A RECIPROCAL PHENOTYPIC INSTABILITY AFFECTING DEVELOPMENT IN *ASPERGILLUS NIDULANS*

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Received 3.ii.66

1. INTRODUCTION

ASEXUAL cloning of a homokaryotic fungus with uninucleate haploid asexual spores, such as in the homothallic Ascomycete, *Aspergillus nidulans*, might be expected to give rise to a uniformity within the clones. However, lines with respectively high and low perithecial (cleistothecial) densities have been reported to arise as a result of selection among asexually produced monospore clones in *A. nidulans* (Jinks 1954, 1956). Heterokaryon transfer tests indicated that the differences produced by selection were under a non-chromosomal control. Further, Mahoney, and Wilkie (1958, 1962) have reported non-mendelian segregation of perithecial and aperithecial (*alba*) colonies among the sexual progenies of a number of homokaryotic lines of *A. nidulans*. The results of a sexual cross indicated that a single gene difference controlled the proportion of perithecial to *alba* colonies recovered among the ascospore progeny of a homokaryotic line.

In the present report the segregation of perithecial (*p*) and aperithecial (*ap*) colonies among the sexual and asexual progenies of one wild isolate of *A. nidulans* is described.

2. MATERIAL AND METHODS

Birmingham isolate 37 was used throughout these experiments. It was collected from the wild during 1962. The yellow spored mutant of this isolate, 37*y*, was obtained by ultra-violet irradiation. This mutation had no visible effect other than the change in conidial pigmentation and a slight change in the background pigmentation of the whole colony.

All experiments were carried out upon Czapek minimal medium (containing K_2HPO_4, giving a *p*H of 7·2) solidified with New Zealand agar (G. T. Gurrs Ltd). Incubation was at 25° C. Standard 90 mm. diameter Petri dishes contained from 22 to 25 ml. of medium. Less than 20 ml. per dish tended to depress perithecial production and therefore to mask the segregations. Unless otherwise stated, all conidial transfers were made from ten day old parental colonies. Suspensions of conidia or ascospores in a solution of " Tween 80 " (Koch-Light Laboratories Ltd.) were diluted and plated in the normal way. Hülle cell plates were prepared by carefully sampling groups of hülle cells under the dissecting microscope, placing these in a drop of " Tween 80 " on a plate and spreading (see Raper and Fennell 1965; Schwartz 1928). Colony densities were kept at from 25 to 35 colonies per plate.

3. EXPERIMENTS AND RESULTS

(i) *Segregation among conidial progenies of extreme* p *and* ap *colonies*

When the conidia of a single colony of isolate 37, itself derived from a single conidium, are sampled and plated the resultant colonies show

a segregation for perithecial production. The majority of these colonies fall into two classes, those producing no perithecia (*ap* type) and those completely and densely covered with perithecia (*p* type). The remaining colonies are mosaics of varying proportions of *p* and *ap* regions. (Plate 1*b*, *c* and *d*.)

