_3 w/v						
		0	0.001	0.005	0.01	0.05	0.1	1
0	Growth	10	14	14	17	14	18	23
	Fruiting	-	-	+	++	+++	+++	+++
0.001	Growth	18	20	18	14	27	16	21
	Fruiting	-	-	++	++	+++	+++	+++
0.005	Growth	21	23	21	24	18	21	23
	Fruiting	-	-	+	+	+++	+++	+++
0.01	Growth	21	16	21	21	23	16	24
	Fruiting	-	-	+	+	+++	+++	+++
0.05	Growth	16	23	23	23	24	25	25
	Fruiting	-	-	-	-	-	+	+++
0.1	Growth	27	27	25	23	25	27	20
	Fruiting	-	-	-	-	-	-	+
1	Growth	34	35	35	34	34	34	25
	Fruiting	-	-	-	-	-	-	-

Table 12 The effects of providing different varieties and amounts of carbon sources on growth and fruiting. Cultures were grown on Westergaard and Mitchell's (1947) medium.

Figures in the columns headed 'growth' represent the increase in diameter (mm) of the colony during 24 hours while growth was linear. The number of fruiting structures was classified by eye into one of four categories (—, +, ++ or +++).

All cultures were regularly inspected for the appearance of protoperithecia. It was found that every fertile culture had no protoperithecia earlier than 16 hours after it reached the plate edge but that these were present within a further 12 hours.

% Carbon source added (w/v)	Glucose		Sucrose		Fructose		Galactose		Glycerol	
	Growth	Fruiting	Growth	Fruiting	Growth	Fruiting	Growth	Fruiting	Growth	Fruiting
0	16	-								
0.1	17	+	18	+	18	+	16	-	16	+
0.5	19	++	17	+	19	+++	17	-	19	++
1.0	22	+++	20	+	18	+++	14	-	17	++
2.0	25	+++	19	+	20	+++	16	-	14	+++
5.0	23	++	21	+++			19	-		
10.0	20	-	19	+++			15			

Table 13 The effects of three different carbon sources on growth and fruiting on CMA from which the usual carbon sources had been omitted.

Figures in the column headed 'growth' represent the increase in diameter (mm) of the colony during 24 hours while growth was linear. The number of fruiting structures was classified by eye into one of four categories (—, +, ++ or +++).

All cultures were regularly inspected for the appearance of protoperithecia. It was found that every fertile culture had no protoperithecia earlier than 16 hours after it reached the plate edge but that these were present within a further 12 hours.

% Carbon source added (w/v)	Glucose		Sucrose		Galactose	
	Growth	Fruiting	Growth	Fruiting	Growth	Fruiting
0	37	++				
0.1	39	++	36	+	30	++
0.5	35	++	34	++	30	++
1.0	45	++	41	++	31	-
2.0	45	-	41	++	34	-
5.0	41	-	45	++	39	-
10.0	33	-	41	++	28	-

higher concentrations. Galactose does not permit any protoperithecial production. The results also show, ~~however~~, that protoperithecial production is not induced as a response to any particular nutrient level in the medium. All cultures first showed protoperithecia at about the same time, i.e. within 24 hours of reaching the dish wall. Growth on defined media was always slower and thinner than that on normal CMA.

The distribution of protoperithecia and perithecia was not the same for all nutrient sources or amounts. Plate 3 shows crosses of one isolate on four different carbon sources. The cross on 2% glucose had large numbers of perithecia. These cannot be seen in the photograph due to a pronounced darkening of the mycelium. This phenomenon was a feature of crosses grown on glucose medium and will be mentioned later in connection with tyrosinase activity.

Internal controls - medium transfer experiments

The preceding section dealt with experiments designed to test the hypothesis that protoperithecial production in S. brevicollis was induced by changes in the availability or proportions of carbon and nitrogen sources in the medium. The results suggested that these factors were not of major importance to the time of onset of fruiting.

An alternative hypothesis was that the composition of the medium was critical as a result of the excretion of some metabolic product from the organism. The concentration of this product in the medium might increase to a threshold value at which protoperithecial production would be initiated. This would be an internal controlling mechanism in which the time of onset of fruiting would be determined by factors which were not solely dependent on the external environment in which

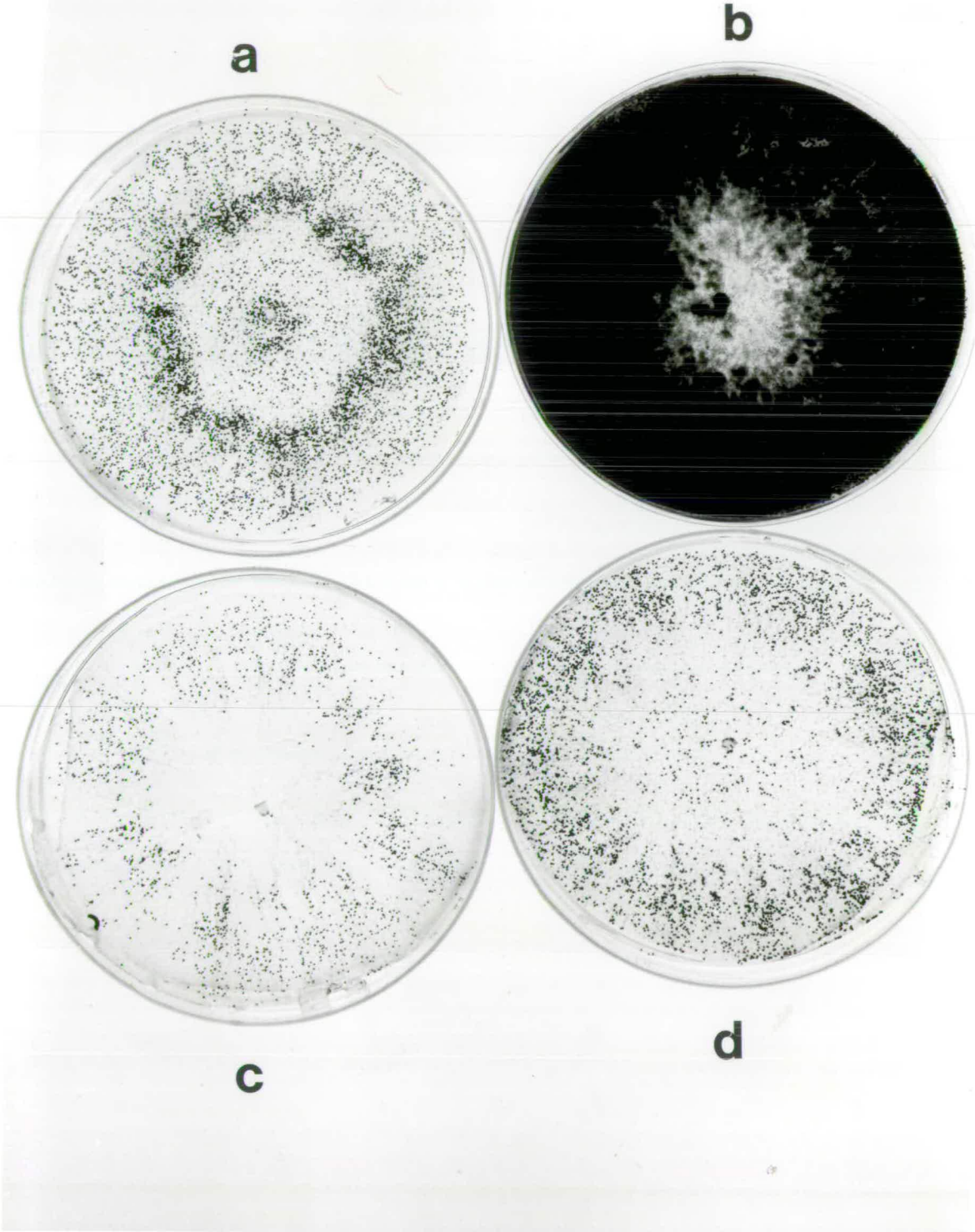
Plate 3 Crossing phenotype of a wild type culture grown in 9cm dishes on Westergaard and Mitchell's medium supplemented with different carbon sources.

(a) +2% glycerol

(b) +2% glucose

(c) +2% sucrose

(d) +2% fructose


a
b
c
d

the organism happened to be growing. The volume and constitution of the medium would still excercise some control over fruiting as it would effect the growth rate and rate of accumulation of the stimulatory substance. The existence of fruit inducing substances has been postulated in several species of fungi, (see page 40). In Chaetomiun globosum the formation of perithecia is associated with the accumulation in the medium of certain phosphorylated intermediates of carbohydrate metabolism (Buston and Khan, 1956).

The experiments designed to test the hypothesis of medium conditioning are described below. They also provide a further test of the hypothesis that the original composition of the medium is important and that protoperithecial production is induced by changes in the concentration of some nutrient, the concentration of which had not been varied in the experiments recounted in the preceding section.

The technique of growing cultures on dialysis membrane placed on the medium surface provided a means of testing the hypothesis that the medium, although initially unfavourable for sexual reproduction, was conditioned by some aspect of the metabolic activity of the fungus. The membrane, with the culture attached, could be lifted off the medium and placed on different medium so that various combinations of cultures and media of different ages could be made. For example a culture could be grown for three days, when it would be on the point of producing protoperithecia, then transferred to fresh medium. Similarly a fresh inoculum could be placed on medium which had already supported three days growth.

The simplest predictions of the medium conditioning hypothesis are as follows.

1) Just prior to protoperithecial production the medium supporting a culture will be almost fully conditioned and in a state conducive to fruiting. A fresh inoculum on such a medium should, therefore, start to produce perithecia earlier than a fresh inoculum placed on unconditioned ~~unprepared~~ medium.

2) If a culture on the point of protoperithecial production is transferred to fresh medium there will be a delay in this production as the medium must first be conditioned.

When these predictions were tested experimentally it was found that neither of them were borne out. The results appear in Table 14. They show that the time which elapsed between inoculation and protoperithecium production was the same whatever transfers were made between media and mycelia of different ages. There was no requirement for the medium to have supported growth before it would sustain fruiting. Neither would medium which had previously sustained growth favour early fruiting in a fresh inoculum. This excluded the hypothesis that protoperithecial production was induced by the accumulation of excreted metabolites. The results also further support the finding that protoperithecial production was not induced by alterations in the amounts and proportions of nutrients available to the organism.

Protoperithecial production on different sizes of petri dishes

One of the most striking features of the experiments involving altered nutrient supply and medium transfer was the constancy of the crossing phenotype under different conditions. Except in the cases where normal growth was limited by an extreme shortage of an essential nutrient all cultures began to produce protoperithecia at about the same stage of growth. Variation in the rate of growth,

Age of culture (days) when transferred	Age of medium (days			
	0	1	2	3
0	80-88	78-88	80-90	79-87
1	80-88	80-88	80-90	79-87
2	80-88	80-88	80-88	79-87
3	80-88	80-88	80-88	80-88

Table 14

Time of first appearance of protoperithecia in a wild type isolate. Cultures were grown on fresh CMA for 0, 1, 2 or 3 days then transferred to CMA which had previously supported 0, 1, 2 or 3 days of growth. The figures in the table are the number of hours elapsed between the time a culture was first inoculated onto fresh CMA and the first appearance of protoperithecia. For example a culture was grown for 2 days on fresh CMA then transferred to CMA which had already supported 3 days of growth. It was inspected 79 and 87 hours after the original inoculation on fresh CMA. Protoperithecia were visible after 87 hours but not after 79 hours.

for example when different sources of carbon were used, meant that cultures of the same age were of different sizes. A feature of all cultures, however, was that the first protoperithecia appeared within 24 hours of the time at which the whole of the medium surface was covered by the mycelium. This observation suggested that protoperithecial production was induced in response to one of two stimuli. First it might be a response to colony size. This would be an entirely internal control mechanism, mediated for example by the accumulation of some metabolic product in the mycelium which reached a threshold value after the colony reached a certain size. The second possibility was the environmental stimulus required to initiate protoperithecial production was provided by the wall of the petri dish. This would suppose that by acting as a barrier to further radial growth the dish wall would interact with an advancing mycelial front in such a way as to produce a morphogenetic response in the whole of the culture. The stimulation of fruiting in fungi by a barrier is a well known phenomenon, some examples are mentioned below (page 47).

These possibilities were tested by growing cultures on petri dishes of different diameters, (5, 9 and 14cm). If colony size was the critical factor protoperithecia would be expected to form at the same time on a 14cm dish as on a 9cm dish. If, however, the dish wall was critical then the first protoperithecia would be expected to appear at a constant time after the mycelium reached the wall on different sizes of dish. The results of these experiments appear in Table 15. On all sizes of dish there was no fruiting until the edge of the dish was reached. Protoperithecia first

	Diameter of petri dish (cm)		
	5	9	14
Time to reaching dish wall	35 hrs	52 hrs	75 hrs
Time for reaching wall to appearance of first protoperithecia	20-28 hrs	20-28 hrs	20-28 hrs

Table 15

Time taken by a wild type isolate to reach the dish wall on different sizes of dish and time elapsed between reaching the wall and the first appearance of protoperithecia. Plates were examined 20 and 28 hours after the edge was reached. Protoperithecia were visible after 28 hours but not after 20 hours.

appeared about 24 hours after this event in all cases. The results clearly favour the hypothesis that protoperithecial production is initiated by an interaction between the petri dish wall and the colony.

Robinson (1926) observed that reproductive structures in Pyronema confluens arose after the growth of the fungus had been checked by the wall of the containing vessel.

In other species of fungi in which the stimulation of fruiting by a physical barrier has been reported the effects are localized (Hawker, 1957; Raper, 1966). Buston and Rickard (1956) showed that in Chaetomium globosum the presence of a physical barrier was associated with an increase in the concentration of perithecia in its neighbourhood. They explained this observation by proposing that the barrier prevented free diffusion of organic phosphates away from the mycelium. These substances stimulate perithecial formation in C. globosum and the localized increase in their concentration resulted in an associated increase in perithecial concentration. In Sordaria brevicollis the edge effect is not confined to the immediate area of the dish wall, nor is there necessarily a tendency for the density of protoperithecia to decrease as the distance from the edge increases. Apparently some interaction occurs between the wall and the colony as a result of which the entire culture is stimulated to produce protoperithecia. The extent of this stimulation, as measured by the number of protoperithecia produced, varies in different parts of the colony.

Genetic control of protoperithecial production

In the course of a series of routine crosses a female sterile strain of *S. brevicollis* was isolated (Bond and MacDonald, manuscript submitted for publication). The vegetative growth of this strain was normal but no protoperithecia were produced. There were normal microconidia so the strain could be used as the male parent in crosses.

Genes affecting the morphogenesis of fruiting structures have been described in a number of species of Ascomycetes (review in Esser and Keunen, 1967). An analysis of mutant blocks to sexual development in *Sordaria macrospora* was carried out by Esser and Straub (1958). They found eighteen sterile mutants blocked in a total of four developmental steps leading to protoperithecial production. Wheeler and McGahen (1952) found a locus determining the production of perithecia in *Glomerella cingulata*. A second locus determined whether the perithecia were scattered or clumped on the mycelium.

The female sterility found in *S. brevicollis* was shown to be an interaction of alleles at two loci. The loci were called perithecial-1 (per-1) and perithecial-2 (per-2). There were four combinations of the alleles of per-1 and per-2. Per 1^- per 2^- was female sterile. Per 1^+ cultures were indistinguishable from wild type whatever per 2 allele they were coupled with. Per 1^- per 2^+ cultures had a distinctive phenotype characterized by the production of a ring of perithecia just in from the edge of the petri dish. Crosses of the eight cultures derived from an ascus from a cross segregating at both loci are shown in Plate 4.

This two gene interaction, and the altered distribution of perithecia in the per 1^- per 2^+ genotype suggested that female sterility in this case might have been the result of mutations in

Plate 4 Crosses of cultures derived from the eight spores of a single ascus from a cross of per 1^- per 2^+ a x per 1^+ per 2^- A

(a) per 1^- per 2^- spore pair - female sterile

(b) per 1^- per 2^+ spore pair - produces perithecia just in from the plate edge

(c) and (d) per 1^+ per 2^+ and per 1^+ per 2^- spore pairs - phenotypically wild type

All crosses on CMA in 9cm dishes

a
b
c
d

pathways affecting the control of the induction of protoperithecial production rather than of a complete block in a protoperithecium synthesizing pathway.

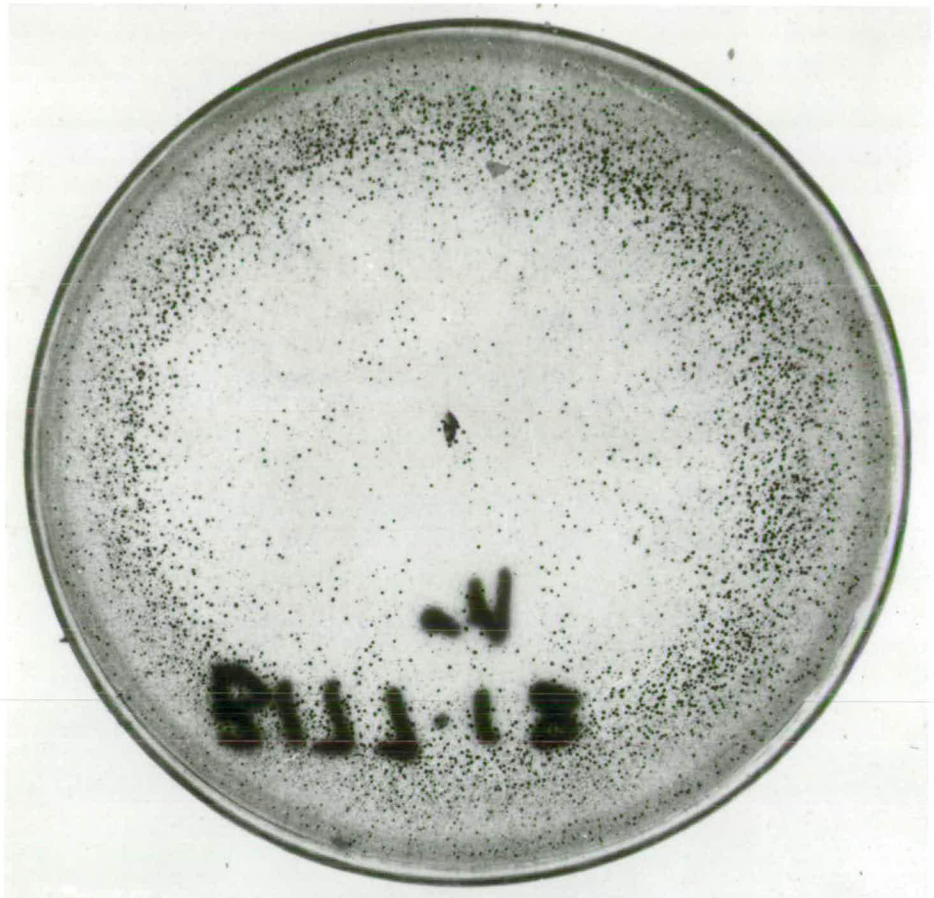
When the four combinations of the per 1 and per 2 alleles were grown on dialysis membrane an interesting effect on one of the crossing phenotypes was observed. Per 1^- per 2^- cultures remained sterile. The phenotype of Per 1^- per 2^+ cultures was altered in a striking manner, illustrated in plate 5. Perithecia were produced in large numbers in the centre of the plate but there was an almost completely sterile margin to the culture, about 15mm wide. This pattern was highly reproducible, no per 1^- per 2^+ cultures which did not show it were found.

