GENETIC AND EPIGENETIC FACTORS CONTROLLING FEMALE STERILITY IN *NEUROSPORA CRASSA*

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1. INTRODUCTION

THE production of protoperithecia, the female reproductive structures, in *Neurospora crassa* is influenced by environmental and genetic factors. Westergaard and Mitchell (1947) developed a synthetic culture medium, which was optimal for protoperithecial formation and subsequent maturation of perithecia. A temperature of 25° C. gave rapid and strong development of protoperithecia and perithecia, while a temperature of 35° C. inhibited the formation of both, findings confirmed by Hirsch (1954). Knowledge of how to control protoperithecial production makes *N. crassa* particularly suitable for genetic studies of their formation. Genetic factors influencing the development of protoperithecia have been recorded in *Neurospora* and other ascomycetes. Dodge (1946) found that lack of protoperithecia in *N. sitophila* was caused by a single gene. Protoperithecial abnormalities and lack of protoperithecia in strains of *N. crassa* with long histories of vegetative propagation, found by Westergaard and Hirsch (1954), appeared to be caused by different, non-allelic, mutant genes. Jinks (1954, 1956) demonstrated a cytoplasmic basis for reduced and permanent loss of perithecial formation in the homothallic *Aspergillus nidulans* and *A. glaucus* after continued asexual propagation by conidia and after ageing.

Failure to develop protoperithecia in a strain of *Neurospora crassa* is shown in this paper to be caused by the joint action of genetic factors and a cellular control system having an epigenetic action (Nanney, 1958).*

The strain of *N. crassa* concerned, originated from an Emerson *A* × Emerson *a* cross, was prototrophic and of mating type *A*. It showed normal fertility during several years of vegetative propagation, but protoperithecial production subsequently weakened, and eventually, all isolations failed to develop protoperithecia. Subcultures of these isolations likewise did not form protoperithecia. Crosses with other normal strains were completely sterile when these isolations were used as female parents, but normal perithecia and ascospores resulted when they were used as male parents.

2. METHODS

Prototrophic strains were involved throughout the investigation, and these were cultured at 25° C. on the minimal medium of Westergaard and Mitchell (1947), which promotes protoperithecial production.

* The use of the term " epigenetic ", and alternatives, is discussed by Catcheside (1959).

In the serial subcultures, inocula from a number of stock lines were incubated at 35° C. for rapid growth on minimal agar slopes, and after 24 hours, hyphal tips were removed and used to inoculate fresh slopes. These were again incubated at 35° C. for 24 hours and the subculture repeated successively. After removal of the hyphal tips, the cultures were incubated at 25° C. for two or more weeks. This allowed protoperithecial development, and any phenotypic differences at successive stages of the treatment could be detected. Some cultures were incubated completely at 35° C. This treatment allowed rapid growth of young hyphæ for several successive 24 hour periods, and created a series of discontinuities between the newly formed hyphæ and possible influences of the old hyphæ.

3. GENETICS OF THE FEMALE STERILITY

(i) *The wild-type × female sterile progeny*

Eighteen asci were isolated from a cross between wild-type and female-sterile strains. Four of the asci, from two perithecia, contained cultures with abnormal growth or melanin production and were not

TABLE 1

Progenies of the crosses : wild-type × female-sterile, and small × breakdown.

Parents	Ascus types in progeny								Numbers of asci
Wild type *a* × female-sterile A	La	La	La	La	Sm	Sm	Sm	Sm	4
	La	La	La	La	Sm	Sm	Bk	Bk	3
	La	La	La	La	St	St	Bk	Bk	4
	La	La	La	La	St	Sm	Bk	Bk	1
	La	La	La	La	St	St	Sm	Sm	2
	Abnormal asci								4
3-6A × 14-8*a* *small × breakdown*	Sm	Sm	Bk	Bk	Bk/Sm	Bk	Sm	Sm	3
	Sm	Sm	Bk	Bk/Sm	Sm	Sm	Bk/Sm	Bk/Sm	1
	Sm	Sm	Sm	Sm	Bk/Sm	Bk	Bk/Sm	Sm	1
	Sm	Sm	Sm	Sm	Sm	Sm	Bk	Bk	4
Ascus 16	Sm	Sm	Bk	Bk	Bk/Sm	Bk	Sm	Sm	
16-1A × 16-6*a*	—	Sm	Sm	Sm	Bk	Bk	Sm	Sm	1
16-8*a* × 16-3*A*	Sm	Sm	Bk	Bk	Bk	Bk	Sm	Sm	2
	Sm	Sm	Sm	Sm	Sm	Sm	Bk	—	1

Phenotypes are indicated as follows :—La, *large* ; Sm, *small* ; St, *sterile* ; Bk, *breakdown*.

scored. The remaining fourteen asci, from six other perithecia, contained four phenotypes as follows: the normal wild-type with large protoperithecia covering most of the culture surface (phenotype *large*); small, pale-coloured yet functional protoperithecia mostly confined to the mycelium at the edges of the culture medium (phenotype *small*); a female-sterile strain without protoperithecia (phenotype *sterile*); a female-sterile strain producing many small, discrete, dark-brown

coloured patches on and in the agar medium at the time protoperithecia are normally differentiated, and accompanied, as are protoperithecia, by a general darkening of the culture medium due to melanin formation. Microscopic examination showed that the dark-brown patches had a fine granular structure, and occurred where cell contents escaped through ruptures in the walls of enlarged hyphal cells. Cells swollen with dark-brown granular contents but as yet unbroken also occurred. As these dark patches appeared to result from a breakdown in the normal differentiation of protoperithecia, this phenotype was called *breakdown*.