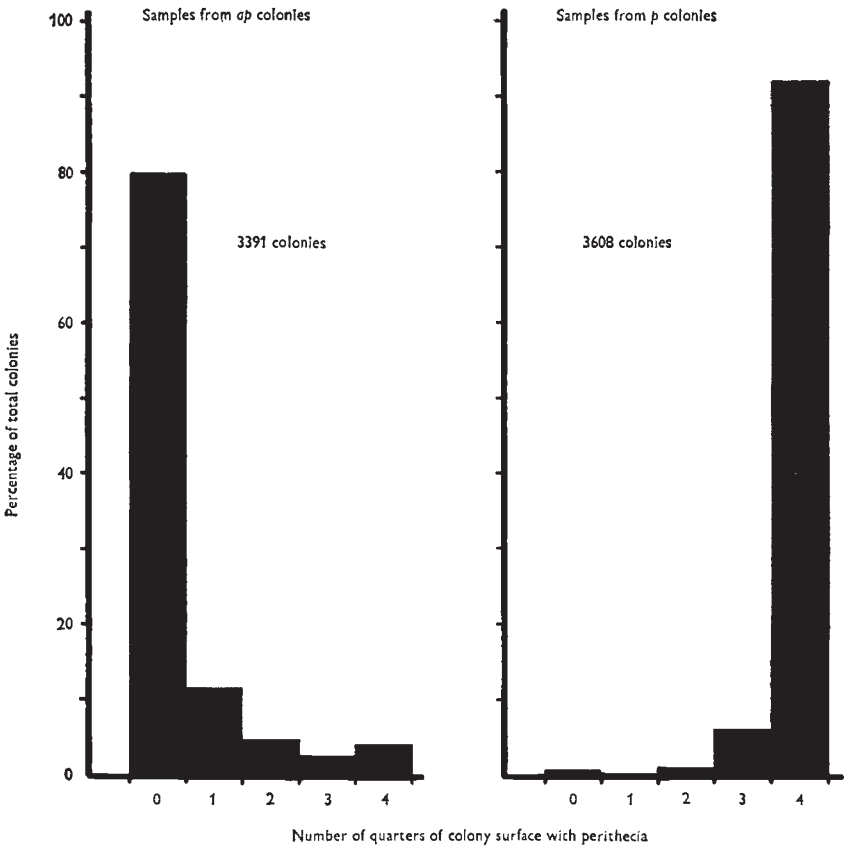


FIG. 1.—Segregation of *p*, *ap* and mosaic colonies among the conidial progenies of *p* and *ap* parental colonies. Conidia were sampled from 10 *p* colonies and 10 *ap* colonies, all of which were derived from one colony grown from a single conidium.

Conidia from extreme *p* and *ap* colonies give the full range of perithecial production in their progenies. However, the proportion of each class of segregants varies according to the phenotype of the " parental " colony. Figure 1 shows that conidia from extreme *ap* colonies gave mainly *ap* progeny while conidia from extreme *p* colonies gave mainly *p* progeny. The intermediate mosaic colonies present in both cases were scored according to the area of the colony surface (to the nearest quarter colony) which was covered with perithecia. Similar segregation distributions were produced when the conidia were obtained from *p* and *ap* colonies derived from single ascospores.

Conidia taken from the *p* and *ap* regions of a mosaic colony repeat

the segregation distributions of conidia taken from extreme *p* and *ap* colonies respectively. Also, although it has not been fully investigated, it has been noticed that a single conidial head from a *p* or *ap* colony repeats the segregation patterns of the conidia taken from the whole of each respective type of colony.