Analysis of the crossing phenotype of per 1^- per 2^+ cultures

Figure 19 shows the distribution of perithecia and protoperithecia on successive days during the growth and crossing of a per 1^- per 2^+ isolate. Apart from the perithecial distribution the crossing phenotype bore many similarities to wild type. Protoperithecia first appeared about 24 hours after the colony reached the plate edge. They appeared simultaneously in all parts of the culture. It seemed likely from these observations that, as in wild types, protoperithecial production was initiated by an interaction with the plate edge. The hypothesis was tested by growing per 1^- per 2^+ cultures on different sized dishes. This experiment also investigated the factors controlling the formation of the distinctive pattern of perithecial distribution. It was thought that the extent of the fertile part of the colony could be determined by the inoculum, in which case a similar size of fertile zone would be found surrounding

Plate 5 Crossing phenotype of a per 1^{-} per 2^{+} culture on dialysis membrane (above), and without membrane (below), on CMA in 9cm dishes.

Both crosses have characteristic phenotypes. With membrane there is an almost sterile margin to the colony and a sharp distinction between fertile and sterile zones. Without membrane perithecia form in a ring just in from the plate edge.



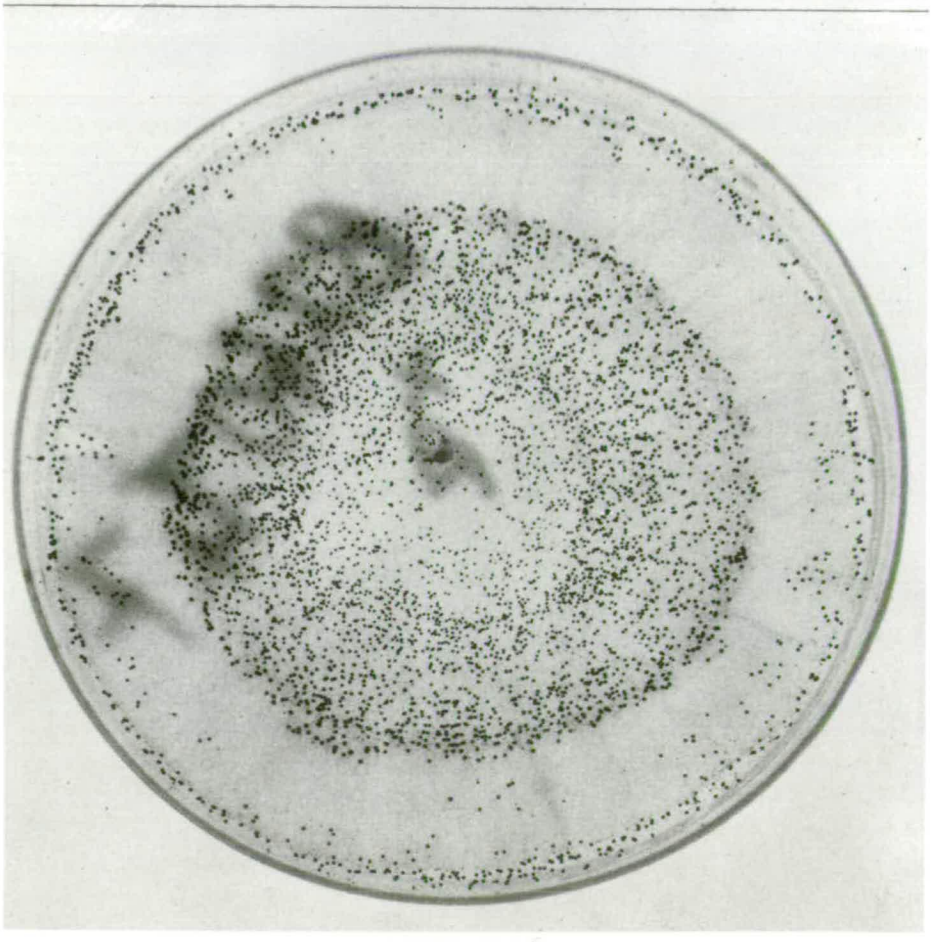
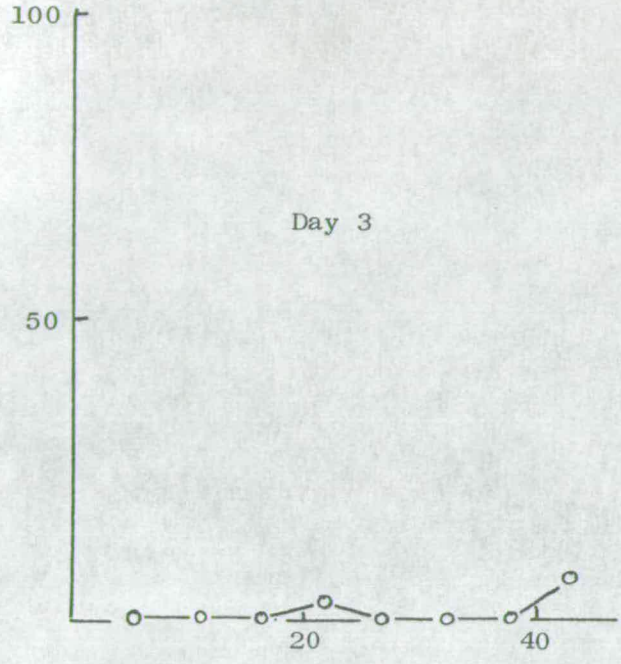
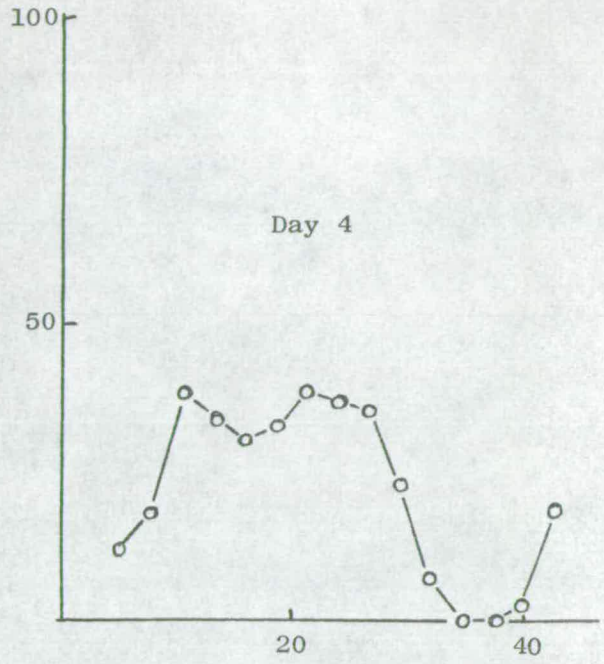
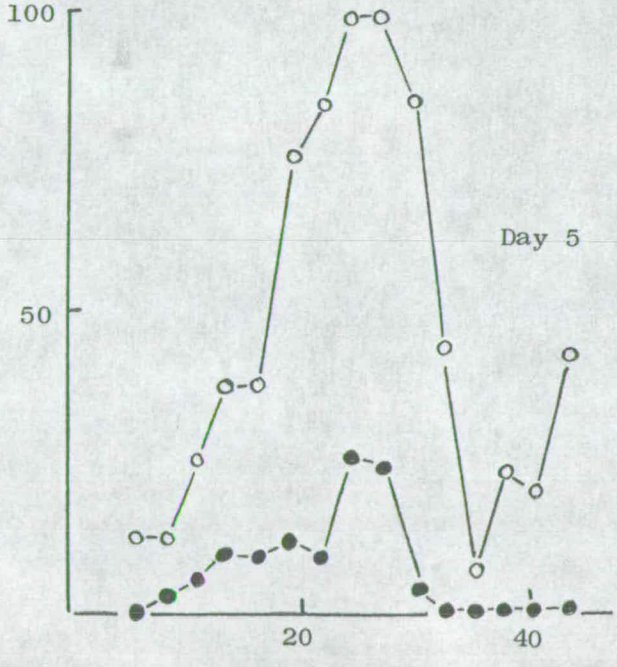


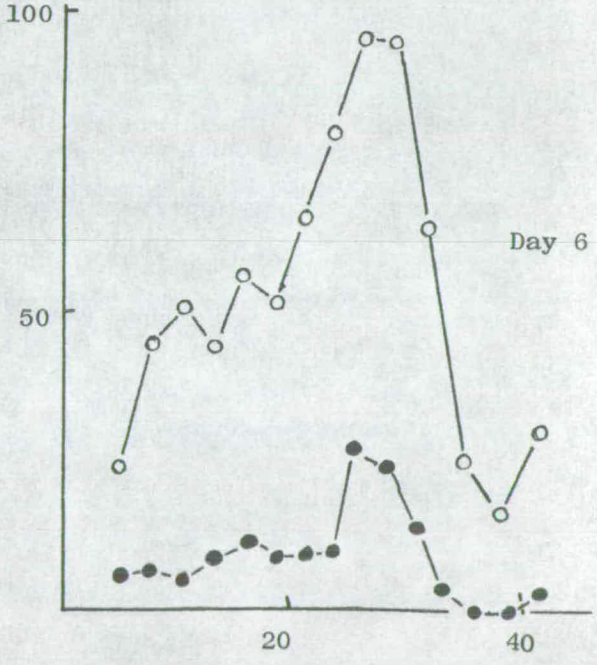
Figure 19 Number and distribution of fruiting structures in a per 1^- per 2^+ culture on CMA with dialysis membrane

Numbers of protoperithecia (o —— o) and perithecia (• —— •) along radii of a wild type culture between 3 and 6 days after inoculation. Distance from the inoculum (mm) is plotted on the abscissa. Number of fruiting structures per microscope field ($\approx 6mm^2$) is plotted on the ordinate. Numbers were determined each day in successive microscope fields along one randomly chosen radius.


100
50
Day 3
20
40


100
50
Day 4
20
40


100
50
Day 5
20
40


100
50
Day 6
20
40

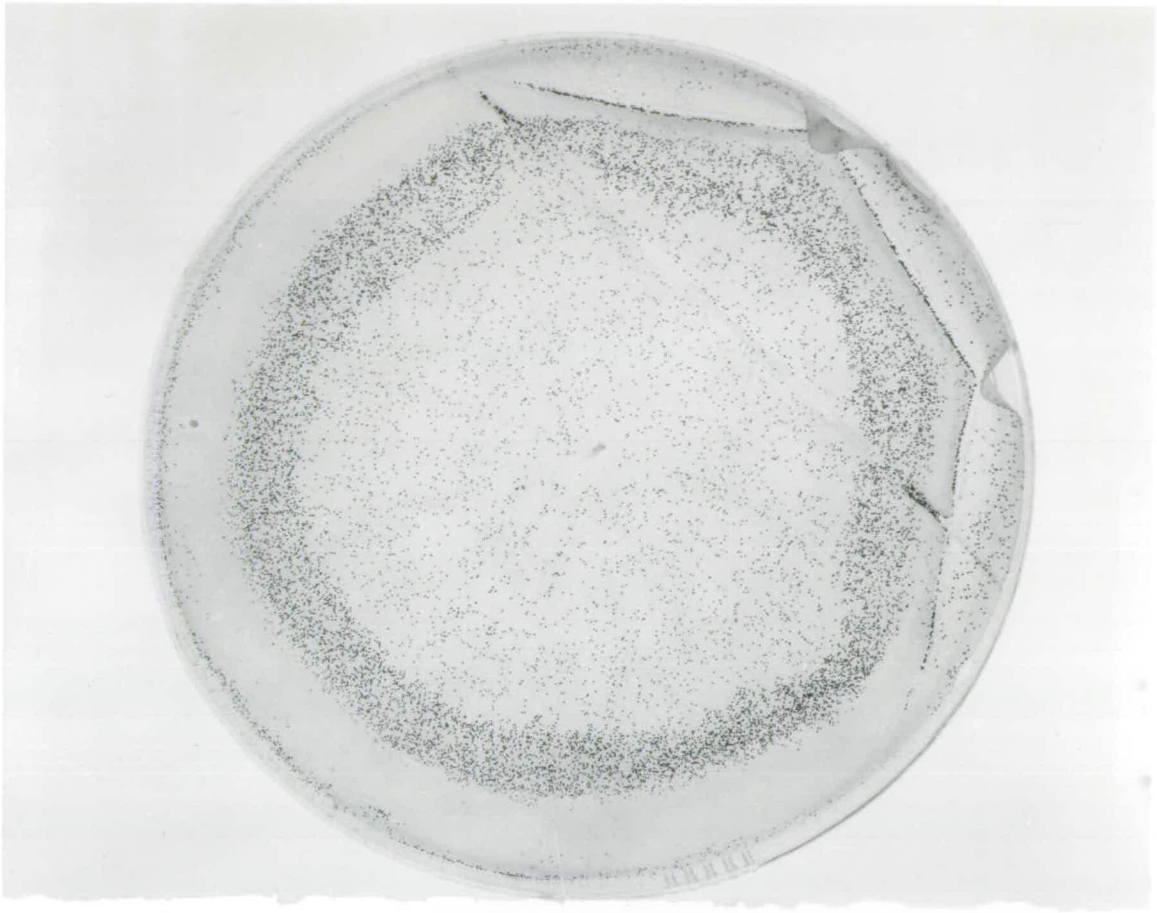
the inoculum on any size of plate. Alternatively the limit of the fertile zone could be determined by the plate edge. In this case the fertile zone would be expected to start a constant distance from the edge whatever size of plate was used. Square petri dishes were also used to show whether the pattern was determined by the inoculum or the edge. The results of these experiments are shown in Plates 6 and 7.

On both 9cm and 14cm dishes protoperithecia first appeared about 24 hours after the cultures reached the plate margin. The crossing phenotype on 14cm dishes was similar to that on 9cm dishes in that there was a sterile margin surrounding the fertile centre of the colony. There was a sharp distinction between the fertile and sterile zones. The width of the sterile margin was relatively constant on both sizes of dish.

Cultures grown on square plates clearly showed that the pattern of protoperithecial distribution was, in this case, determined by the edge of the petri dish rather than the position of the inoculum. Further evidence to support this observation was obtained by inoculating dishes eccentrically with per 1^- per 2^+ cultures. A series of such dishes appears in Plate 8. The position of the fertile colony centre is not affected by displacement of the point of inoculation. In extreme cases, where the inoculum is placed very close to the dish edge, there is some distortion of the normally circular fertile zone. On 14cm plates this distortion is more marked as is shown in Plate 9. In the vicinity of the inoculum the sterile margin typical of per 1^- per 2^+ is present. Further from the inoculum this phenomenon vanishes though the reasons for this have not been investigated. An interesting feature of Plate 9 is the presence of

Plate 6 Per 1^- per 2^+ culture grown on dialysis membrane in a 14cm diameter petri dish

Plate 7 Per 1^- per 2^+ culture grown on dialysis membrane in a square petri dish (10 x 10cm)



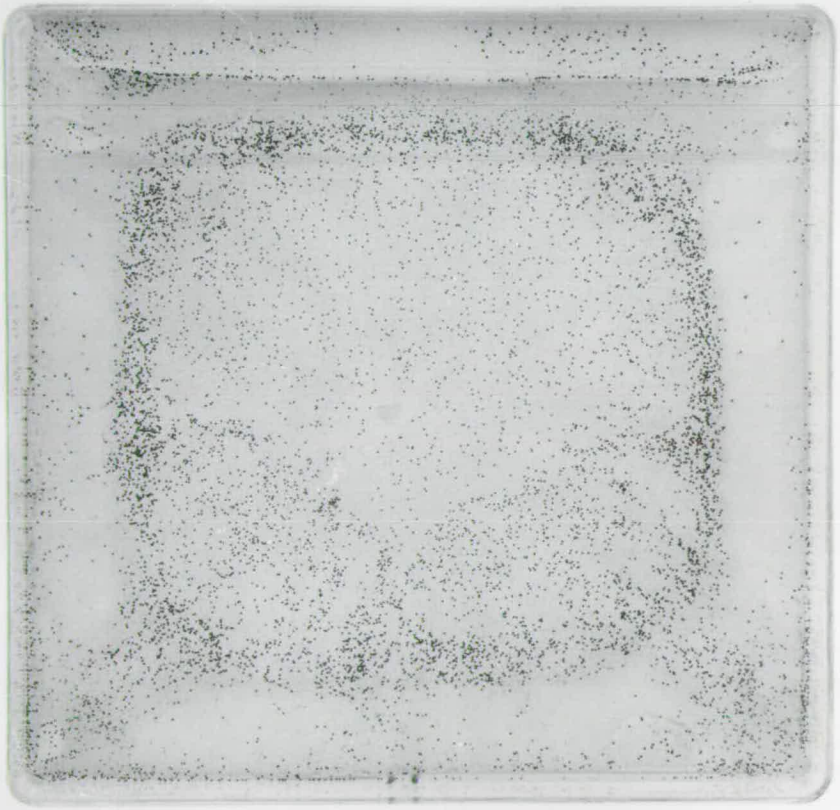


Plate 8 Crossing phenotype of eccentrically inoculated per 1^{-} per 2^{+} cultures on dialysis membrane on CMA in 9cm dishes.