The patterns of these four phenotypes in the asci, and the frequencies of different ascus types are shown in table 1. *Large* and *non-large* spore pairs segregated at the first division in all asci. One spore pair showed both phenotypes *small* and *sterile*. All four phenotypes were obtained from single perithecia, and did not therefore result from heterocaryosis in the parent strains.

When subcultured on the same medium, *large* strains produced protoperithecia by the sixth day, and *small* subcultures produced their characteristic protoperithecia in ten to sixteen days. Subcultures of the *sterile* strains likewise produced protoperithecia at this time and could not be distinguished from the *small* phenotype, while subcultures of *breakdown* strains did not form dark-coloured centres of hyphal breakdown, and instead developed a few *small* protoperithecia. The change of phenotypes *sterile* and *breakdown* to *small* was persistent in serial subcultures, and was also observed in nearly all subcultures of *sterile* and *breakdown* strains in later progenies. Intercrosses of the progeny strains are considered in the following sections.

(ii) *Crosses between* small *and* breakdown *strains*

Six *small* strains were crossed by subcultures of *breakdown* strains which had changed to the *small* phenotype, and the progeny of one of these crosses 3-6 $A \times$ 14-8 *a* is shown in table 1. *Breakdown* reappeared in the progenies. Some *breakdown* cultures also produced *small* protoperithecia and showed both phenotypes at the same time. These cultures occurred in spore pairs with either *breakdown* or *small* cultures, and appeared to be an earlier manifestation of the change to *small* observed previously in subcultures of *breakdown* strains. The progeny contained, in general, asci with 3 *small* and 1 *breakdown* spore pairs, and asci with 2 *small* and 2 *breakdown* spore pairs. The cultures showing both *breakdown* and *small* occurred only in the latter asci. Subcultures of the *breakdown* ascospore cultures showed no sign of *breakdown* and developed *small* protoperithecia or, in a few cases, no protoperithecia. Reciprocal crosses using *breakdown* subcultures as female parents gave no progeny, probably because of their imperfectly developed protoperithecia.

The *small* and *breakdown* strains from one of the 2 *small* : 2 *breakdown* asci (ascus no. 16) were intercrossed, and the progenies (table 1),

although small because of poor ascospore germination, showed 2 *small*: 2 *breakdown* asci and 3 *small* : 1 *breakdown* asci, and so were similar to the progenies already described. Subcultures of the *breakdown* cultures in these progenies all developed phenotype *small*.

These results suggested that more than one gene distinguished *small* and *breakdown*. The presence of two or more major genes was tested by analysing three asci containing 3 *small* and 1 *breakdown* spore pairs. These three analyses gave similar results and one of them (ascus no. 21) is shown in table 2. Because of poor ascus maturity in these and previous crosses, the progenies were obtained from random ascospores. The high germination percentages in many of these

TABLE 2

Analysis of ascus 21 (random spore progenies)

Ascus 21 Spore No.	3	4	6	7
Phenotype	Sm	Sm	Sm	Bk
Mating type	*A*	*a*	*a*	*A*
Deduced genotype	*bk*	*bk*+	*bk*+	*bk*

Parents	Progeny			Total	Ascospore viability
	Sm	St	Bk		
21-2 × 21-6	46	4	29	79	0·75
21-2 × 21-4	52	1	25	78	0·80
21-6 × 21-7	42	3	34	79	0·82
21-4 × 21-7	52	—	28	80	0·83
21-2 × 16-6	—	—	80	80	0·61

progenies suggested that the ascospores ripened after discharge from the perithecia. The three *small* strains in ascus 21 were intercrossed as their mating types allowed, and all three were crossed by a *breakdown* strain. Because of mating type differences, *small* strain 21-2*A* was crossed by *breakdown* strain 16-6*a* from another ascus. Crosses 21-2*A* × 21-6*a* and 21-2*A* × 21-4*a* involved *small* strains only, yet the progenies of both contained *breakdown* cultures. This result was impossible if two or more genes determined *small*, when at least one of the crosses would have given an all *small* progeny, and indicated a single gene difference. The strain common to both crosses 21-2*A* presumably did not carry the gene determining *small*, as is further shown by the lack of *small* segregants from the *small* × *breakdown* cross 21-2*A* × 16-6*a*. This progeny was the same as would be expected from a *breakdown* × *breakdown* cross, and indicated that 21-2*A*, although phenotypically *small*, had the same genotype as *breakdown* strains, that is it carried a mutant gene *bk*. Likewise, 21-4*a* and 21-6*a* carried a factor *bk*+, determining phenotype *small*. The progenies of the first four crosses analysing ascus 21 varied little from each other ; and the deviation from the 1