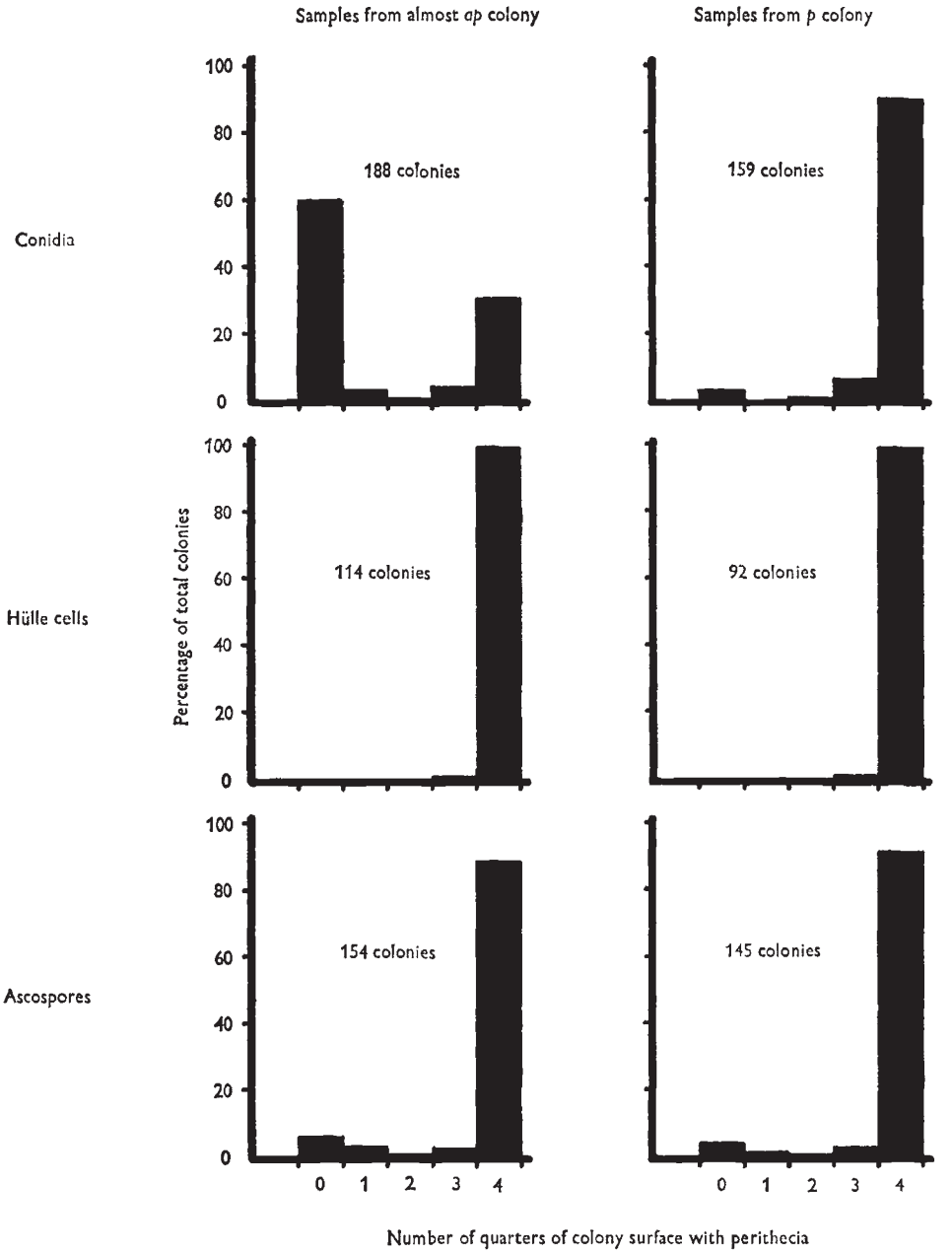


FIG. 2.—Segregation of *p*, *ap* and mosaic colonies among conidial, hülle cell and ascospore progenies of an almost *ap* colony and of a *p* colony.

(ii) *Segregation among conidial, ascospore and hulle cell progenies*

The segregation of *p* and *ap* colonies among the conidial, ascospore and hülle cell progenies of an extreme *p* and of an almost *ap* mosaic colony was examined. (The extreme *ap* colony has no perithecia at all). These three types of spore were sampled when the two parental colonies were 14 days old. The results, illustrated in fig. 2, show that ascospores and hülle cells gave similar segregation patterns, both producing mainly *p* colonies and irrespective of whether they originated from the *p* or almost *ap* colony. As before the conidial progeny did vary according to the phenotype of the parental colony. The segregation distribution of the conidial progeny of the low perithecial parental colony illustrates the typical manner in which a mosaic colony gives rise mainly to both extreme classes of colony and only to a few intermediate mosaics (see section iii).

(iii) *Inheritance of the* p *and* ap *states within a population*

The results of the previous sections show that the genotype of isolate 37 can give rise to two alternative phenotypic states. In order to examine the transmission of the *p* and *ap* states from one asexual generation to another under non-selective conditions random samples of asexual progeny were taken over two generations and the colony phenotypes of the second generation were scored.

All of the conidia were removed from a randomly selected 10-day old colony and a sample plated out. Nineteen of the resultant colonies were selected at random and their conidia were similarly sampled and plated. When the colonies constituting these 19 *asexual* " families " were three days old a further 20 colonies were chosen at random within each family. The resultant 380 small colonies were then inoculated in duplicate by cutting each into two halves and using each half as one inoculum. The 760 inocula were placed in a regularly spaced arrangement, 10 to each plate, the position of any one inoculum being determined at random. This regular, low density arrangement permitted the resultant colonies to be scored according to the number of sixths of their surface which were covered with perithecia.