The point of inoculation is marked with an X.

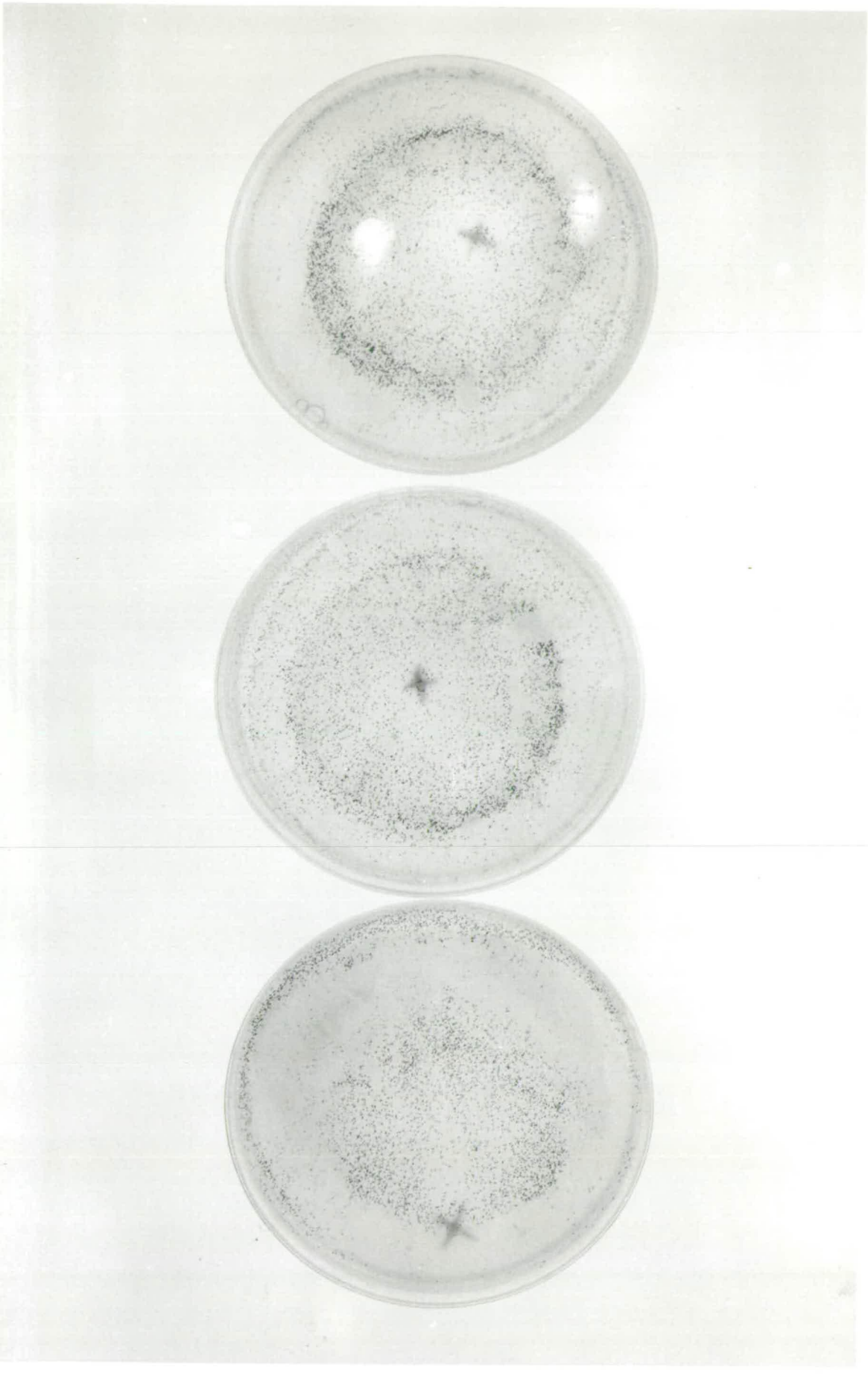


Plate 9 Per 1^- per 2^+ culture inoculated at the edge of a 14cm dish. The point of inoculation is marked with an X. Cross on CMA with dialysis membrane.

a periodicity around the inoculation point, manifested as a series of concentric bands of perithecia centred on the inoculum. This observation suggests that the distribution of perithecia may be influenced by both the dish edge and the position of the inoculum.

Effects of nutrient availability on the phenotype of per 1^- per 2^+ cultures

As with wild type cultures it was found that alterations in the composition of the medium did not affect the time of onset of proto-perithecial production (i.e. 24 hours after reaching the dish wall). The characteristic phenotype of per 1^- per 2^+ isolates, however, disappeared on defined media and no combination of carbon and nitrogen supply was found to restore it. Cultures transferred to fresh medium after 2.5 days of growth on CMA still showed the phenotype, as did fresh inocula placed on CMA which had already supported growth. It was therefore thought unlikely that the per 1^- per 2^+ phenotype arose primarily as the result of any particular state of the medium.

Conclusion

Experimental evidence has been obtained which suggests that the primary stimulus to protoperithecial production in S. brevicollis under specified conditions was the interaction between the culture and the wall of the petri dish. Protoperithecia were produced within 24 hours of the mycelial front reaching the wall. Alterations in the composition of the medium which permit normal growth did not affect the time of onset of protoperithecial production, although they may have had minor effects on numbers and distribution. Protoperithecial production was not induced by the excretion of any metabolite into the medium.

Alleles have been found at two loci which have marked effects on protoperithecial production. Per 1^- per 2^- cultures are female sterile and per 1^- per 2^+ cultures on CMA have a crossing phenotype consisting of a fertile colony centre and a sterile margin. It was proposed that these phenotypes were probably the result of mutations affecting the control of protoperithecial production rather than complete developmental blocks.

Fruiting in some species of fungi is known to be correlated with biochemical changes in the mycelium. With this in mind it was decided to look at some biochemical events during growth and fruiting in the hope of gaining further insight into the stimulus for protoperithecial production and the associated metabolic changes it induced.

CHAPTER 2

Biochemical changes during growth and crossing

Tyrosinase activity, melanin and protoperithecium production

Introduction

Fruiting in Neurospora crassa has been linked with melanin production and tyrosinase activity, (Hirsch, 1954; Horowitz, 1951; Horowitz and Shen, 1952). Hirsch postulated this connection on the grounds that protoperithecia are differentiated abundantly on a mycelium which turns dark brown, that a female sterile mycelium does not darken in this way, and that dark pigments are contained in protoperithecia, perithecia and ascospores. Hirsch showed that the dark pigments were melanins and proposed a causal relationship between their formation, tyrosinase activity and protoperithecium production. He demonstrated a peak of tyrosinase activity concurrent with the onset of protoperithecial production. He also found that no protoperithecia were produced if tyrosinase activity was suppressed by p-aminobenzoic acid, sodium thioglycolic acid, cysteine or phenylthiourea.

Material and Methods

Tyrosinase activity was assayed according to the method of Horowitz et al. (1960). The production of dopachrome from DL Dopa was measured spectrophotometrically at 475mμ in the presence of crude extracts of Sordaria mycelium.

Cultures were grown on dialysis membranes placed on the surface of Westergaard and Mitchell's medium modified to contain a low concentration of

sulphate (0.06mM). 2% sucrose and 1.5% agar were added. The mycelium was scraped off the membrane, weighed, and homogenized in sodium phosphate buffer (pH 6, 5mls of buffer per gram wet weight of mycelium). Crude extract was obtained by centrifuging the homogenate for ten minutes at 10,000g. The reaction mixture contained 0.5ml of the supernatant; 4.0ml of 0.1M sodium phosphate buffer, pH 6; 0.5ml of 0.03M 3,4 dihydroxy-DL-phenylalanine (DL Dopa) in 0.1N HCl. The assay was conducted at 25°C. Tyrosinase activity was measured in tyrosinase units, one unit being the amount of tyrosinase required to produce a change of 0.001 in Optical Density per minute at 25°C. The addition of measured amounts of purified tyrosinase (Sigma) in place of crude mycelial extracts showed that the system gave an accurate estimate of tyrosinase activity.

Assays were carried out on crude extracts of mycelia from wild type and female sterile strains. Cultures were harvested and assayed at several stages of their growth, before reaching the dish wall, immediately after reaching the wall and at the time of first appearance of protoperithecia. Assays were also performed on cultures grown on CMA as these were more fertile than those grown on low sulphate medium. It was thought that the darkening of the medium observed in some cultures grown on Westergaard and Mitchell's medium with glucose as the source of carbon might be an indication of high tyrosinase activity so cultures grown under these conditions were also added.

The effect of the addition to the medium of the tyrosinase inhibitions cystein and p-amino benzoic acid (paba) was investigated at concentrations of 10^{-1}M, 2×10^{-2}M, 10^{-2}M, 10^{-3}M and 10^{-4}M.

Results

No tyrosinase activity was detected in any strain of S. brevicollis on any medium or at any stage of its life cycle. Using the techniques employed in Sordaria the tyrosinase activity of the Em_a strain of Neurospora crassa was assayed. The activity detected was of the same order as that detected by Horowitz et al. (1960) i.e. approximately 1 unit per gram wet weight of mycelium.

At concentrations at which vegetative growth was normal the addition of tyrosinase inhibitors to the growth medium did not have any obvious effect on protoperithecial production in S. brevicollis. Hirsch (1954) found inhibition of fruiting by 10^{-2}M cystein and 3.6×10^{-2}M paba in Neurospora whereas a tenfold increase in these concentrations had no effects on growth or fruiting on S. brevicollis.

It was concluded that no correlation could be demonstrated between tyrosinase activity and protoperithecial production.

Organic phosphates and protoperithecial production

Introduction

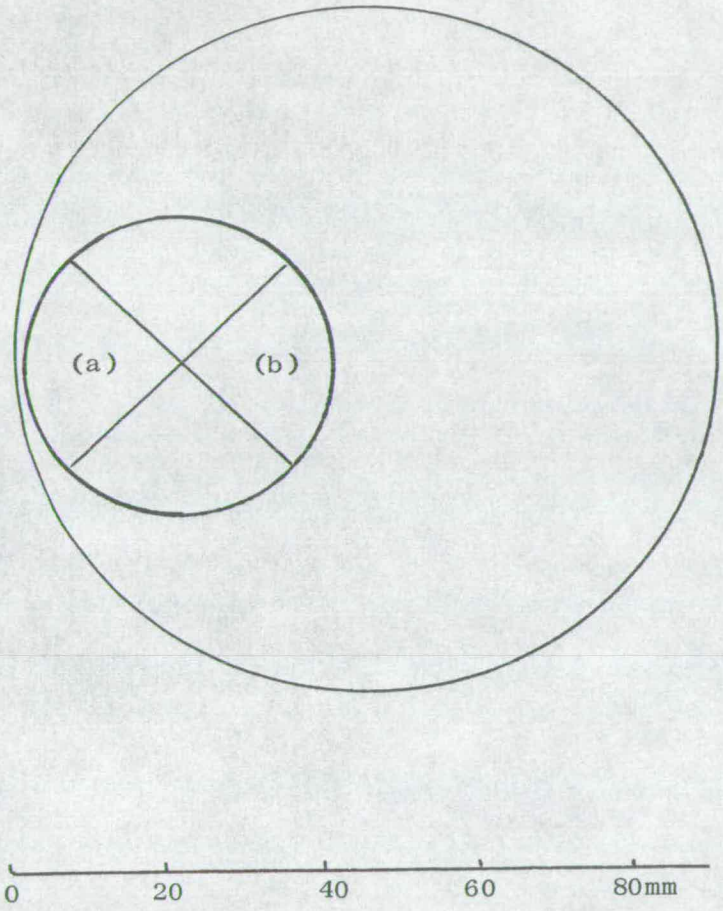
It has been suggested that fruiting in *Chaetomium globosum* is stimulated in the presence of a physical barrier to growth (Buston and Rickard, 1956). They also showed that the increase in perithecial density near a barrier was associated with an increased concentration in the medium of certain phosphorylated intermediates of carbohydrate metabolism, specifically of glucose-6-phosphate (G6P), fructose-1,6-diphosphate (FDP), and 3-phosphoglyceric acid. There is evidence that fruiting in some fungi is stimulated by the addition of organic phosphates to the medium (Buston and King, 1951; Hawker, 1948).

Buston and Rickard have proposed that organic phosphates produced in the mycelium diffuse outwards from a growing culture and that "the distribution of perithecia is such as might be expected if free diffusion of the excreted phosphates is impeded by a physical barrier." They apply this hypothesis to eccentrically inoculated cultures in which perithecia are more abundant in region (a) (see Fig. 20) than in region (b) of the colony. If organic phosphates are excreted by the mycelium it is to be expected that they are more concentrated in region (a), from which diffusion is restricted, than in region (b). A somewhat similar hypothesis is proposed by Chet, Henis and Mitchell (1966), who suggest that sclerotium formation in *Sclerotium rolfsii* is induced when an inhibitor is inactivated by an increased concentration of metabolites in the colony margin following cessation of growth at a barrier. The latter hypothesis is favoured by Pollock (1975) in his explanation of perithecium production at a barrier in *Sordaria fimicola*.

Figure 20 Diagrammatic representation of areas in which perithecia were counted and organic phosphate was estimated, (from Buston & Rickard, 1956).

The inner circle represents the margin of a fungal colony, the outer circle represents the rim of a petri dish.

Differences between the quadrants (a) and (b) are referred to in the text.


(a)
(b)
0
20
40
60
80mm

Buston and Rickard's hypothesis cannot satisfactorily be applied to S. brevicollis as no evidence has been obtained to suggest that fruiting in this organism is stimulated by excreted metabolites. In addition their hypothesis depends on a simple correlation between medium with a high organic phosphate concentration and areas of high perithecial density, a direct and localised effect. The edge effect observed in S. brevicollis however is not simply that protoperithecia are most dense at the plate edge but that their production is induced over the entire colony surface simultaneously. The effect is evidently more complex than can be accounted for on the Buston and Rickard hypothesis and involves the transfer of information from the colony margin to the colony centres through the mycelium as opposed to physiological changes induced by changes in the immediate external environment of different parts of the culture.

A hypothesis along the lines proposed by Chet et al., is more attractive as a possible explanation of the edge effect in S. brevicollis. A localised change in the colony margin, brought about by the dish wall causes either the production of an inducing substance which diffuses throughout the mycelium, or inhibits the production of a repressor of protoperithecium production during vegetative growth. The nature of the localized interaction might be an increase in metabolite concentration in the colony margin when further growth is blocked, as Chet et al., proposed, or some other effect of a change in the internal environment of the mycelium on the cessation of growth.

Whatever the mechanism of the effect, the fact that phosphorylated intermediates of carbohydrate metabolism had been linked to fruiting in other fungi prompted an investigation of its possible role in

protoperithecium production in S. brevicollis. The effect of adding organic phosphates to the medium was investigated. In addition evidence of a correlation between organic phosphate concentration in mycelium and the onset of protoperithecium production was sought.

Materials and Methods

All the experiments in this section were performed on cultures of S. brevicollis grown on corn meal agar (CMA).

The effect of the addition of glucose-6-phosphate (G6P), fructose-6-phosphate (F6P), fructose-1,6-diphosphate (FDP), adenosine diphosphate (ADP) and adenosine triphosphate (ATP) to the medium was investigated. These supplements were added at concentrations of 0.1mM, 1mM and 10mM and were also added as crystals placed on the surface of a growing culture.

The internal concentrations of a number of glycolytic intermediates were assayed. Mycelial extracts were prepared by harvesting cultures grown on dialysis membrane and homogenizing in ice-cold TCA (5% w/v solution). 10mls of TCA were added for each gram wet weight of mycelium. The homogenate was centrifuged for 10 minutes at 10,000g and the assays were carried out on the supernatant. The supernatant was extracted five times with two volumes of ether to remove the TCA. Assay techniques were spectrophotometric, based on the oxidation or reduction of nucleotide coenzymes.

1) Assay of G6P, F6P and ATP.