small : 1 *breakdown* ratio expected from a one gene difference was similar to that in the original *small* × *breakdown* progeny (table 1) where it was caused by some *bk* strains showing phenotype *small*. It was found that such pseudo-*small* strains could be distinguished from true *small* strains by their later development of protoperithecia and by a general darkening of the medium as they aged, as was characteristic of *breakdown* strains. When such strains were taken into account, the random ascospore progenies showed good 1 : 1 ratios. The genotypes of fifteen of these pseudo-*small* strains were checked by crossing them to strains known to carry *bk*+. All gave progenies containing *breakdown* cultures, whereas four *small* strains, which did not darken with ageing and showed an earlier development of protoperithecia, gave progenies of *small* cultures only.

All of the *small* × *breakdown* crosses involved *breakdown* strains which had developed phenotype *small* as a result of being subcultured. The results therefore showed that the change from *breakdown* to *small* did not involve a gene change. Indeed, the phenotypic expression of allele *bk* was most variable, and the ease with which it could be changed, as for example by subculture, and the occurrence of different phenotypes in the same spore pair, both indicated that it was largely controlled by environmental or other non-genetic factors.

(iii) Crosses between large and breakdown strains

Two types of progenies resulted from crosses between *large* strains and *breakdown* strains which had changed to the *small* phenotype. The first type, obtained from eight of the *large* strains, contained only asci with a clear segregation of two *large* spore pairs and two spore pairs showing phenotypes *breakdown*, pseudo-*small* and *sterile*. The progeny of one of these crosses 10-3*a* × 6-8*A* is shown in table 3 (*a*). Seventeen of the pseudo-*small* and *sterile* cultures from different progenies were crossed to true *small* tester stocks and all produced some *breakdown* progeny. One gene determined phenotype *large* in these asci and *bk* showed variable phenotypic expression, including differences within spore pairs, as in the *small* and *breakdown* progenies.

In the second type of progeny, which resulted from four of the *large* strains, the asci likewise contained two *large* spore pairs, whereas the other two spore pairs showed the true *small* phenotype as well as *breakdown*, pseudo-*small* and *sterile*. The progeny of cross 6-4*a* × 6-8*A* which was of this type is shown in table 3 (*b*). The true *small* strains gave progenies of *small* cultures only, when crossed to tester strains. Allele *bk*+ determining phenotype *small* was segregating in this progeny, and the parental strains differed by two genes.

Large strains were of two genetic types: those differing from *breakdown* by one gene *s*+ determining *large*, and those differing from *breakdown* by genes *s*+ and *bk*+. Genotypes *s*+*bk*+ and *s*+*bk* determined phenotype *large*, *s bk*+ determined *small*, and *s bk* produced phenotypes

ranging from *breakdown* through intermediate *sterile* forms to pseudo-*small* according to the degree of its expression. The equal segregation of *large* and non-*large* spore pairs in all asci showed that s^+ was epistatic to the alleles at the *bk* locus. Random spore progenies from crosses of the twelve *large* strains by *breakdown* gave similar results to these.

Seven of the *large* $(s^+bk) \times$ *breakdown* crosses were repeated from cultures of parental strains which had been subjected to serial subculture under the conditions, described in section 4, leading to the

TABLE 3

Progenies of large × breakdown *crosses*

	Parents	Ascus types in progeny								Numbers of Asci
a	10-3*a* × 6-8*A* .	La	La	La	La	Sm′	Sm′	Sm′	Sm′	2
		La	La	La	La	Sm′	St	Sm′	Sm′	1
		La	La	La	La	Sm′	Bk	Sm′	Sm′	2
		La	La	La	La	Sm′	Sm′	Bk	Bk	2
		La	La	La	La	St	St	Bk	Bk	1
		La	La	La	La	Bk	Bk	Bk	Bk	2
b	6-4*a* × 6-8*A* .	La	La	La	La	Sm	Sm	Sm	Sm	1
		La	La	La	La	Sm	Sm	Sm′	Sm′	1
		La	La	La	La	Sm′	Sm′	Sm′	Sm′	4
		La	La	La	La	St	St	Sm′	Sm′	2
		La	La	La	La	St	St	St	St	1
		La	La	La	La	Bk	Bk	Sm	Sm	2
c	10-3*a* × 6-8*A* . (after serial subculture)	La	La	La	La	Bk	Bk	Bk	Bk	1
		La	La	La	La	Bk	Bk	Bk	—	5
		La	La	La	La	Bk	—	Bk	—	4
		La	La	La	La	Bk	Bk	—	—	1

Phenotypes are as indicated in earlier tables ; also Sm′ denotes pseudo-*small*, and a dash denotes an inviable ascospore. First and second division segregation differences are not shown.

induction of *breakdown* in subcultures of *s bk* strains. A marked effect on the expression of *s bk* was observed in the progenies. The asci in these showed *large* and *breakdown* cultures only, with considerable ascospore inviability among the *s bk* segregants. The progeny of cross 10-3*a* × 6-8*A* (table 3 (*c*)) can be compared with the progeny of this cross already described in table 3 (*a*). Only 2 of the 44 s^+bk ascospores were inviable compared with 15 of the 44 *s bk* ascospores. Progenies of the other crosses showed near complete viabilities of s^+bk segregants coupled with viabilities of *s bk* segregants ranging from 4 to 50 per cent. Many spore pairs contained one inviable ascospore and one *breakdown* culture. The numbers of *s bk* spore pairs containing 2, 1 and 0 viable *breakdown* ascospores agreed, in each progeny (probabilities ranging from 5 per cent. to 90 per cent.), with the numbers expected if inviability was distributed randomly in the population of *breakdown* ascospores and was not influenced by the pairs of genetically identical ascospores.

The two series of *large* × *breakdown* crosses showed that a deficiency of *breakdown* in a progeny could result from inviability of *s bk* ascospores and from incomplete expression of this genotype giving pseudo-*small* cultures. The occurrence of both these expressions of *s bk* in progenies from different crossings of the same parental strains, differing only in pretreatment, indicated that they were epigenetic rather than genetic effects.

(iv) *Genetic relationship of* large, small *and* sterile *strains*

Random ascospore progenies from three crosses between *small* and *sterile* strains are shown in table 4. The progeny of cross 3-6*A* × 5-6*a*

TABLE 4

Parent strains and random ascospore progenies of crosses between large, small *and* sterile *strains*

Parents	Genotypes of parents	Progeny				
		La	Sm	St	Bk	Total
small* × *sterile						
3-6*A* × 5-6*a*		—	49	3	26	78
1-7*a* × 1-5*A*		—	76	2	—	78
1-7*a* × 6-5*A*		—	76	2	—	78
large* × *sterile						
4-3*a* × 1-5*A*	$s^+bk^+ \times s\ bk^+$	44	36	—	—	80
5-2*A* × 5-6*a*	$s^+bk^+ \times s\ bk$	44	16	2	16	78
5-4*A* × 5-6*a*	$s^+bk^+ \times s\ bk$	44	26	1	6	77
3-4*a* × 1-5*A*	$s^+bk \times s\ bk^+$	55	18	1	6	80
3-4*a* × 6-5*A*	$s^+bk \times s\ bk^+$	52	15	2	11	80
1-3*a* × 1-5*A*	$s^+bk \times s\ bk^+$	37	18	13	9	77
1-1*A* × 5-6*a*	$s^+bk \times s\ bk$	47	7	6	19	79
3-1*A* × 5-6*a*	$s^+bk \times s\ bk$	63	6	—	9	78
small* × *small						
3-6*A* × 1-7*a*		—	53	11	—	64
3-6*A* × 3-8*a*		—	77	3	—	80
4-5*A* × 1-7*a*		—	56	—	—	56
large* × *large						
5-2*A* × 1-3*a*	$s^+bk^+ \times s^+bk$	78	—	—	—	78
5-2*A* × 4-3*a*	$s^+bk^+ \times s^+bk^+$	79	—	—	—	79
1-1*A* × 1-3*a*	$s^+bk \times s^+bk$	79	—	—	—	79
large* × *small						
5-2*A* × 3-8*a*	$s^+bk^+ \times s\ bk^+$	31 (9)	35	5	—	80
4-3*a* × 3-6*A*	$s^+bk^+ \times s\ bk^+$	41	30	8	—	79
1-3*a* × 3-6*A*	$s^+bk \times s\ bk^+$	46	16	—	18	80

was the same as the random spore progenies obtained from *small* × *breakdown* crosses. Of six asci also isolated from this cross, three contained 3 *small* and 1 *breakdown* spore pairs and three contained 4 *small* spore pairs. Crosses to *small* tester stocks revealed that, in the latter asci, two of the *small* spore pairs were *s* bk^+ whereas the other two segregated *breakdown* strains and were genetically *s bk*. The identity of these results to those of the *small* × *breakdown* crosses indicated that

the *sterile* strain 5-6*a* was genetically *s bk*. The progenies of the other two *small* × *sterile* crosses consisted almost entirely of *small* cultures, and were the same as obtained from *small* × *small* crosses, indicating that the *sterile* strains 1-5*A* and 6-5*A* were genetically *s bk*$^+$. The *sterile* phenotype did not represent a distinct genotype, but resulted from either failure to produce *breakdown* in a *s bk* strains, or from failure to develop protoperithecia in *s bk*$^+$ strains.