Reagents:

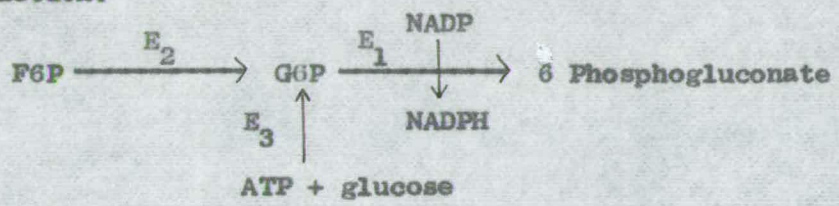
0.8mls Buffer - Glycyl glycine 0.4M
$MgCl_2$ 0.15M
Glucose 0.10M
} pH 7.4

0.1mls NADP 20mM

2.1mls sample

Enzyme 1	-	Glucose 6 phosphate dehydrogenase	10 units
Enzyme 2	-	Phosphoglucose isomerase	50 units
Enzyme 3	-	Hexokinase	10 units

Reaction:

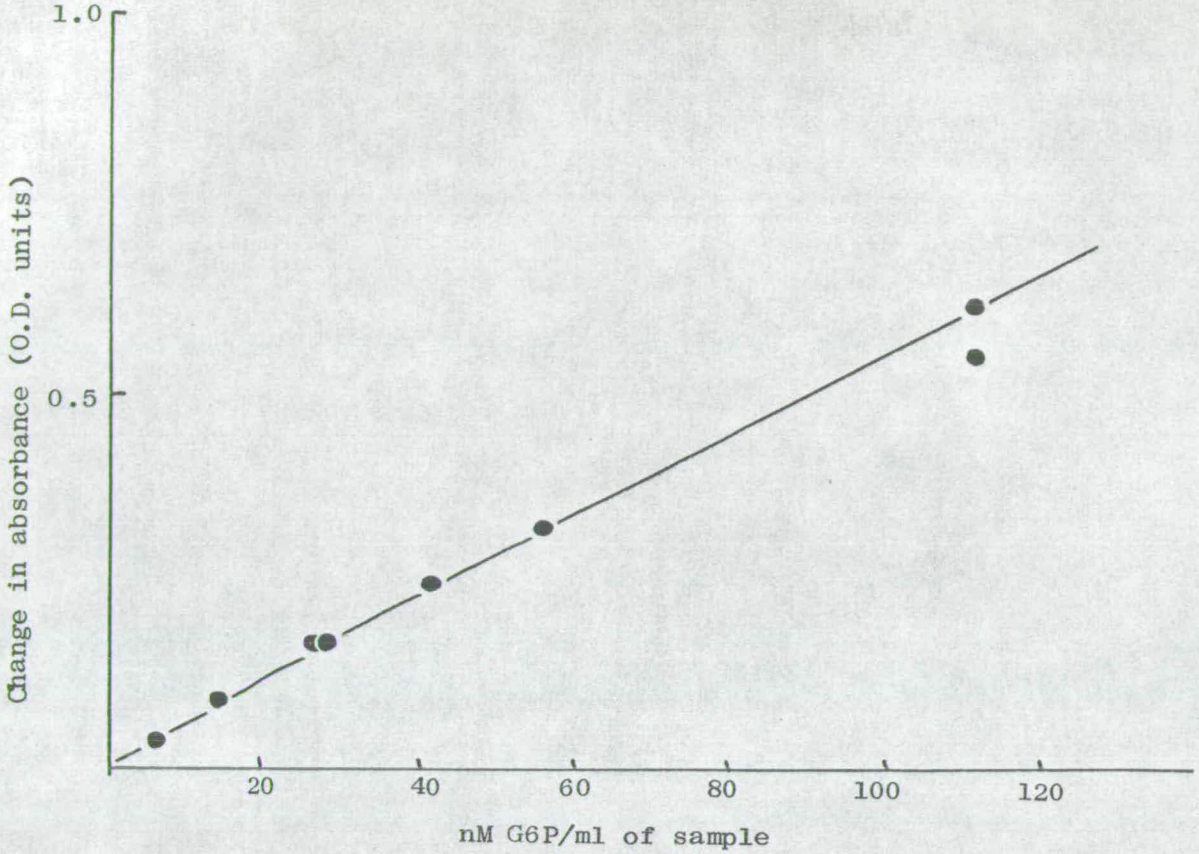


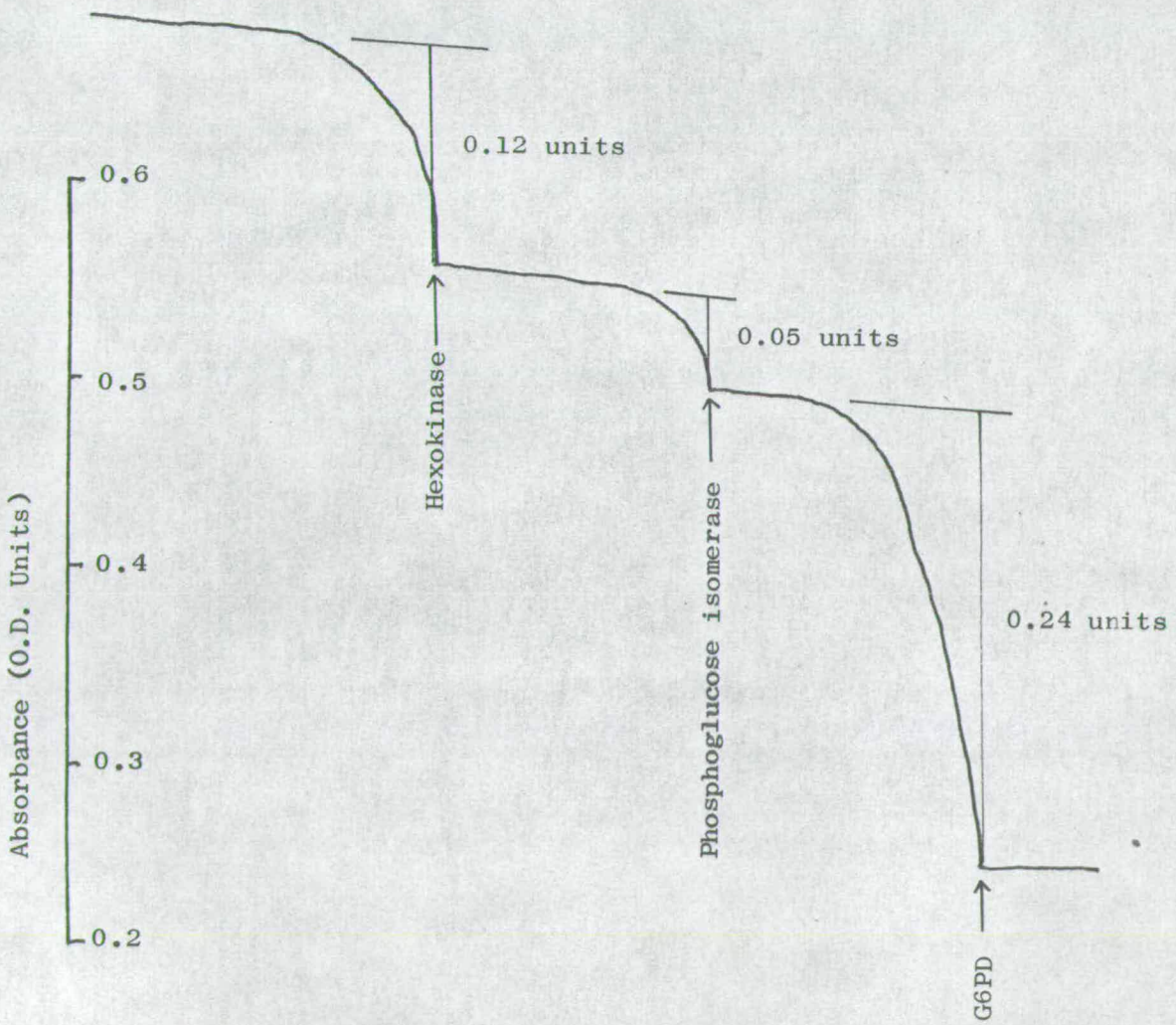
The production of NADPH was measured spectrophotometrically at 340mμ. The reagents were preincubated for 5 minutes at 25°C and enzyme 1 (G6PD) added. On completion of the reaction the change in O.D. was a function of the amount of G6P in the sample. Enzyme 2 was then added to assay the F6P concentration. When the second step of the reaction was completed enzyme 3 was added to assay ATP. The change in O.D. for known concentrations of G6P was measured to obtain a standard curve against which the unknown concentrations were calibrated. The standard curve and the trace obtained from a typical unknown are shown in Figure 21. As only very small volumes of enzymes were added (0.005ml) it was not deemed necessary to correct the results of F6P and ATP assay steps to compensate for dilution of the sample.

Assays (2) and (3) involved the measurement of NAD^+ produced from NADH at 340mμ. The basic technique was identical to that employed in assay (1).

Figure 21 a) Standard calibration curve for G6P assays. The change in absorbance of known solutions of G6P was measured on the addition of G6PD to the cuvette. The known concentrations of G6P were dissolved in 5% TCA and extracted with ether to compensate for any loss of G6P which might occur during the preparation of the unknown samples.

b) Typical recorder trace obtained from wild type mycelium assayed for G6P, F6P and ATP. The arrows indicate the time of addition of enzymes. Concentrations of the three phosphates were calculated by comparing the change in absorbance on the addition of enzyme with the standard curve above. The recorder was run at 0.2 inches/minute.


1.0
0.5
Change in absorbance (O.D. units)
20
40
60
80
100
120
nM G6P/ml of sample


0.6
0.5
0.4
0.3
0.2
Absorbance (O.D. Units)
0.12 units
Hexokinase
0.05 units
Phosphoglucose isomerase
0.24 units
G6PD

2) Assay for FDP and Triose phosphates.

Reagents:

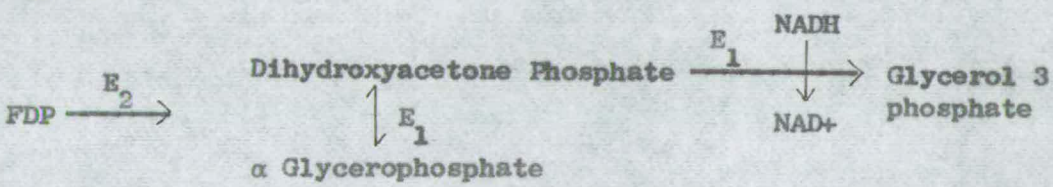
0.8mls buffer -	Glycyl glycine	0.25M	pH 7.4
	EDTA	10mM	
	Ammonium acetate	0.8M	

0.05mls NADH 7mM

2.15mls sample

Enzyme 1	α Glycerophosphate dehydrogenase	2 units
	Triosephosphate isomerase	20 units
Enzyme 2	Aldolase	5 units

Reaction:



3) Assay for Pyruvate, 2-Phosphoglycerate (2PGA), and Phospho (Enol) Pyruvate (PEP)

Reagents:

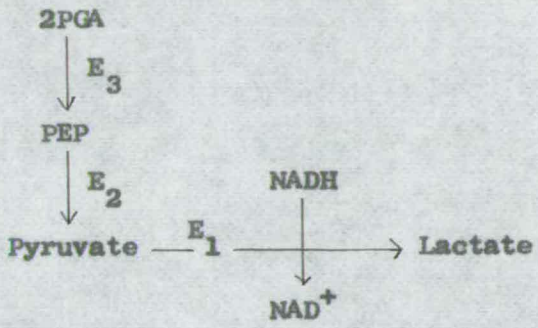
0.8mls buffer	Glycyl glycine	0.5M	pH 7.4
	EDTA	0.01M	
	KCl	0.6M	
	$MgCl_2$	0.01M	
	ADP	5mM	

0.05mls NADH 7mM

2.15mls sample

Enzyme 1	Lactic dehydrogenase	2 units
Enzyme 2	Pyruvate kinase	5 units
Enzyme 3	Enolase	1 units

Reaction:



All enzymes were obtained from Sigma.

When assays were performed on unknown samples it was found that the highest concentrations of the substrates measured in assays (2) and (3) were not very much above the lowest levels detectable. These assays are therefore mentioned only briefly in the results and were not performed on most samples.

Results

The addition of G6P, F6P, FDP, ADP or ATP to the medium, either in known concentration or as crystals placed on the mycelium had no effect on either the production or distribution of protoperithecia. This may have been the result of failure of the compounds to be taken up by the mycelium, or it may be that variations in their concentrations within the mycelium do not exercise any influence on the processes controlling differentiation of protoperithecia.

Figure 22 shows the concentration of G6P, F6P and ATP in the mycelium of a wild type culture during vegetative growth and fruiting. The margin of the colony was assayed separately and compared with the remainder.

During vegetative growth the margin of the colony has a slightly higher concentration of the organic phosphates measured. This was

Figure 22 a) Concentration of three organic phosphates in the mycelium of a wild type culture during growth and fruiting on CMA in a 9cm petri dish. The arrows represent the time at which the culture reached the dish wall (1) and the time of first appearance of protoperithecia (2)

● —— ● G6P

o —— o F6P

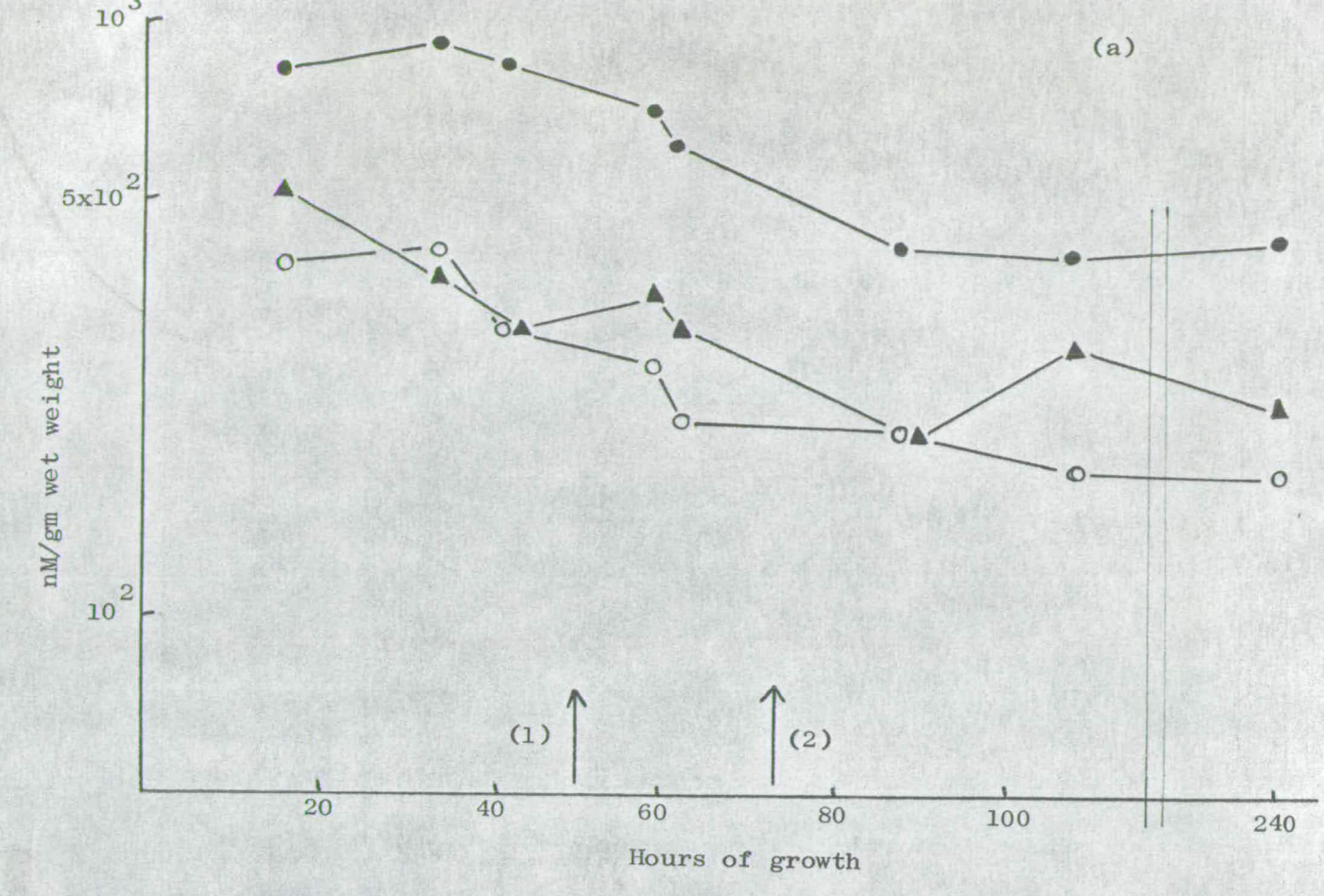
▲ —— ▲ ATP

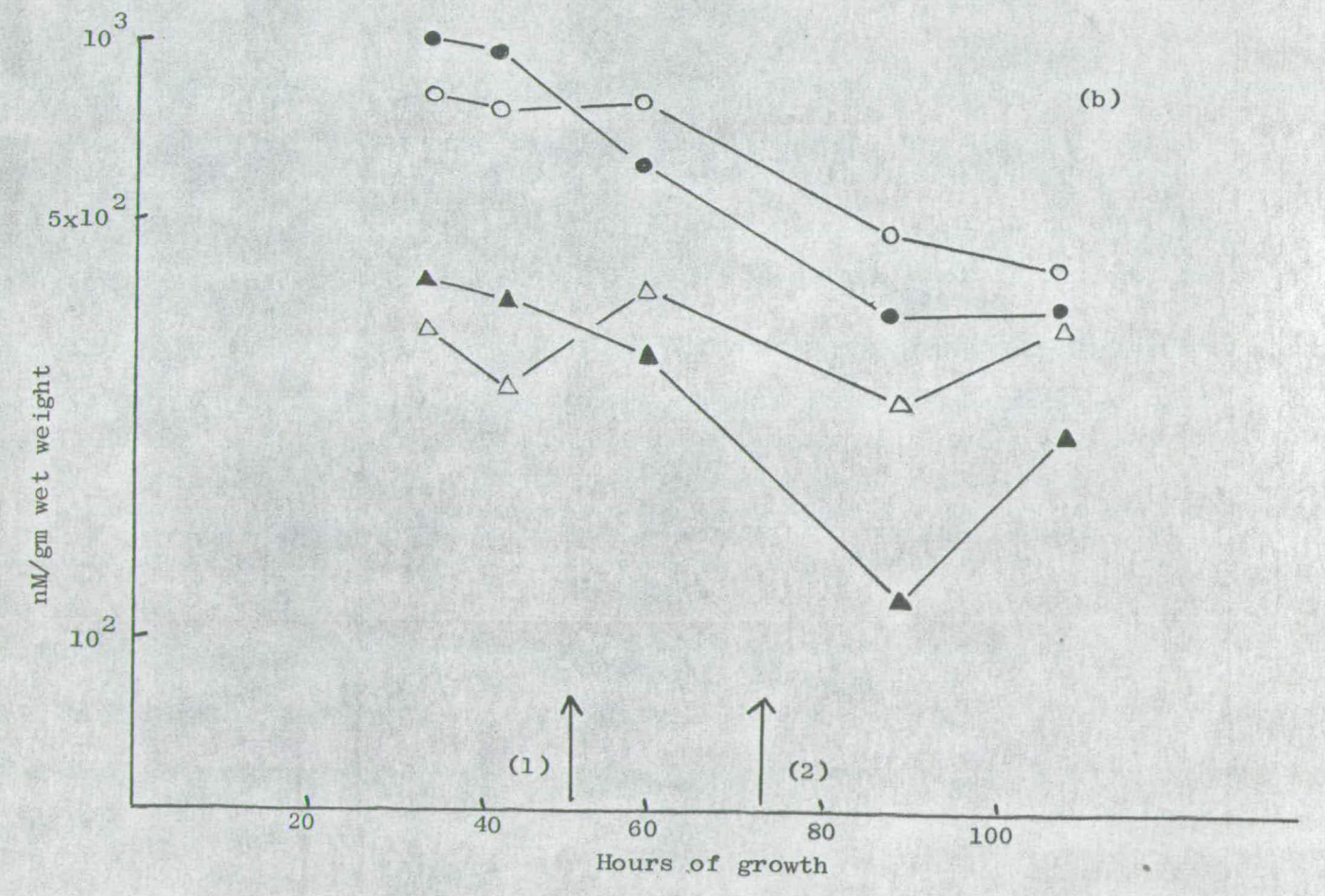
b) Concentration of G6P and ATP in the centre and margin of the same culture as above. The margin of the colony was the outer 2cm, the centre being the remainder

G6P { ● —— ● colony margin
o —— o colony centre

ATP { ▲ —— ▲ colony margin
△ —— △ colony centre

Concentrations in nM/gm wet weight of mycelium


(a)
10^3
$5x10^2$
10^2
nM/gm wet weight
(1)
(2)
20
40
60
80
100
240
Hours of growth


(b)
10^3
$5x10^2$
10^2
nM/gm wet weight
(1)
(2)
20
40
60
80
100
Hours of growth

probably the result of a higher rate of metabolism at the growing hyphal tips. There was a drop in the concentration of the margin when the edge of the dish was reached though the difference between margin and centre was never very large. The concentration per gm wet weight of the culture declines by about 50% within 40 hours of the edge being reached. There was no sudden change in the concentration of any of the substances assayed corresponding with the onset of protoperithecium production.