The parent strains and progenies from eight *large* × *sterile* crosses are presented in table 4. The progenies showed a good correlation with the parental genotypes—as indicated by the results of previous crosses—after allowances were made for deficiencies of *breakdown*. These appeared to be due largely to inviability of *breakdown* ascospores in crosses 5-4*A* × 5-6*a*, 3-4*a* × 1-5*A*, 3-4*a* × 6-5*A*; and to reduced expression of *s bk*—appearing as *sterile*—in 1-3*a* × 1-5*A*. The presence of some *small* cultures in the two *s*$^+$*bk* × *s bk* progenies, where an equal segregation of *large* and *breakdown* was expected, suggested reduced expression of *s bk* although true and pseudo-*small* strains were not distinguished in these progenies. The results of these crosses in general agreed with the conclusion that *sterile* strains were genetically either *s bk*$^+$ or *s bk*.

Small cultures were predominant in the progenies from three intercrosses of *small* strains (table 4) from the progeny of the original wild-type × female-sterile cross. Some *sterile* strains, presumably the result of failure to develop protoperithecia, were also present. These progenies agree with the earlier findings that phenotype *small* was determined by genotype *s bk*$^+$.

Progenies from three intercrosses of *large* strains (table 4) all contained *large* cultures only. The crosses represented all three combinations of the two *large* genotypes, and their results supported the earlier findings that *s*$^+$, determining phenotype *large*, was epistatic to the two alleles at the *bk* locus.

The progenies of the first two *large* × *small* crosses (table 4) conformed in general to the equal segregation of *large* and *small* expected from their genotypes, although the expression of the segregating genotypes was not always clear. The *sterile* cultures were probably *s bk*$^+$ in which full protoperithecial development was not realised. Nine cultures in the first progeny showed characters of both *large* and *small* phenotypes, and could not be classified into either group. The progeny of the third cross contained *large*, *small* and *breakdown* cultures in a ratio approximating 2 : 1 : 1, which confirmed that the *large* and *small* phenotypes were determined by non-allelic factors, and that *s*$^+$ was epistatic to the *bk* locus.

This analysis of the genetic relationship of the female-sterile and wild-type strains showed that two different genes determined protoperithecial formation, and that the expression of the double mutant genotype was subject to non-genetic variation. The genetic constitution of the six asci from the original wild-type × female-sterile progeny

which were used in the analysis are shown in table 5. Asci 1, 3 and 10 showed one ditype segregation, ascus 5 the other ditype segregation, and asci 4 and 6 showed the tetratype segregation. Alleles s^+/s and bk^+/bk segregated at the first division in 89 per cent. (of 114 asci) and 39 per cent. (of 14 asci) respectively. The two loci were not likely to be linked to each other, and there was no evidence of linkage in

TABLE 5

Phenotypes and genotypes of asci 1, 3, 4, 5, 6 and 10 from the progeny of the original wild-type × female-sterile cross

Ascus no.	Ascospore no.	Phenotype	Genotype	Mating type
1	1-1	*large*	s^+bk	A
	1-3	*large*	s^+bk	a
	1-5	*sterile*	$s\ bk^+$	A
	1-7	*small*	$s\ bk^+$	a
3	3-1	*large*	s^+bk	A
	3-4	*large*	s^+bk	a
	3-6	*small*	$s\ bk^+$	A
	3-8	*small*	$s\ bk^+$	a
10	10-1	*large*	s^+bk	a
	10-3	*large*	s^+bk	a
	10-5	*small*	$s\ bk^+$	A
	10-7	*small*	$s\ bk^+$	A
4	4-1	*large*	s^+bk	a
	4-3	*large*	s^+bk^+	a
	4-5	*small*	$s\ bk^+$	A
	4-8	*breakdown*	$s\ bk$	A
6	6-2	*large*	s^+bk	a
	6-4	*large*	s^+bk^+	a
	6-5	*sterile*	$s\ bk^+$	A
	6-8	*breakdown*	$s\ bk$	A
5	5-2	*large*	s^+bk^+	A
	5-4	*large*	s^+bk^+	A
	5-6	*sterile*	$s\ bk$	a
	5-7	*breakdown*	$s\ bk$	a

crosses where it would be detected. While these two genes account for the different types of protoperithecial formation in the progeny strain, the results of the wild-type × female-sterile cross were unusual in that the stable sterility which characterised the female-sterile parent was not present in the progeny.

4. SERIAL SUBCULTURE

(i) *Phenotypic expression of* s bk

Serial subculture, details of which are given in section 2, was maintained for twenty successive subculture generations on twelve *s bk* strains showing the *small* and *sterile* phenotypes. The *breakdown* phenotype reappeared in all of the strains, usually in the first generations

treated, and, after a number of subculture generations which varied between different strains, it increased in intensity, showing a much greater production of the brown pigment. The appearance of *breakdown* was accompanied by a reduced growth rate, and as it intensified the growth rate dropped further. Two strains, in which *breakdown* was strongly developed, showed no growth at all during the first 24 hours of subculture generations 12 and 14 respectively, and only after this time was slow hyphal growth observed. In four other cases, involving three strains, inocula from cultures showing intense brown pigmentation and decreased growth rate were inviable. Intensification of *breakdown* reduced the viability of hyphæ.

The marked reappearance of *breakdown* in these treated strains contrasted with the results of previous continuous incubation at 35° C. of four of them. Only two showed *breakdown*, and it did not persist in successive subcultures at 35° C.