Organic phosphate content of mutant strains

The G6P, F6P and ATP content of female sterile ($per1^-$ $per2^-$) and $per1^-$ $per2^+$ cultures was determined by the methods previously described. It was found that the G6P content of a female sterile mycelium dropped from a level similar to that of wild type cultures to a barely detectable level within 24 hours of the plate edge being reached. This effect is graphed in figure 23. The concentrations of the substances measured in assays (2) and (3) (See Materials and Methods) dropped from barely detectable to undetectable levels at the same time as the G6P content of the mycelium dropped. This was not caused by exhaustion of the medium as cultures grown on 10, 20 and 30 mls of medium all showed a similar drop at the same time without any difference in the wet weight of mycelium produced (figure 24). The decline in G6P content bore the same relationship to the time at which the mycelium reached the dish wall on both 9cm and 14cm plates (figure 25).

There is an obvious similarity between the biochemical response to the dish wall in female sterile cultures and the morphogenetic response in fertile cultures. In both cases an interaction between

Figure 23 Concentration of G6P (● —— ●) and ATP (▲—— ▲) in female sterile (per 1^{-} per 2^{-}) mycelium on CMA in a 9cm petri dish. The arrow indicates the time at which the culture reached the dish wall.

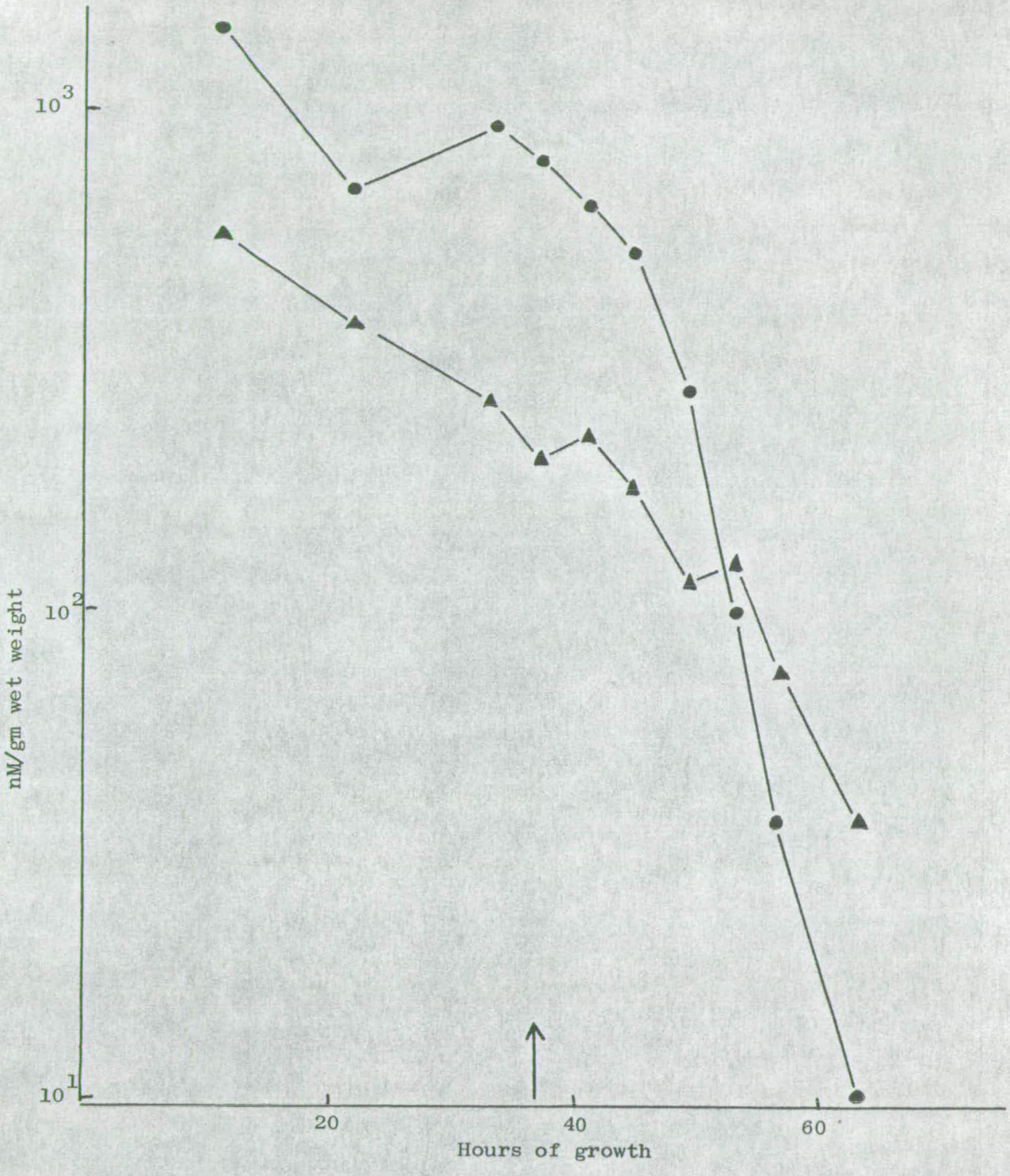
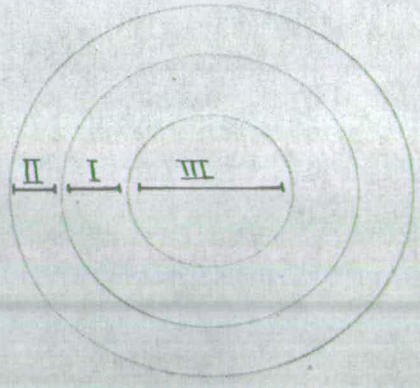

10^3
10^2
10^1
nM/gm wet weight
20
40
60
Hours of growth

Figure 24 a) Decline in G6P content of a female sterile isolate grown on 30mls (o —— o), 20mls (● —— ●) and 10mls (▲——▲) of CMA on 9cm petri dishes.

The mean weight per culture of the mycelium on the three different media was,

30ml dish ——— 0.74gms
20ml " ——— 0.80gms
10ml " ——— 0.65gms

b) Decline in G6P content of a female sterile isolate on CMA in a 9cm petri dish. The colony was divided into three regions, as illustrated below.



G6P in each region was assayed separately

● —— ● region I
o —— o " II
▲ —— ▲ " III

The arrows indicate the time at which the culture reached the dish wall.

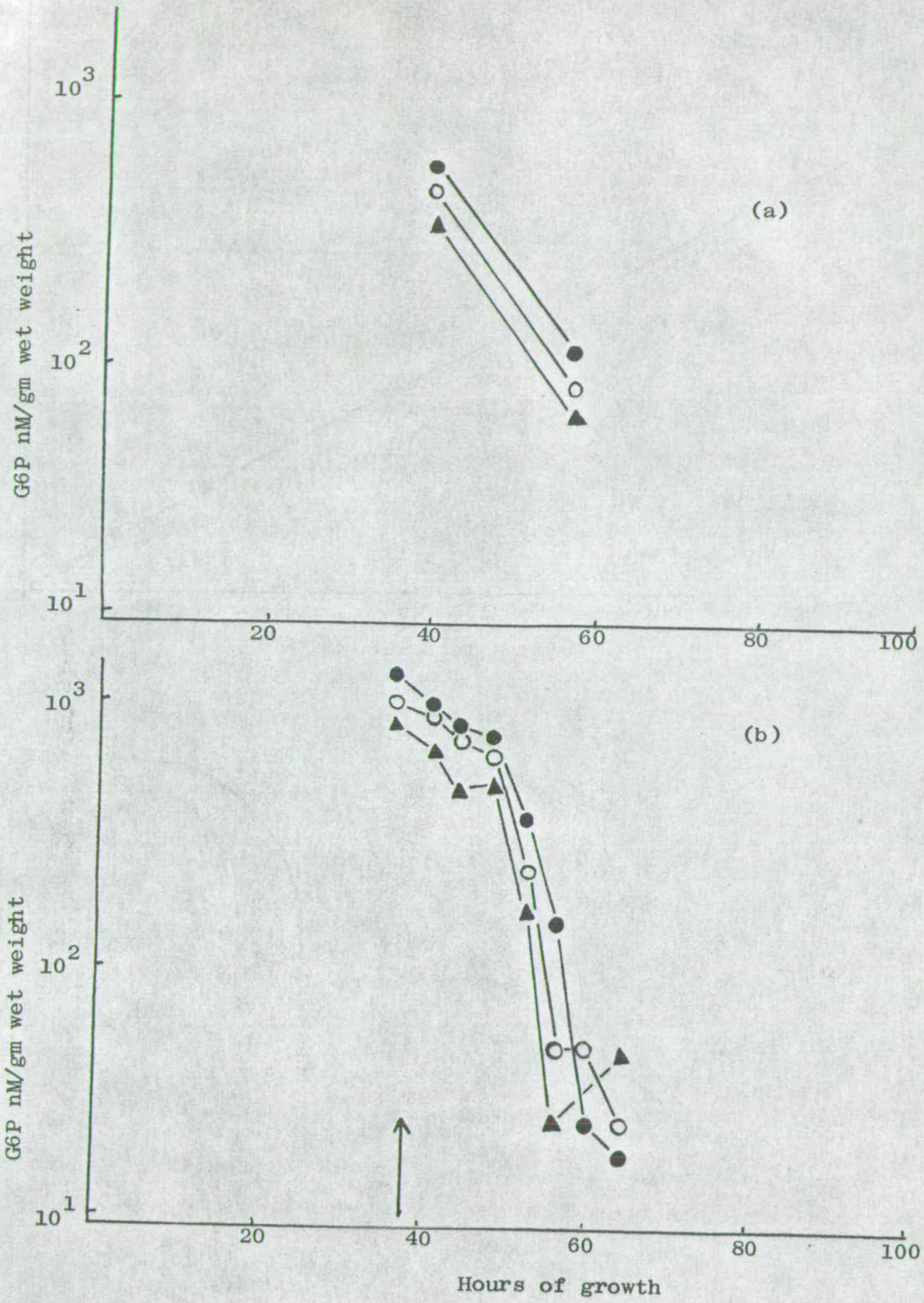
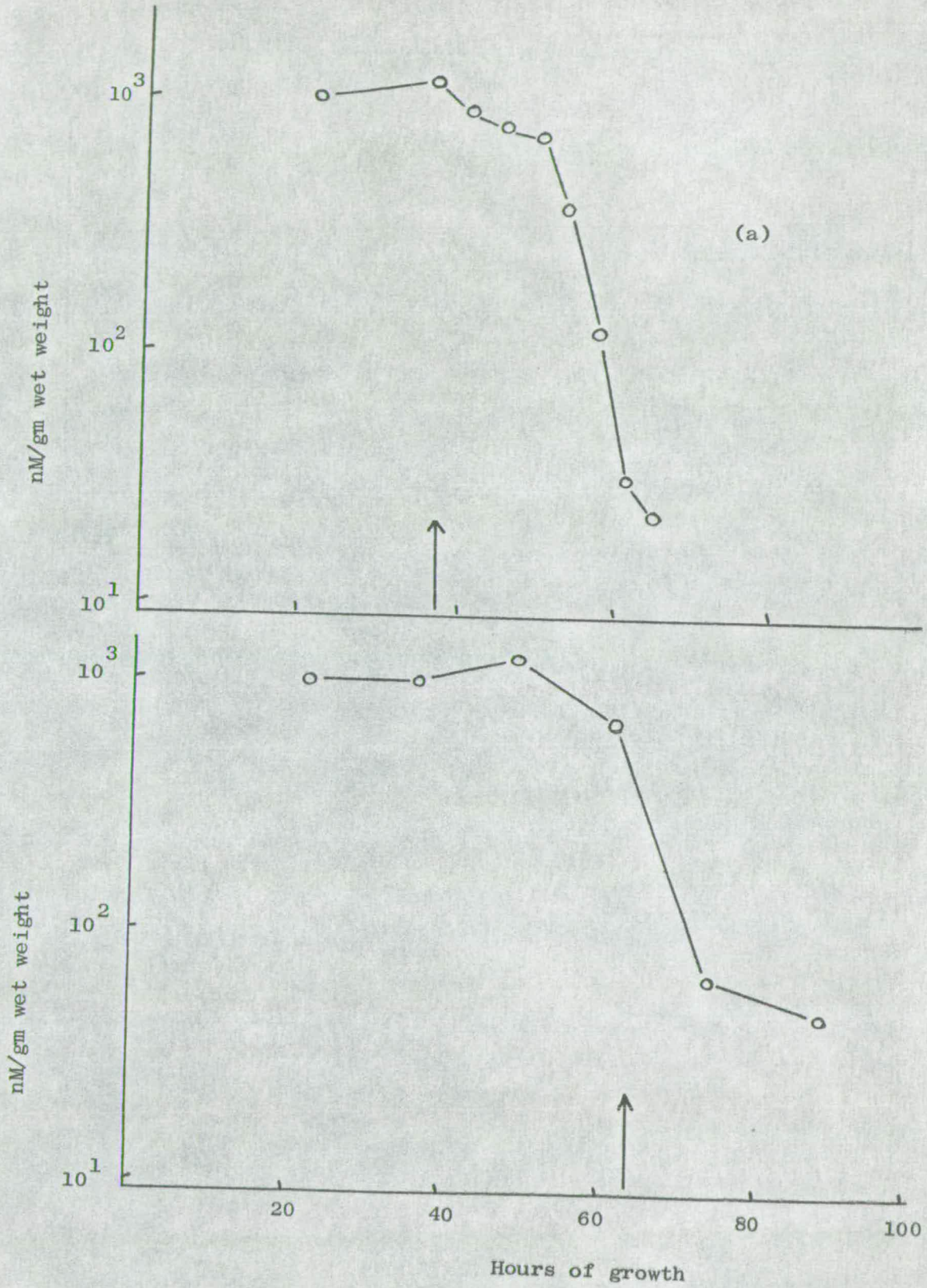

(a)
(b)
G6P nM/gm wet weight
10^3
10^2
10^1
20
40
60
80
100
Hours of growth

Figure 25 G6P content of a female sterile isolate grown on CMA in a 9cm petri dish (a) and a 14cm petri dish (b). The arrows indicate the time at which the culture reached the dish wall.


(a)
nM/gm wet weight
10^3
10^2
10^1
nM/gm wet weight
10^3
10^2
10^1
20
40
60
80
100
Hours of growth

the mycelium and the wall stimulates a change in the whole colony. It is curious that the biochemical response to the wall should ~~be manifested~~ occur in the culture which shows no morphogenetic response, i.e. which does not produce protoperithecia. Vice versa, protoperithecial production in fertile cultures is not correlated with changes in the sugar phosphate levels.

Another similarity between the two phenomena is their simultaneous effect on the whole colony. The decline in G6P content of the mycelium was measured in three regions by cutting a culture into concentric rings. The result in figure 24 shows that there was no tendency for the G6P level in any part of the colony to drop earlier than that in any other part.

Organic phosphate levels were assayed in $per1^{-}$ $per2^{+}$ cultures. The levels in a typical isolate are shown in figure 26. After the colony reached the plate edge a drop in the G6P content of the colony margin was found. The remainder of the colony did not show a similar effect. Although the decline observed in the margin was smaller than that in female sterile cultures there did appear to be a positive correlation between the decline and the failure of cultures, or parts of cultures, to produce protoperithecia.

Discussion

The relationship between the edge effects in fertile and sterile cultures is open to debate. The female sterile phenotype may have been a consequence of an aberrant response to the stimulus produced by the mycelium/wall interaction. Alternatively the stimulus itself may have been altered in sterile cultures.

Figure 26 Concentration of G6P (a) and ATP (b) in a per 1^- per 2^+ isolate grown on CMA in a 9cm petri dish.

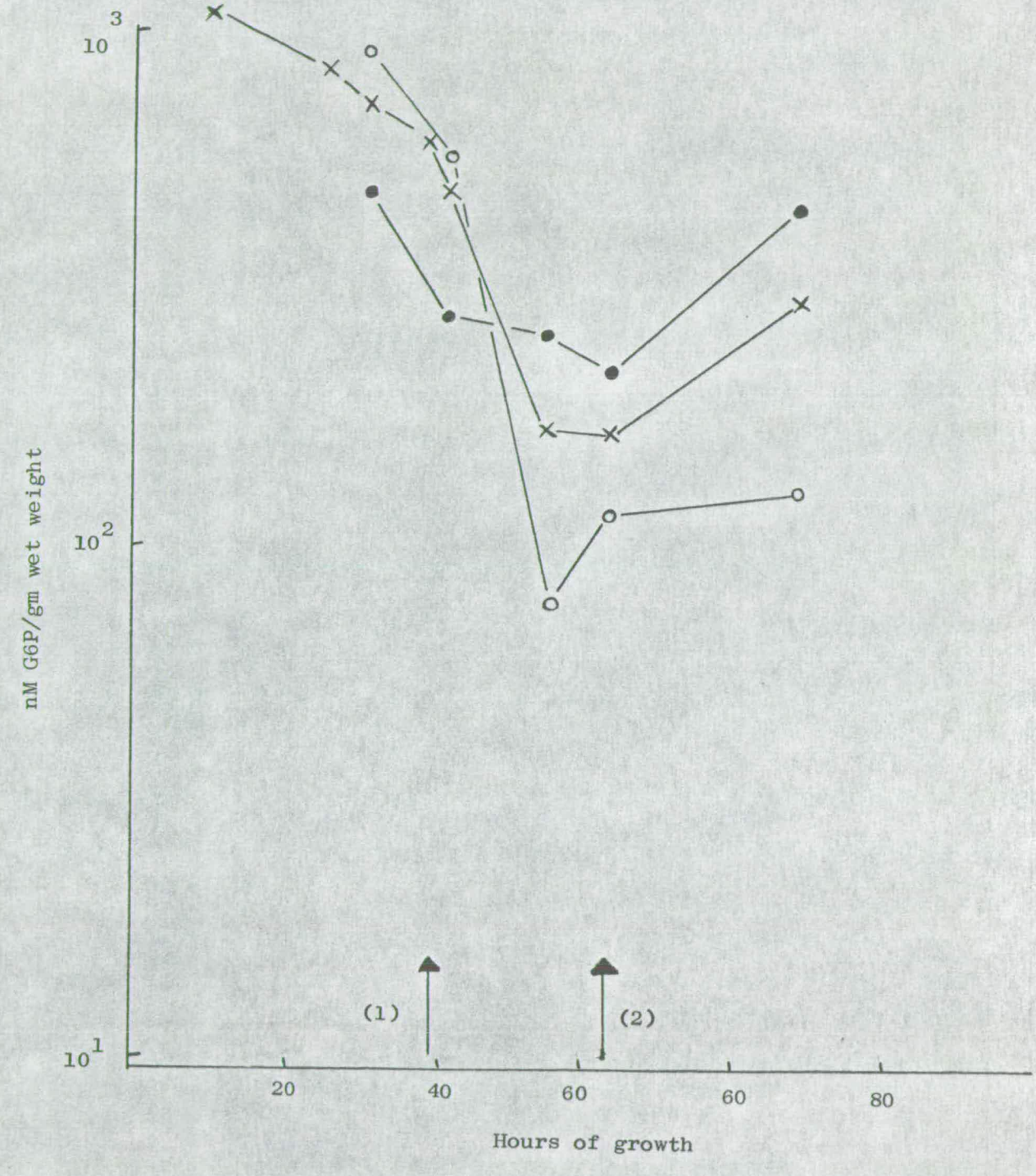
The outer 1.5cm of the culture was assayed separately from the remainder

X —— X —— whole colony

o —— o —— colony margin

● —— ● —— colony centre

The arrows indicate the time at which the culture reached the dish wall (1) and the time of first appearance of proto-perithecia (2).


10^3
10^2
10^1
nM G6P/gm wet weight
(1)
(2)
20
40
60
60
80
Hours of growth

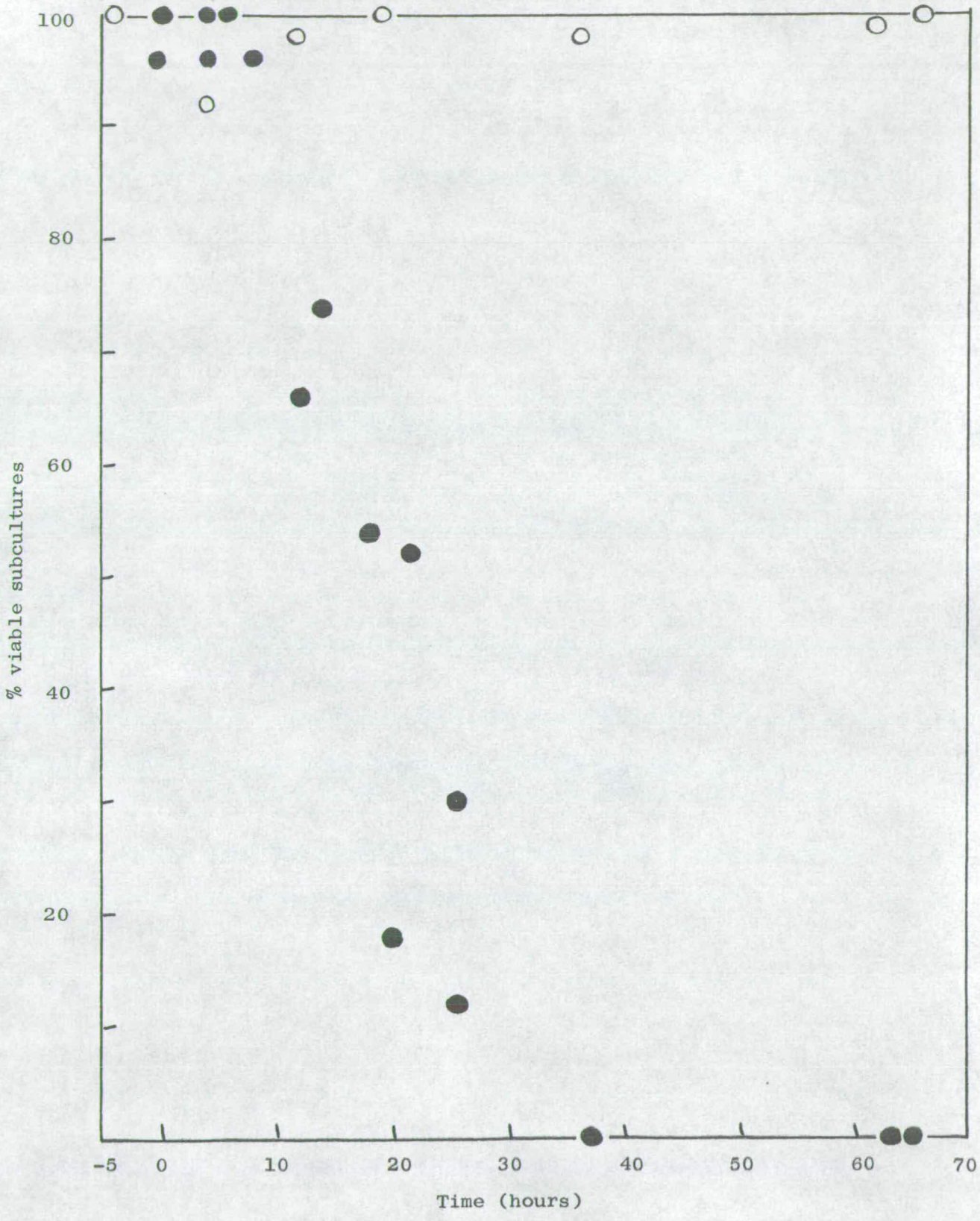
It has been found (MacDonald & Bond, manuscript submitted for publication) that subcultures taken from a per 1^- per 2^- culture often fail to grow. Their viability decreases rapidly over a 24 hour period immediately after the parent culture reaches the petri dish wall. Figure 27 shows this decline in ~~subculturability~~ viability with time. Wild type and per 1^- per 2^+ cultures do not show any corresponding decline. Apparently a sterile culture dies after it has covered the available medium surface and reached the physical limits of its environment. The drop in organic phosphate content of the mycelium may be symptomatic of a general decline in metabolic processes. If this is ~~indeed the case~~ so, then the sterility of these cultures is understandable, protoperithecia are not produced because the culture is not metabolically active at the time when protoperithecial production would otherwise be induced.

The results do not suggest that the levels in the mycelium of organic phosphates tested were correlated with the onset of protoperithecial production.

Figure 27 Percent of viable subcultures taken from a wild type (o) and a female sterile (•) isolate at various times before and after reaching the edge of a 9cm petri dish (t = 0), on CMA.

The standard subculture was a plug of agar and mycelium punched out with a Pasteur pipette tip. The position of subcultures was chosen at random. A subculture which grew in a tube of Vogels medium was defined as viable.

This data was obtained by D.J. Bond.


100
80
60
40
20
% viable subcultures
-5
0
10
20
30
40
50
60
70
Time (hours)

Cyclic AMP and Protoperithecial production

Introduction

Cyclic adenosine 3', 5'- monophosphate (cAMP) has in recent years been shown to have regulatory function in a wide variety of organisms. For example it has been shown to mediate the intracellular actions of hormones that interact with the cell membrane (Robison, Butcher & Sutherland, 1971), to control catabolite repression in bacteria (Pastan & Perlman, 1970) and to play an important role in the migration and aggregation of cellular slime moulds (Bonner, 1969). Together with the related nucleotide cyclic guanosine monophosphate (cGMP) it has been implicated in the modulation of growth and development of a large number of prokaryotic and eukaryotic cell types.

In fungi it has been shown that cAMP acts as a fruit inducing substance in Coprinus macrorhizus (Uno and Ishikawa, 1971). Leonard and Dick (1968) extracted a diffusable substance from a number of fungal species and showed that it was capable of inducing fruiting in Schizophyllum commune. Uno and Ishikawa found the active ingredient to be a heat stable substance with a molecular weight similar to cAMP. The addition of cAMP to the growth medium induced fruiting in C. macrorhizus.

In view of these observations and the ubiquity of cAMP in regulatory processes, its role in fruiting in Sordaria brevicollis was investigated. This was done by adding cAMP to the medium to see if it acted as a fruit inducing substance and by investigating the intracellular levels of cAMP at different stages of the life cycle.

Materials and Methods

cAMP (Sigma) was incorporated in corn meal agar (CMA) at five concentrations from 5×10^{-1} to 5×10^{-5}M. Wild type, sterile and $per1^{-}$ $per2^{+}$ cultures were grown on each concentration. cAMP was also added as crystals placed on the mycelium.

The levels of cAMP in mycelium were determined in cultures grown on the normal CMA medium. Assays were performed using a cAMP assay kit obtained from the Radiochemical Centre, Amersham (Tovey et al., 1974). The assay was based on the competition between unlabelled cAMP and a fixed quantity of tritium labelled cAMP for binding to a protein with a high specificity and affinity for cAMP. Measurement of the protein bound radioactivity enabled the amount of unlabelled cAMP in the sample to be calculated.

Mycelial extracts were obtained by homogenizing cultures in ice-cold TCA (5% w/v solution, 10 mls per gram wet weight of mycelium), followed by centrifugation for 10 minutes at 10,000g. The TCA was removed from the supernatant by extracting five times with two volumes of ether.

The reagents used were as follows:

1) Tris/EDTA buffer:- 0.05M solution at pH 7.5 containing 4mM EDTA.

2) Binding protein, purified from bovine muscle and containing Tris/EDTA buffer, pH 7.5.

3) [8-^{3}H] Adenosine 3',5'-cyclic monophosphate - 18μM solution in Tris/EDTA buffer, pH 7.5.

4) Unlabelled cAMP in Tris/EDTA buffer pH 7.5. This was used to obtain a standard curve of concentration of unlabelled cAMP against scintillation counts per minute. Five concentrations of unlabelled cAMP were used in the standard curve, 1, 2, 4, 8 and 16 p mol per assay tube (0.2 mls).

5) Charcoal absorbsorbent.

The assay protocol is given in table 16.

	Blank		Zero		Standards									Unknowns		
Tube No.	1	2	3	4	5	6	7	8	9	10	11	12	13 14	15	16	17 etc
Buffer sln	150	150	50	50												
1 pmol cAMP					50	50										
2 pmol cAMP							50	50								
4 pmol cAMP									50	50						
8 pmol cAMP											50	50				
16 pmol cAMP													50 50			
Unknown 1														50	50	
Unknown 2																50 50
c[^{3}H] AMP	←								50							→
Binding protein			←						100							→

Table 16

cAMP assay protocol. All volume in microlitres

The reagents were added to plastic haematocrit tubes (capacity 0.5mls) kept at 0^{o}C in an ice/water bath. The buffer was added first, followed by the unlabelled cAMP or unknown, labelled cAMP and binding protein in that order. The tubes were vortex mixed for approximately 5 seconds then incubated in the ice bath for 2 hours. Twenty minutes before the end of the incubation time distilled water was added to the charcoal reagent which was stirred continuously at

0°C with a magnetic stirrer. 100µl of charcoal suspension was added to as many tubes as could be centrifuged in one batch (8) and vortex mixed for approximately 5 seconds. Immediately after this step the tubes were centrifuged for 5 minutes at 10,000g and 200µl samples removed to scintillation vials for counting. Counts were carried out in a water-compatible scintillant mixture, (Packard Insta-Gel).

Calculation of results: The blank counts per minute (cpm) for the assay were subtracted from the zero cpm to obtain the protein bound cpm in the absence of unlabelled cAMP (C_o). The cpm bound in the presence of standard or unknown unlabelled cAMP (Cx) were obtained by subtracting the blank cpm from that of each sample tube. A plot of Co/Cx against concentration of cAMP should give a stright line with an intercept of 1.0 on the ordinate (see figure 28). This graph, obtained from the five standard concentrations, can be used to determine the concentration (pmol per assay tube) of cAMP in the unknown samples by using the formula

$$x = b\ (Co/Cx - a)$$

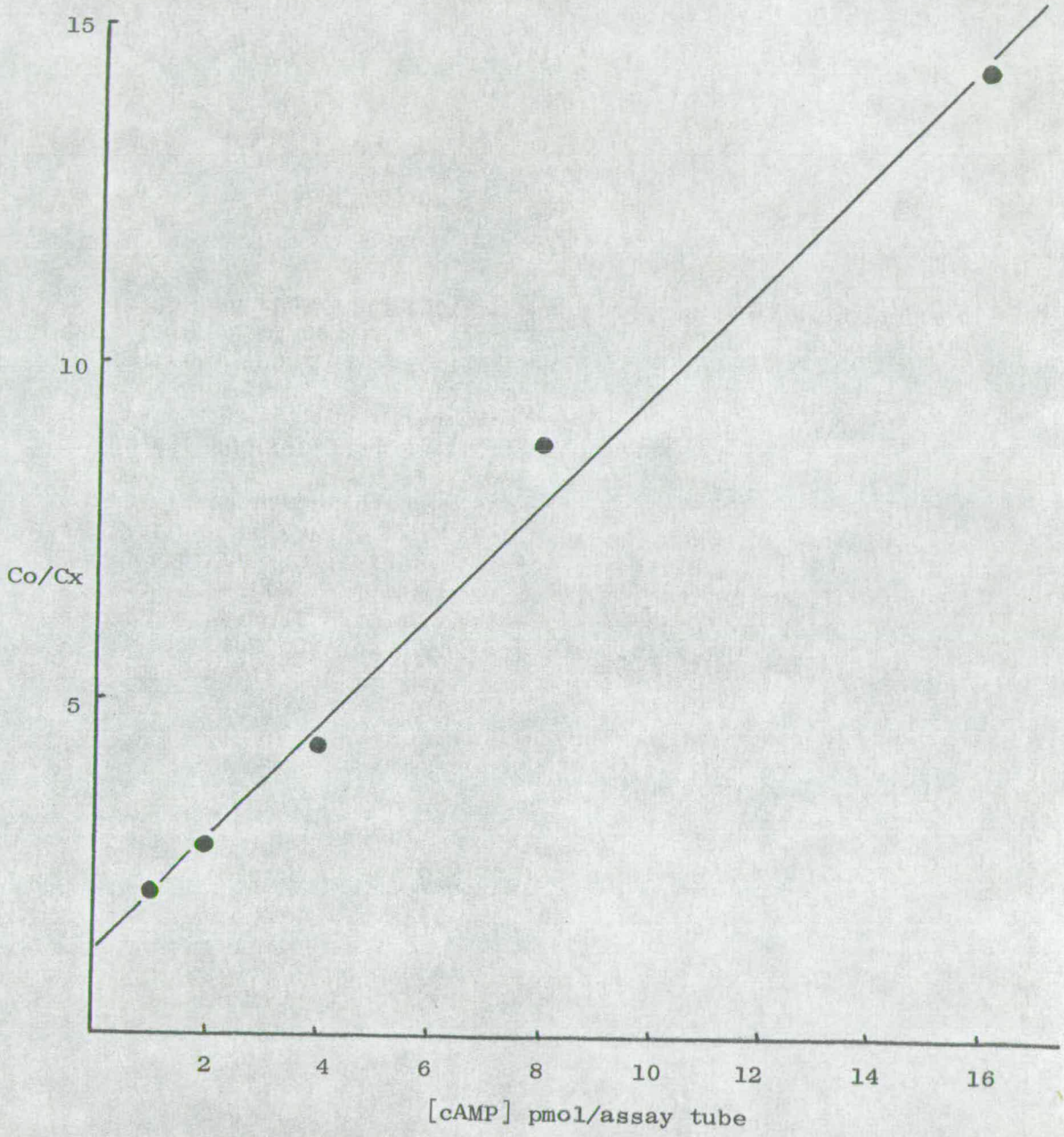
where b is the regression coefficient of y on x and a is the intercept of the regression line on the y axis.

Assays were performed on wild type and sterile cultures at a variety of times before and after protoperithecia were visible.

Results

The addition of cAMP to CMA either in known concentrations or as crystals placed on the mycelium had no visible effect on the numbers of protoperithecia or their time of production. The only effect of

Figure 28 Standard curve used to estimate the concentration of cAMP in unknown samples. The data from which this curve was calculated appears in Table 17.


15
10
5
Co/Cx
2
4
6
8
10
12
14
16
[cAMP] pmol/assay tube

cAMP observed was on hyphal morphology when crystals were placed on the surface of the culture. Within about 2mm of the crystals there were a large number of aerial hyphae which looked as if they bore microconidia. A possible explanation for the failure to find any action of cAMP on fruiting was that the compound was not taken up by the organism. It may be that the extracellular cAMP supplied was rapidly inactivated enzymatically. An important property of cAMP is metabolic lability which allows rapid fluctuations in concentration as a response to environmental changes. The observation that exogenous cAMP did not affect fruiting was not therefore, taken to exclude the possibility that intracellular fluctuations in cAMP were important to protoperithecial production.