Subcultures of the twelve strains were taken from generations 18 and 20 of the series by a mass inoculum of conidia and hyphæ, and incubated continuously at 25° C. for about 30 days when they in turn were subcultured. This was repeated for five subculture generations. Under these normal culture conditions, the twelve strains mostly retained the abnormal development of *breakdown* induced by the serial subculture treatment. Four of the strains showed no indication of losing *breakdown*, but in some of the others its intensity was reduced. Prior to this treatment, *breakdown* was rarely expressed in subcultures incubated at 25° C., but was an unstable condition found only in ascospore cultures, from which subcultures developed phenotype *small*.

The increased intensity of expression of *breakdown* after several treated generations, and its maintenance in some strains, for several subculture generations after the return to normal culture conditions showed that the changes in expression of *s bk* from *breakdown* to *small* and from *small* to *breakdown* were not direct environmental effects. Rather it would appear that a physiological or cytoplasmic system, intervening between genotype *s bk* and its phenotype, could exist in different states, some of which were self-perpetuating to some extent, and that these different states led to the different phenotypic expressions of *s bk*.

(ii) *Expression of the female-sterile strain*

The serial subculture treatment was maintained for ten successive subculture generations on the parent strains, and related strains, from the original wild-type × female-sterile cross, and on twelve *large* progeny strains. The phenotypes developed by successive stages of these strains showed the following characters. (*a*) Two separate lines of the female-sterile parent both developed *breakdown* strongly in the first treated stages, as did *s bk* strains in the earlier series. However, in later stages of the treatment, *breakdown* disappeared and *large* protoperithecia developed. These persisted in five further subculture generations under normal culture conditions. (*b*) *Breakdown* did not appear in

a female-fertile strain isolated asexually from the wild-type *A* stock before it became female-sterile. (*c*) *Breakdown* did not appear in any of six separate lines of the wild-type *a* stock used in the original cross. (*d*) None of the twelve *large* strains showed *breakdown* in cultures incubated at 25° C. after the first 24 hours at 35° C., but eight of them showed *breakdown* in cultures incubated at 35° C. after this time. These eight strains were of genotype s^+bk, while the four strains which did not show *breakdown* were s^+bk^+. The conditions of the serial subculture treatment allowed the expression of allele *bk* in these *large* cultures.

This behaviour of the *large* strains indicates that *breakdown* did not appear in the cultures of the wild-type *a* parent of the original cross because this stock did not carry *bk*. This allele was introduced into the wild-type × female-sterile progeny by the female-sterile parent in which *breakdown* was well developed. Its absence in a female-fertile strain derived earlier from the same stock as the sterile strain suggested that development of the sterility was associated with the appearance of *breakdown*, and hence with the mutant *bk*.

(iii) *Crosses involving the female-sterile strain*

Cultures of the female-sterile strain showing phenotypes *breakdown* and *large*, from early and later stages respectively, of the serial subculture treatment, were crossed to a wild-type *a* strain. There was little difference between the progenies from the two crosses (table 6 (*a*)) in spite of the different phenotypes of one of the parents. Also the

TABLE 6

(a) *Random spore progenies from the female-sterile strain showing phenotypes* breakdown *and* large

Parents	Progeny				
	La	Sm	St	Bk	Total
Wild-type *a* × female-sterile *A* (*breakdown*)	59	—	9	10	78
Wild-type *a* × female-sterile *A* (*large*)	68	—	3	6	77

(b) *The formerly female-sterile strain used as a female parent (random spores)*

Parents	Genotype of male parents	Progeny				
		La	Sm′	St	Bk	Total
Female-sterile *A* × 5-7 *a* .	*s bk*	31	1	5	42	79
Female-sterile *A* × 1-3 *a* .	s^+bk	76	—	1	—	77
Female-sterile *A* × 4-3 *a* .	s^+bk^+	74	—	3	3	80

numbers of *breakdown* segregants in the two progenies were similar to those obtained from the original wild-type × female-sterile cross (table 1). The development of phenotype *large* by the female-sterile strain did not therefore appear to involve genetic changes such as back mutation or the action of modifying genes. Neither of the progenies in table 6 (*a*) showed any differentiation between the *large* and *small* phenotypes, which, because of the high ascospore viabilities of these crosses, can only mean a breakdown in the distinction between these phenotypes.

The female-sterile strain which developed protoperithecia as a result of the serial subculture treatment was used as a female parent in three crosses made by the spermatisation technique (table 6 (*b*)). The male parent of the first cross 5-7*a* was genetically *s bk* and the progeny contained *large* and *breakdown* cultures. The formerly female-sterile strain clearly passed on the ability to form *large* protoperithecia to some of the progeny cultures. The male parent of the second cross, 1-3*a*, was a *large* strain with genotype s^+bk, and the progeny was typical of that expected from an intercross of two *large* strains, apart from one culture which probably had genotype *s bk*. In the third cross the male parent 4-3*a* was a large strain with genotype s^+bk^+, and yet there were three *breakdown* cultures in the progeny. As 4-3*a* did not carry *bk*, these must have originated from the newly female-fertile strain. This supports the view that back mutation was not involved in the change from female-sterile to the *large* phenotype. The lack of *small* segregants in this progeny, and in those shown in table 6 (*a*), also shows that the change in phenotype was not due to modifying genes. From these results, it appeared that the female-sterile strain had gained a non-genetically determined ability to form *large* protoperithecia in much the same manner as *s bk* strains produced *small* protoperithecia.