Intracellular cAMP: The known concentrations of cAMP gave a reasonably good calibration curve (Figure 28). Table 17 shows the data from one experiment in which cultures of wild type and female sterile ($per1^-$ $per2^-$) cultures were harvested before and after reaching the petri dish edge. A series of dilutions of each sample was made as the assay was only accurate over the range 1-16 pmol cAMP per tube. Maximum accuracy is in the range 2-8 pmol. If the results of the 1:25 and 1:125 dilutions of sample 9 are ignored, the range of concentrations of cAMP found varied from 8.1 to 31.0 pmol per assay tube. Agreement between dilutions of the same sample was poor in some cases, e.g. samples 5 and 9.

There did not appear to be any large fluctuations in the intracellular levels of cAMP between any of the samples. The only aspect of the results which was thought worthy of further investigation was the slightly higher cAMP levels in samples 2 and 3. These were wild

Table 17 Concentration of cAMP in wild type and female sterile cultures. t refers to the time in hours relative to reaching the dish wall, at which cultures were harvested.

Three dilutions of each sample were assayed and the figures in the final column are corrected for these dilutions.

Co = Zero - Blank = 6152-314

= 5838

		cpm	Cx	Co/Cx	x	cAMP in undiluted sample pmol/tube
Blank		314				
Zero		6152				
Standards	(16pmol	722	408	14.3	15.7	
	(8 "	1001	687	8.5	8.7	
	(4 "	1659	1349	4.3	3.7	
	(2 "	2402	2088	2.8	1.4	
	(1 "	3146	2832	2.1	1.0	
S1		858	544	10.7	11.4	11.4
wild type	1:5	2034	1720	3.4	2.6	13.0
t = -12	1:25	3323	3009	1.9	0.8	-
	1:125	4616	4302	1.4	0.2	-
S2		723	409	14.3	15.7	15.7
wild type	1:5	1523	1209	4.8	4.2	21.0
t = -4	1:25	4015	3701	1.6	0.4	-
	1:125	3791	3477	1.7	0.6	-
S3		709	395	14.8	16.3	-
wild type	1:5	1341	1027	5.7	5.4	27.0
t = +8	1:25	3474	3160	1.8	0.7	-
	1:125	4845	4531	1.3	0.1	-
S4		830	516	11.3	12.1	12.1
wild type	1:5	2156	1842	3.2	2.4	12.0
t = +12	1:25	3594	3280	1.8	0.7	-
	1:125	4396	4082	1.4	0.2	-
S5		805	491	11.9	12.8	12.8
wild type	1:5	1227	913	6.4	6.2	31.0
t = +36	1:25	3038	2724	2.1	1.0	25.0
	1:125	3537	3223	1.8	0.7	-
S6		1045	731	8.0	8.1	8.1
female sterile	1:5	4219	3905	1.5	0.3	-
t = -12	1:25	4138	3824	1.5	0.3	-
	1:125	4655	4341	1.3	0.1	-
S7		662	348	16.8	18.7	-
female sterile	1:5	1364	1050	5.6	5.2	26.0
t = 0	1:25	3067	2753	2.1	1.0	25.0
	1:125	3879	3565	1.6	0.4	-
S8		767	453	12.9	14.0	14.0
female sterile	1:5	2128	1814	3.2	2.4	12.0
t = +12	1:25	3505	3191	1.8	0.7	-
	1:125	3873	3559	1.6	0.4	-
S9		683	369	15.8	17.5	-
female sterile	1:5	1296	982	5.9	5.6	28.0
t = +24	1:25	2345	2031	2.9	2.0	50.0
	1:125	2777	2463	2.4	1.4	175.0

type mycelium harvested just before (t = -4) and just after (t = +8) reaching the dish edge. Sample 3 exhibits about a two-fold higher level than samples 1 or 4. The possibility that these samples were indicative of a pulse of cAMP production concurrent with reaching the plate edge was investigated by assaying a series of samples at short time intervals immediately before and after the edge was reached. The results appear in Table 18. Again they showed poor agreement between dilutions of the same sample and also between replicate tubes. However there was no suggestion that cAMP levels varied significantly in response to meeting the dish wall.

Conclusions

The results of the assays to determine the intracellular levels of cAMP did not suggest that the fluctuations in the concentration of this substance were correlated with any morphogenetic changes. The poor quality of the results was probably the result of lack of expertise in performing the assay. As with most experimental systems, it would have been optimistic to expect perfect results the first few times the experiment was performed.

The results were not considered sufficiently encouraging to warrant continuation of the expensive assays, particularly in view of the fact that the addition of cAMP to the medium had no effects on protoperithecial production.

Table 18 Concentration of cAMP in wild type and female sterile cultures. t refers to the time in hours, relative to reaching the dish wall, at which cultures were harvested.

Two dilutions of each sample were assayed and the figures in the final column are corrected for these dilutions.

Co = Zero - Blank = 3353-176

= 3177

		cpm	Cx	Co/Cx	x	cAMP in undiluted sample pmol/tube
Blank		182 170				
Zero		3553 3153				
S1		1001	825	3.9	3.1	3.1
		617	441	7.2	7.2	7.2
t = -2	1:5	1379	1203	2.6	1.7	8.5
	1:5	1570	1394	2.3	1.3	6.5
	1:25	2972	2796	1.1	-	
	1:25	3110	2934	1.1	-	
S2		872	696	4.6	4.0	4.0
		814	638	5.0	4.5	4.5
t = 0	1:5	956	780	4.1	3.4	17.0
	1:5	993	817	3.9	3.2	16.0
	1:25	1658	1482	2.1	1.1	27.5
	1:25	2147	1971	1.6	0.4	
S3		560	384	8.3	8.4	8.4
		576	400	7.9	8.0	8.0
t = +2	1:5	1156	980	3.2	2.4	12.0
	1:5	1406	1230	2.6	1.6	8.0
	1:25	2335	2159	1.5	0.3	
	1:25	2416	2240	1.4	0.2	
S4		494	318	10.0	10.5	10.5
		453	277	11.5	12.3	12.3
t = +4	1:5	924	748	4.2	3.6	18.0
	1:5	962	786	4.0	3.4	17.0
	1:25	2009	1833	1.7	0.6	
	1:25	2008	1832	1.7	0.6	
S5		511	335	9.5	9.9	9.9
		684	508	6.3	6.0	6.0
t = +7	1:5	1512	1336	2.4	1.4	7.0
	1:5	1457	1281	2.5	1.5	7.5
	1:25	2617	2441	1.3	0.1	
	1:25	2831	2655	1.2	-	

General Conclusion

In this part of the thesis some environmental and genetic factors which affect the production of protoperithecia in Sordaria brevicollis have been described. While the composition of the medium on which a culture was grown was important in determining the numbers and distribution of protoperithecia evidence has been obtained which suggests that this was not the primary stimulus initiating protoperithecial production. This stimulus was an interaction between a growing culture and the edge of the dish in which it was grown. Fruiting was induced simultaneously on all parts of the culture, the first protoperithecia being visible about 24 hours after contact with the edge.

From these observations it seems that information about events occurring at the margin of a colony (i.e. reaching the dish wall) was transmitted to all parts of a culture where it interacted with the genetic component of the organism inducing differentiation of hyphae into fruiting structures. It is not clear how this information was transmitted. If a chemical 'message' was involved two mechanisms for its action spring to mind.

First there could be a specific inducing substance produced at the colony margin in response to the cessation of growth or some other aspect of the colony/edge interaction. This substance would have to spread rapidly throughout the entire culture, perhaps transported by cytoplasmic steaming. The observation that different parts of a culture respond to the fruiting stimulus to different extents suggests that the mycelium is not a "well stirred space", as it would presumably be if such rapid transport were possible.

A second form of chemical messenger could be a repressor of fruiting produced only by growing hyphal tips. The cessation of growth on reaching the plate edge could then result in derepression of the entire colony. Again this would require the transport of the repressor throughout the mycelium. In this case, however, the transport need not be so rapid in order to induce simultaneous protoperithecial production in all regions. This theory supposes that within less than 24 hours of the cessation of linear growth the level of repressor falls below a critical value.

A second 'edge effect' was found in mutants with altered protoperithecial production and distribution. This took the form of a sudden drop in the G6P content of the mycelium which occurred when female sterile cultures reached the plate edge. G6P fell to an undetectable level within 24 hours of this event. It was thought likely that the failure of such cultures to produce protoperithecia was a consequence of the general cessation of metabolic processes.

A mutant strain (per 1^- per 2^+) has been found which shows responses characteristic of both edge effects. The centre of the colony produces many protoperithecia within 24 hours of the margin reaching the plate edge whereas the colony margin is virtually sterile and shows a decline in G6P content.

It is not clear whether there are two different interactions with the plate wall which stimulate different morphogenetic events. Protoperithecial production and G6P decline may be the products of the same stimulus, the mutant strains having an aberrant response to the interaction with the wall.

Presumably the differentiation of protoperithecia is accompanied

by biochemical changes in the fruiting mycelium. No correlations with fruiting were found for any biochemical processes investigated.

Further investigations of the induction of protoperithecial production would be facilitated if mutants could be isolated which fruited before the plate wall was reached, i.e. which were constitutive for fruiting without requiring the stimulus of the interaction with the wall. Similarly, strains which show no edge effect and are consequently female sterile would be useful in such investigations. The mutants described in this work were discovered more or less accidentally in the laboratory stocks. In fact it was this discovery which was to a large extent responsible for stimulating this investigation of morphogenesis in S. brevicollis. To isolate morphogenetic mutants of the two types mentioned above is extremely difficult. It is hard to think of any satisfactory screening process which could be used to avoid a laborious and costly search through thousands of isolates in order to find the desired type of mutant.

Further experiments to investigate morphogenetic control could, in the absence of these mutants, involve looking at other biochemical pathways or other environmental factors. One approach would be to devise experiments which would distinguish between the induction and repression models of protoperithecial production.

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APPENDIX I

Composition of Media

1) Corn Meal Agar (CMA)

Corn Meal Agar (Difco)	17g
Sucrose	3g
Glucose	2g
Yeast Extract Powder (Oxoid)	1g
Distilled Water to 1000 mls	

2) Vogel's minimal medium

Bacto Agar (Difco)	15g
Glucose	20g
Vogel's (1956) salt solution (x50)	20 mls
Distilled water to 1000 mls	

3) Westergaard & Mitchell's minimal medium

Bacto Agar (Difco)	15g
Glucose	20g
W & M's (1947) salt solution (x10)	100 mls
Distilled water to 1000 mls	

Vogel's (1956) salt solution (50x working strength)

Sodium citrate	123g
KH_2PO_4	250g
NH_4NO_3	100g
$MgSO_4\ 7H_2O$	10g
$CaCl_2\ 2H_2O$	5g
Trace elements (see below)	5 mls
Biotin solution (25µgm/ml)	10 mls
Distilled water to 1000 mls	

APPENDIX II

Preferential Ascus Discharge During Cross Maturation in *Sordaria brevicollis*

by

D.J. MacDonald and D.J. Bond

Reprinted from Genetics 76: 245-253

February 1974

Vogel's trace elements solution

Citric acid	5g
$ZnSO_4\ 7H_2O$	5g
$Fe(NH_4)_2(SO_4)_2\ 6H_2O$	1g
$CuSO_4\ 5H_2O$	0.25g
$MnSO_4\ H_2O$	0.05g
H_3BO_3	0.05g
$Na_2MoO_4\ 2H_2O$	0.05g
Distilled water to 100 mls	

Westergaard & Mitchell's (1947) salt solution (x10 working strength)

[1] KNO_3	10g
KH_2PO_4	10g
[2] $MgSO_4\ 7H_2O$	5g
$CaCl_2\ 2H_2O$	1g
NaCl	1g
Trace elements solution (see below)	2.5 mls
Biotin solution (1μgm/ml)	16 mls
Distilled water to 1000 mls	

W & M's trace element solution

Na_3BO_3	0.01g
$CuSO_4\ 5H_2O$	0.1g
$FePO_4$	0.2g
$MnSO_4\ H_2O$	0.02g
$NaMoO_4\ 2H_2O$	0.02g
$ZnSO_4\ 7H_2O$	2.0g
Distilled water to 250 mls	

[1] KNO_3 was normally omitted and added separately when making up media

[2] For low sulphate (0.06mM) medium substitute 0.15g of $MgSo_4\ 7H_2O$

Media were sterilized by autoclaving for 20 minutes at 20lb/sq in

PREFERENTIAL ASCUS DISCHARGE DURING CROSS MATURATION IN *SORDARIA BREVICOLLIS*

D. J. MacDONALD AND D. J. BOND

Institute of Animal Genetics, King's Buildings, Edinburgh University, Edinburgh, EH9 3JN Scotland

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ABSTRACT

Crosses involving spore color mutants of *Sordaria brevicollis* all showed a decline in the frequency of second division asymmetric asci (2:2:2:2's) as the cross matured. This decline was due to the preferential maturation and/or discharge of these asci. The proportion of spindle overlap and recombinational asci within the group did not change as shown by ascus dissection. The preferential discharge was also found to occur in two-point crosses where the asci did not contain wild-type spores.

IN ascomycetes with a linear ascus it is possible to infer a gene to centromere distance from the sequence of spores within the ascus. First-division segregation for any locus results in a 4:4 distribution of spores, whilst a crossover between gene and its centromere results in second-division segregation and one of two spore sequences. Second-division asci are identifiable either by a 2:2:2:2 (asymmetric) or a 2:4:2 (symmetric) sequence. In the absence of complicating factors therefore the frequency of 4:4 asci will depend on the centromere distance and 2:2:2:2 and 2:4:2 asci are expected to be equal. Biased distribution (BERG 1966) is the term used to describe observed deviations from these expectations. Biases have been attributed to several factors.

SHAW (1962), CHEN and OLIVE (1965) and BERG (1966) analyzed crosses involving spore color mutants of *Sordaria brevicollis*. They found inequalities in the frequencies of the two asymmetric spore sequences (2+:2m:2+:2m and m:2+:2m:2+) compared to the two symmetric spore sequences (2+:4m:2+ and m:4+:2m). The asymmetric asci were in excess of the symmetric. This is formally accounted for by invoking the concept of spindle overlap or nuclear passing. The consequences of partial overlap of the second-division spindles are illustrated in Figure 1.

Factors other than spindle overlap have been shown to result in a biased distribution of ascospores. In *Neurospora crassa* spindle overlap is rare or absent but in this organism polarized segregation has been reported. The observational basis of this phenomenon is an inequality of complementary classes which are expected to be equal. For example, within first-division segregation asci an excess of asci with four wild-type spores at one end of the ascus has been reported (NAKAMURA 1961; LAMB 1966). Polarized segregation has also been reported in *Sordaria*

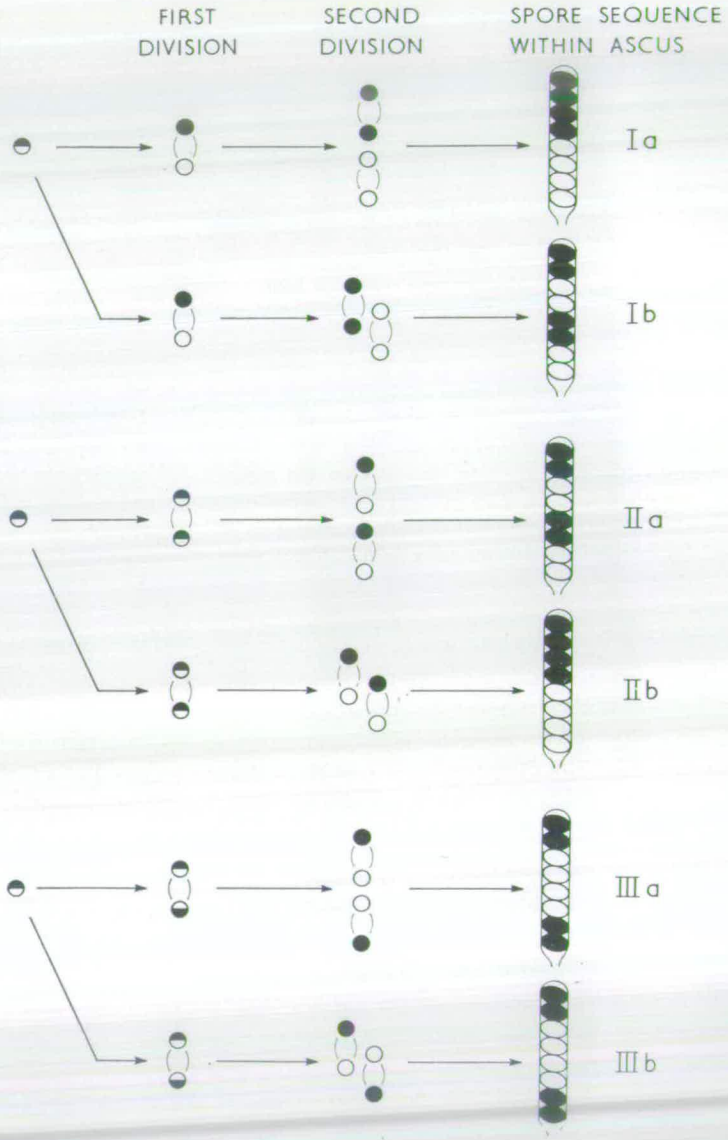

FIGURE 1.—The consequences of partial second-division overlap of spindles in first- and secon division segregation asci.

brevicollis (BERG 1966). This phenomenon has been extensively analyzed b LAMB (1966; 1967), who has shown that the bias is due to the preferential di charge of asci with wild-type spores at the base of the ascus. LAMB has suggeste that this is due to more efficient transportation of nutrients in asci of this so compared to asci with mutant spores at the base. There is consequently a diffe ential maturation of the two types.

In this paper we report large variations in the frequency of the main ascu

rpes during development of any one cross. The experiments arose from obser-ations on control crosses for another set of experiments. These showed large ifferences in the frequency of asymmetric second-division asci and here it is nown that these differences are generated by the preferential discharge of these sci. Consequently, estimates of the frequency of spindle overlap and of recombi-ation vary according to the time of sampling of the cross.

MATERIALS AND METHODS

Strains: Three spore-color mutants were used in this work. *S229* is a member of the y_4 locus CHEN 1965) and is inseparable from the centromere of linkage group V. *c79* is an allele of the locus and was kindly provided by PROFESSOR L. S. OLIVE whilst *S187* is a member of the y_9 ries. Both of these loci are located on linkage group II approximately 4 and 16 units from the ntromere. The morphological mutant (*mo-1*) was isolated and mapped by M. H. V. COOKAY. is located approximately halfway between the b_1 locus and the centromere.

Media: Crosses were carried out on cornmeal agar of the following composition: Cornmeal gar (Difco) 17 gm; glucose 2 gm; sucrose 3 gm; yeast extract 1 gm; distilled water up to 00 ml. Asci were dissected on 4% agar and the ascospores germinated in tubes of VOGEL'S 956) N medium to which 0.7% sodium acetate was added to stimulate germination.

Method of crossing: The crosses were set up using the method described by CHEN (1965). The ale and female parents were grown up separately for 4 days at 25°. Microconidia from the male rent were harvested in sterile water and the resulting suspension used to fertilize the female. he fertilized crosses were incubated at 25° until they were sufficiently mature for scoring.

Scoring techniques: Perithecia from the maturing cross were harvested daily and crushed en in a drop of 10% sucrose solution on a slide. The sugar solution prevents preferential burst-g of asci (LAMB 1966). Clumps of asci were transferred to a clean slide where they were teased art using tungsten needles. The asci were then flattened under a coverslip and scored using a agnification of ×400. Harvesting of perithecia was continued until ascus breakdown and dis-arge made it impossible to continue.

RESULTS

The results of single point crosses involving three spore color loci are presented Table 1. The frequencies of the three main ascus classes were scored on ccessive days. There was a marked decline in the frequency of asymmetric cond division asci as the cross matured. This trend is graphed in Figure 2. The crease in the frequency of 2:2:2:2 asci was smallest, although still highly gnificant, for the mutant *S187* which was furthest from the centromere. This ould be expected if there was a preferential discharge of spindle overlap asci, nce the fraction of overlap asci which are found in 4:4 asci increases with the ntromere distance.

Table 2 contains the results of an experiment which tested in a rather direct ay the idea that overlap asci were being preferentially discharged. The asym-etric asci are comprised of two sorts—those of overlap origin and those of combinational origin. Preferential discharge of the former will result in an crease in the recombinant fraction. Recombinational asci can be distinguished a marker proximal to the spore color locus is included in the cross.

The cross of the *S187* mutant contained a morphological mutant (*mo-1*) close the centromere of the same linkage group. From dissection of asci with a spore

TABLE 1

Number of asci in the three main ascus classes harvested at various times after fertilization in three single point crosses

Cross	Days after fertilization	Ascus classes 4:4	2:2:2:2	2:4:2	Percent 2:2:2:2s	Scorable* asci (%)
S229 x +	5	248	298	6	54.0	73.5
	6	322	230	0	41.7	80.4
	7	372	193	2	34.0	83.8
	8	-	-	-	-	-
	9	481	128	1	21.0	80.4
c79 x +	6	88	147	20	57.6	68.2
	7	108	121	28	47.1	79.1
	8	124	113	14	45.0	84.2
	9	124	97	12	41.6	86.3
	10	180	64	7	25.5	86.6
S187 x +	4	61	91	33	49.2	37.1
	5	100	164	64	50.0	63.2
	6	116	108	31	42.4	83.3
	7	75	69	30	39.7	89.7
	8	157	100	46	33.0	71.6

* Scorable asci are non-discharged asci in which the segregation of spore colors is unambiguou

color pattern of 2:4:2 it is known (BOND, unpublished observations) that approx mately 90% of these asci are recombinant between the *mo-1* mutant and the locus of which *S187* is a member. Assuming that all the 2:4:2 asci are recomb national, and extrapolating to 2:2:2:2 asci, 9/10 of the recombinant asymmetr asci can be detected using this marker.

A sample of asci were dissected on day 4 and another sample dissected 4 da

TABLE 2

The number and frequency of recombinants and non-recombinants amongst 2:2:2:2 asci from ascus dissection of two crosses of

mo-1 S187 + + × +

		Recombinant 2:2:2:2 asci	Non-recombinant 2:2:2:2 asci	% Asci recombinant between *mo-1* + *S187*
Cross 1	Day 5	25	71	26.0 ± 4.5
	Day 9	21	71	22.8 ± 4.4
Cross 2	Day 4	20	76	20.8 ± 4.1
	Day 8	23	81	22.1 ± 4.1

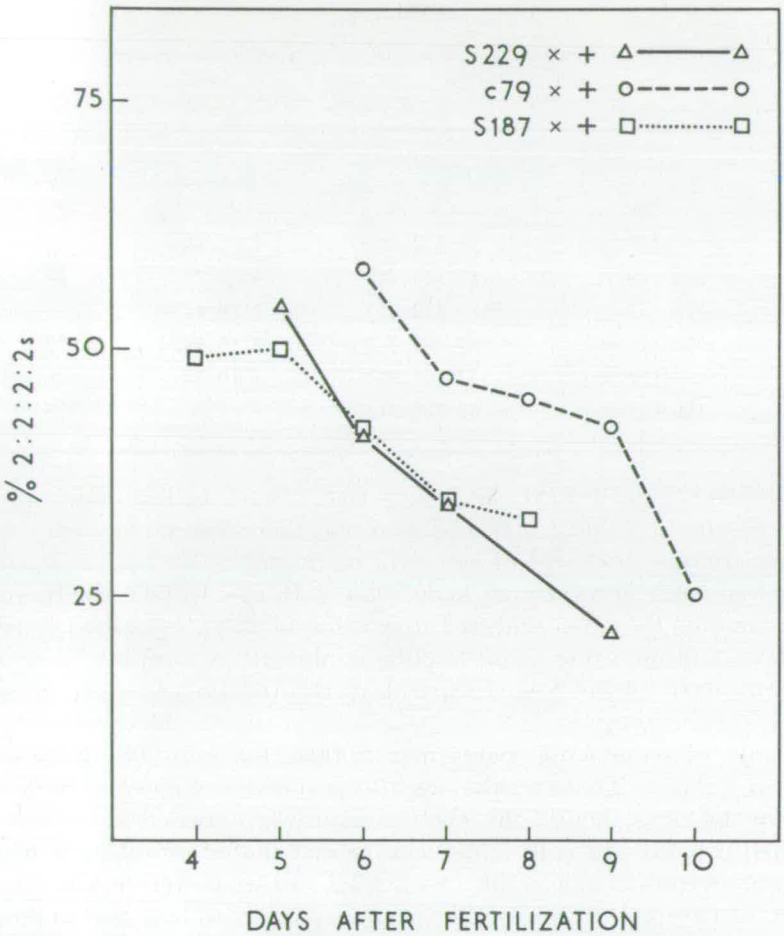

FIGURE 2.—The decline in the frequency of 2:2:2:2 asci with time for crosses of three spore lor mutants.

ter, after the presumed increase in recombinants had taken place. Dissections ere carried out on two separate crosses and on both occasions there was no crease in the frequency of asci recombinant between the markers *mo-1* and *187*, although there had been the customary decrease in the 2:2:2:2 frequency. hat is, there was no enrichment of recombinants due to the discharge of spindle verlap asci.

Preferential discharge of asci was not confined to single point crosses. Tables and 4 contain the results obtained from a two-point cross involving *c79* and *187*. This experiment was designed to test the possibility that there was a prefer- ıtial discharge of asci containing wild-type spores. The rationale of this experi- ent will be considered more fully in the DISCUSSION.

In the cross of *c79* × *S187* there are four possible spore genotypes which are l phenotypically distinguishable. Spores of the genotypes c79$^-$ S187$^+$; c79$^+$

TABLE 3

The number and frequency of ascus types in the cross: c79 + × + S187

Days after fertilization	Parental ditypes	Tetratypes	Non-parental ditypes	Percent tetratypes discharged
5	371 (87.5%)	51 (12.0%)	2 (0.5%)	
6	329 (78.9%)	87 (20.9%)	1 (0.2%)	21.9
7	426 (77.3%)	123 (22.3%)	2 (0.4%)	
8	408 (76.4%)	124 (23.2%)	2 (0.4%)	23.2
9	453 (77.8%)	127 (21.8%)	2 (0.3%)	26.9
10	196 (69.8%)	85 (30.2%)	0	24

S187$^-$; c79$^+$ S187$^+$; c79$^-$ S187$^-$ are, respectively, buff, yellow, black and white From the results in Table 3 it can be seen that there was no tendency for ther to be a preferential discharge of asci with black spores. Such a tendency woul cause the frequency of tetratypes to decrease with age. In fact, the frequency o tetratypes rose as the cross matured to a value of 23%, remained constant fo several days, and then rose again to 30% on day 10. A sample of asci collecte on the petri dish lid for 5 successive days showed no variation in tetratyp frequency.

The results of sequencing spores in asci from the same two-point cross ar recorded in Table 4. These results are also graphed in Figure 3. Both marker involved in the cross showed the phenomenon when crossed separately to wil type. When intercrossed both alleles can be distributed in a 2:2:2:2 manner i the parental ditype asci and in this case 2:2:2:2 asci are preferentially discharged Of course, in parental ditype asci the sequence refers to buff and yellow spore rather than wild type and mutant as for the single-point crosses. In tetratyp asci a 2:2:2:2 distribution of one allelic pair excludes the possibility of a 2:2:2:

TABLE 4

The distribution of the buff allele (c79) in parental ditype and tetratype asci from the cross: c79 + × + S187

Days after fertil-ization	In parental ditypes				In tetratypes				
	4:4	2:2:2:2	2:4:2	Percent 2:2:2:2s	4:4	2:2:2:2	2:4:2	Percent 2:2:2:2s	Scorable asci(%)
5	135	213	23	57.4	15	32	4	62.7	64.9
6	167	145	17	44.1	22	53	12	60.9	72.8
7	217	191	18	44.8	49	66	8	53.7	75.5
8	257	134	17	32.8	64	54	6	43.5	79.9
9	259	168	26	37.1	70	52	5	40.9	88.5
10	126	59	11	30.1	49	33	3	38.8	91.3

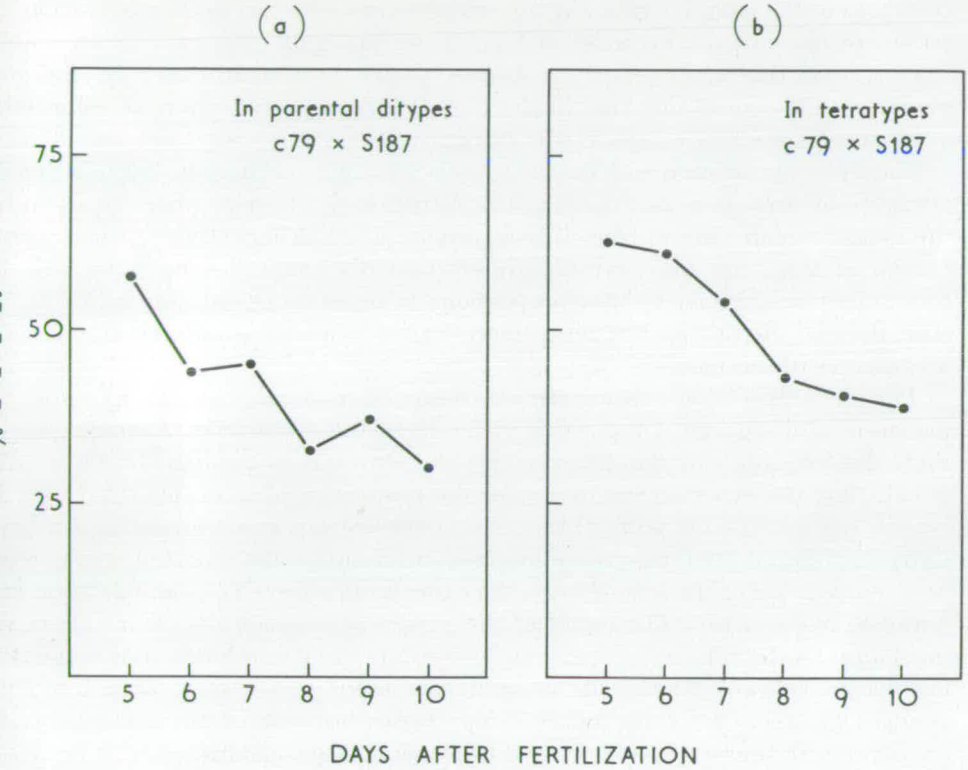

FIGURE 3.—The frequency of 2:2:2:2 distribution of the buff allele in parental ditype and tratype asci from the cross:

$$\text{o}\frac{\text{c79} \quad +}{} \times \text{o}\frac{+ \quad \text{S187}}{}$$

Data taken from that in Table 4.

stribution of the other. In these asci, therefore, the phenomenon can either isappear or be shown by one of the two loci. In tetratypes preferential matura-on and discharge did occur. Asci with a 2:2:2:2 distribution of the buff allele owed the decline in frequency.

The sequence of spores in all asci from all crosses was recorded. There were o major differences between any of the complementary spore sequences within n ascus class. For example, there was no difference in the frequency of 2+:2m: +:2m and 2m:2+:2m:2+ asci. For the two-point crosses there are four possible ositions for the wild-type spore pair within a tetratype ascus. There was no ndency for asci with the black spores in any one position to be discharged efore the others.

DISCUSSION

All crosses studied in *S. brevicollis* have, in our hands, shown a significant ecline in the frequency of 2:2:2:2 asci with increasing age of the cross. The ariations which occurred in the frequencies of the main ascus classes are a

reflection of the complex relationship which exists between ascus maturation an ascospore discharge. The onset of 2:2:2:2 decline coincided with ascospore di charge, and this observation implicates preferential dehiscence in the ph nomenon. Of course the bias in discharge may be a reflection of some oth attribute of these asci, e.g. shorter maturation time.

The early maturation and/or dehiscence of 2:2:2:2 asci may be because of son attribute of this class, *per se*, (e.g. the distribution of spore phenotypes with the ascus) which distinguishes it from other classes. Alternatively, a major com ponent of the class may exhibit preferential discharge. In the latter case th component would have to have proportionally more representation in the 2:2:2 class than in the others. For centromere-linked markers spindle overlap asci a an example of this case.

The ascus dissection experiment was designed to test the specific hypothesis preferential discharge of the overlap asci. By using appropriate formulae to es mate the frequency of spindle overlap (MACDONALD, unpublished) it is possib to calculate the expected frequency for the proportion of recombinants in 2:2:2 asci at any given time point. Thus, if spindle overlap asci were being prefere tially discharged, then in cross 1 24% recombinants were expected on day 5 an 38% on day 9. For the second cross the expectations were 12% and 28% on da 4 and 8, respectively. The result of the experiment was quite clear. There w no change in the relative proportions of overlap and recombinational asci with the 2:2:2:2's. Thus during the cross development there were large frequen changes between the three main ascus classes but within the 2:2:2:2 asci th proportions of the two developmental types remained constant.

Preferential discharge of asci has been reported before, although not of th magnitude reported here. LAMB (1967) has suggested that the position of th wild-type spores in an ascus affects its maturation and consequently its discharg It was possible therefore that the 2:2:2:2 asci were preferentially discharged account of the distribution of the black spores within the ascus. This idea w tested by analyzing a cross of two linked spore color mutants. In a cross of *c79 S187* only recombinant asci contain black spores. If these affect ascus maturatio then recombinant asci should be preferentially discharged. The results of th experiment indicated that there was no selective discharge of recombinants. O the other hand, data from the same cross indicated a preferential discharge 2:2:2:2 asci regardless of whether they contain some black spores or all muta spores.

Two parameters, spindle overlap and recombination, are estimated from th frequencies of the major ascus classes found in a cross. For recombinatio second-division segregation frequencies are required. For spindle overlap th symmetrical and asymmetrical second-division sequences are compared. Clear the preferential discharge of asci in any major class will affect estimates of th frequency of both these parameters. For example, the frequency of spindle ove lap as calculated using the formulae of WHITEHOUSE (1957) or CHEN (196 will apparently vary with the time of sampling asci from the cross.

The ascus dissection data indicate that, despite a changing relationsh

tween classes, within any class the frequency of overlap and recombinant asci constant. This can be used as a basis for estimating spindle overlap frequency d will be the subject of a further communication.

We are indebted to MR. E. D. ROBERTS for skillfully preparing the figures. One of us (D.J.M.) n receipt of an M.R.C. RESEARCH STUDENTSHIP.

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Corresponding Editor: D. STADLER

ABSTRACT OF THESIS

Name of Candidate Douglas John MacDonald

Address 11a Warrender Park Crescent, EDINBURGH, EH9 1EA

Degree PhD *Date* 5th January 1976

Title of Thesis "Ascus analysis and the induction of protoperithecia in Sordaria brevicollis"

The frequency of spindle overlap during ascus development in Sordaria brevicollis has been found by different workers to vary widely between crosses. This variation was confirmed and it was proposed that it can be accounted for largely by assuming that different types of asci have different rates of maturation and discharge. This assumption was confirmed by observation. In single point crosses the frequency of asci with a 2:2:2:2 distribution of wild type and mutant spores declined as the crosses matured. It was proposed that either spindle overlap asci or 2:2:2:2 asci per se matured and were discharged preferentially. Ascus dissection experiments failed to distinguish between these alternatives.

The production and distribution of perithecia and protoperithecia in Sordaria brevicollis is determined by environmental and genetic factors. It was found that the primary stimulus to protoperithecium production under laboratory conditions was an interaction between a growing culture and a physical barrier, such as a petri dish wall. Exhaustion of nutrients and the accumulation of organic substances in the medium did not play a major role in the induction of fruiting. Two genes, per-1 and per-2, which affect fruiting have been found. Per-1$^-$ per 2$^-$ cultures were completely devoid of protoperithecia. Per 1$^-$ per 2$^+$ cultures have a characteristic phenotype with a fertile colony centre and a sterile margin. The concentration of some phosphorylated intermediates of carbohydrate metabolism dropped markedly in the sterile regions of cultures when their growth was impeded by a physical barrier. The nature of the interaction between a culture and a barrier was speculated on. No evidence was found to suggest a role for cyclic AMP in the regulation of these morphogenetic events.

Use other side if necessary.