5. DISCUSSION

(i) *The variable phenotypic expression of* s bk

The most interesting of the four genotypes present in the progeny of the wild-type × female-sterile cross was the double mutant *s bk*, which varied in expression showing phenotypes ranging from different intensities of *breakdown* pigmentation through intermediate *sterile* forms to reduced protoperithecial production (*small*). This phenotypic variation was not caused by gene differences as could be seen from the presence of both phenotypes *breakdown* and *small* in some cultures, the two cultures of *s bk* spore pairs showing different phenotypes, and *breakdown* ascospore cultures being genetically identical with their subcultures which developed phenotype *small*. Successive culture of the phenotypically *small s bk* strains at 35° C. for 24 hours, followed by maturation at 25° C., readily induced *breakdown* to reappear. The appearance of *breakdown* in ascospore cultures or as a result of serial subculture only, suggests that *small* was the normal phenotype of *s bk*, and that *breakdown* was induced by the serial subculture and, to

a lesser degree, by some feature of ascospore cultures. The increased intensity of *breakdown* with continuation of serial subculture, and its maintenance after the return to normal subculture conditions showed that its induction was not a direct environmental effect. Also, the induction of *breakdown* carried over to the progeny of the next sexual generation. Treatment of both parents of *large* × *breakdown* crosses gave a much increased expression of *breakdown* in the progeny, and, also, considerable inviability of *s bk* ascospores. Such dauermodification-like behaviour suggests an epigenetic system of a physiological or cytoplasmic nature was present, and that the different phenotypic expressions of the nuclear genotype *s bk* were functions of different interchangeable, partly autonomous states of this system. The inviability of *s bk* ascospores accompanying the strong expression of *breakdown* would appear to be an extreme manifestation of the cellular condition causing *s bk* to be expressed as *breakdown*. Like *breakdown*, it occurred randomly and regardless of spore pairs; also, the intensification of *breakdown* pigmentation in the serial subculture series was accompanied by slowing down of hyphal growth and eventually death of the hyphæ.

The range of phenotypic expression with several distinct phenotypes shown by *s bk*, and the common reversion of the induced *breakdown* phenotype to *small* in subcultures, together with the immediate induction of *breakdown* by serial subculturing, make it unlikely that the appearance of *breakdown* was due to depletion and loss of controlling cytoplasmic elements. Rather, the change from one phenotype to another, and reversion to the original, would suggest that changes in the equilibrium of cytoplasmic elements were involved. Different equilibrium states of cytoplasmic elements could produce a continuous series of phenotypes, and would allow rapid changes and considerable stability at different times, depending on the factors controlling the equilibrium. The extreme cellular condition, characterised by reduced vitality of hyphæ and inviability of many *s bk* ascospores, could result from an extreme change in the equilibrium by which one of the cytoplasmic elements was either greatly reduced or lost causing death of the cell. An alternative and perhaps more likely explanation of the extreme cellular condition is that the initially induced change in the cytoplasmic equilibrium caused hyphal breakdown, and that further change in the equilibrium increased this abnormal physiological condition so as to eventually cause death of the hyphæ. Ascospores with this extreme equilibrium state would have a high degree of physiological abnormality and would not germinate. This is a physiological limit to phenotypic variation as described by Jinks (1957), and has already received comment (Fitzgerald, 1958).

Distinct cytoplasmic elements are not necessary to explain the phenotypic behaviour of *s bk*. An epigenetic system of gene interaction can do this. Between the initial action of a gene and its ultimate phenotypic effect, a wide field of gene interaction and possibly cytoplasmic influence intervenes. Normally a stable equilibrium of these

interactions results in a definite canalisation which produces a stable phenotype. However, in some cases, especially with mutant genes, a less stable equilibrium might be expected. Small influences inside or from outside the cell would disturb such an equilibrium and lead to different canalisations from which a range of phenotypic effects might be expected. A gene concerned with such an epigenetic system would show variation in its expression, and furthermore, depending on the ability of a particular canalisation to maintain itself once established, examples of limited inheritance might be expected. Such a system could explain the changes in phenotypic expression of *s bk* and the stability of the induced changes. It has similarities to the steady state system of Delbrück (see Ephrussi, 1953; Beale, 1954).

Regarding the feature of ascospore cultures which induced *breakdown*, it was proposed that this might be the 60° C. heat treatment for 50 minutes to initiate ascospore germination. However, this phenotype was present in ascospore cultures germinated by furfural instead of heat (Emerson, 1948); and heating subculture inocula to 60° C. failed to induce *breakdown*.

The behaviour of *s bk* has some similarities to the *nd* and *wd* mutants which initiated degenerative changes in *Neurospora crassa* (Sheng, 1951*a*, *b*). Whereas external stimuli, in the form of ascospore activation or continued serial subculture, caused a slowing down of hyphal growth and eventually hyphal death of *s bk* strains, death or degeneration of strains carrying the *nd* and *wd* mutants occurred spontaneously within a few vegetative transfers. Sheng found that this progressive degeneration involved a cytoplasmic run-down or ageing, possibly due to the accumulation of a self-intoxicating substance in the growing frontier, the evidence being that replacement of the cytoplasm by making heterocaryons or crossing to wild-type temporarily reversed the degenerative phenotypic changes. While the tendency of the *s bk* strains to overcome the adverse induced effects contrasted with the degenerative action in the *nd* and *wd* strains, it is likely that both types of behaviour were functions of similar cellular systems.

(ii) *The nature of the female-sterility*

Four different genotypes, each with corresponding phenotypes, were present in the progeny of the wild-type × female-sterile cross. The wild-type parent corresponded to phenotype *large*, and while the female-sterile parent was like the *sterile* phenotype, it retained its sterility over several subculture generations unlike the progeny cultures which were sterile only in ascospore cultures and developed *small* protoperithecia in subcultures. This failure to fully reproduce the female-sterility in the progeny suggested that it involved more than the gene differences shown to exist between the parent strains. However, the parental female-sterile strain was not completely stable, and as a result of serial subculture it developed *large* protoperithecia which were

maintained subsequently and were fertile. The wild-type parent consistently produced *large* protoperithecia and therefore carried allele s^+, whereas the female-sterile parent presumably carried allele *s*. Serial subculture failed to show *breakdown* in the wild-type strain, but showed it in the female-sterile strain, indicating the presence of allele *bk*. This is supported by the demonstration of *breakdown* only in serial subcultures of *large* strains with the s^+bk genotype. The development of phenotype *large* by the female-sterile strain did not involve back mutation of *s*, and was not likely due to mutation at other loci modifying *s*. The female-sterile strain was therefore genetically similar to the *sterile* strains with genotypes *s bk*, and like them could change to a fertile phenotype as a result of some change other than gene mutation. It differed from the *sterile* strains only in its more stable sterility, and in its development of phenotype *large* instead of *small*. It has already been shown that *s bk* progeny cultures produced a sterile phenotype only when they were coupled with a particular non-permanent cellular state. Likewise, the female sterility of the original strain can be considered to have resulted from the association of *s bk* with a particular cellular state belonging to the same or a similar system as that controlling the expression of *s bk* in the progeny cultures. A likely hypothesis therefore is that the originally wild-type strain developed mutant genes *s* and *bk*, which did not affect its female-fertility in the presence of a normal cellular state. An aberrant, semi-permanent cellular state then gradually became established in the mycelium as a result of continued vegetative reproduction, and possibly from ageing; and as it developed it caused a gradual diminishing of female-fertility, and finally complete failure to form protoperithecia.

Alternative factors interacting with *s bk* to cause the female-sterility can be postulated. During the long period of asexual propagation the original female-sterile strain may have accumulated gene mutations and become heterocaryotic in respect of factors affecting the production of protoperithecia, not all of which were present in the progeny strains; or chromosome unbalance resulting from polysomy or structural hybridity may have arisen. A possible indication of such abnormalities were the four asci in the original wild-type × female-sterile progeny which showed abnormal growth and originated from different perithecia than did the other asci. However, the abnormal growth in all spores of these four asci indicated an overall extreme cytoplasmic modification of genotype expression rather than the effect of segregating genes, and does not support this view. Also, it is difficult to account for the phenotypic behaviour of the sterile parental strain by such genetic mechanisms. It is clear that chromosome unbalance, if it existed in the sterile strain, did not occur in its progeny because the irregular ratios obtained from crosses of progeny strains were not consistent with the ratios expected from chromosome unbalance in a haploid organism (see Emerson, 1956), but were caused by non-genetic variation in the expression of *s bk*.

6. SUMMARY

1. Female-sterility, due to failure to form protoperithecia, was found in a biochemically wild-type strain of *N. crassa*.

2. This sterile strain differed from wild-type by two genes. Genotypes s^+bk^+ and s^+bk were fully fertile wild-type, and $s\ bk^+$ showed reduced protoperithecial development but was fertile. Genotype *s bk* varied in expression with phenotypes ranging from a reduced female-fertile form to a sterile form producing dark pigmentation. This latter phenotype was accompanied by considerable ascospore inviability.

3. The phenotypic variation of *s bk* was not due to gene differences but appeared to be determined by different equilibrium states of a cellular system intervening between *s bk* and its phenotypic effects. Under certain conditions, some of these states were self-perpetuating.

4. The female-sterile strain had the *s bk* genotype, and it appeared that sterility resulted when this genotype was associated with a particular cellular state belonging to the same or a similar system as that which controlled the expression of *s bk* in the progeny cultures.

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