The segregation distributions of the 19 families (fig. 3) illustrate that there was a continuous range of perithecial production from the extreme *ap* family number 18 to the extreme *p* family number 17. They also show that the intermediate families consisted mainly of varying proportions of extreme *p* and *ap* colonies rather than of colonies each with an intermediate score. These results are best explained by assuming that each of the 19 parental colonies was a mosaic composed of varying proportions of *p* and *ap* regions, from the almost completely *p* parental colony of family 17 to the almost completely *ap* parental colony of family 18. The segregation distributions given in fig. 3 are thus proportional to the degree of mosaicism of the parental colonies. However conidial density is negatively correlated with perithecial density ($r = -0{\cdot}955$, $P < 0{\cdot}001$), so that a far greater number of

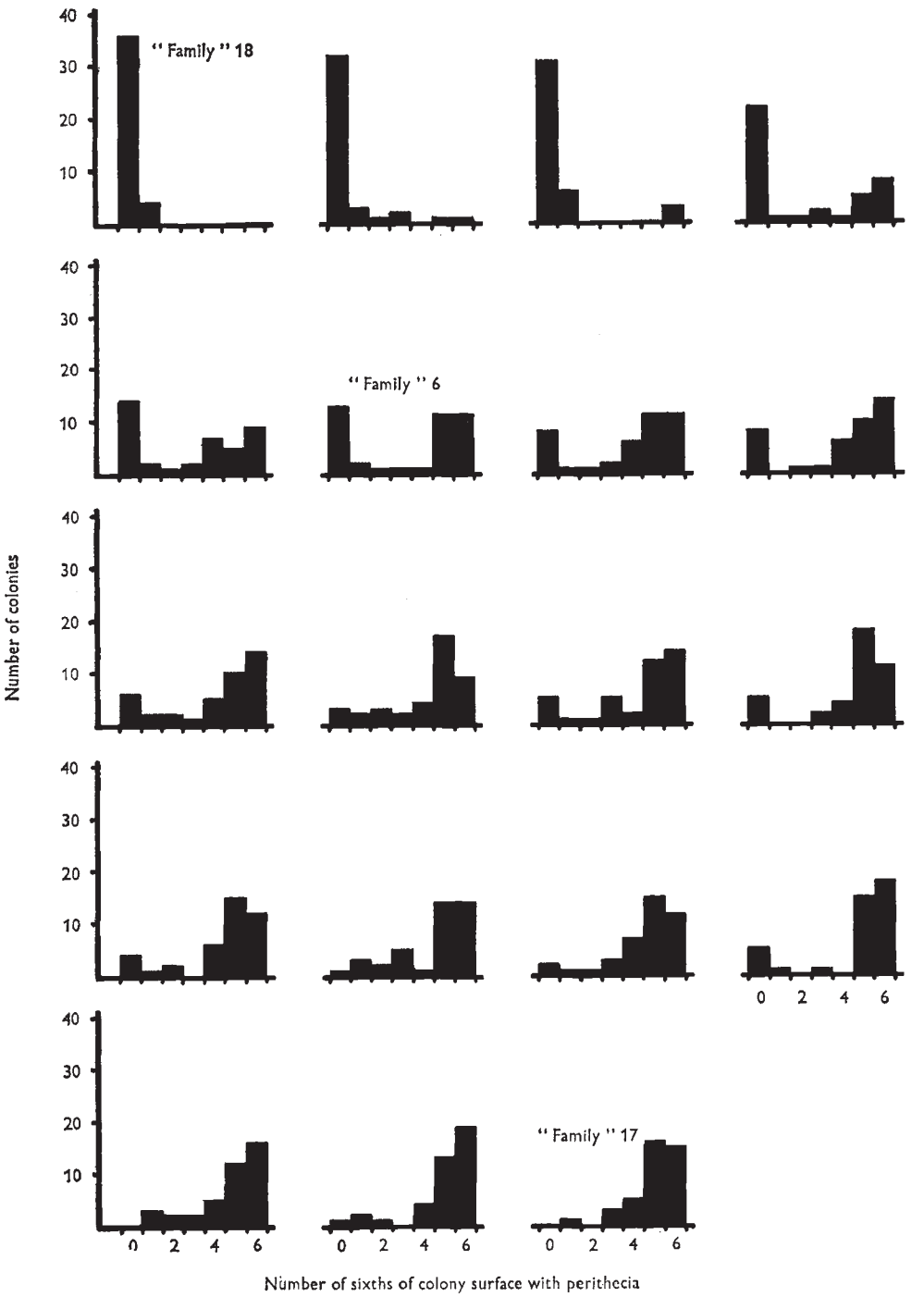


FIG. 3.—Segregation of *p*, *ap* and mosaic colonies among the 19 conidial " families ". The histograms are arranged according to the order of the " family " means.

conidia will be obtained from an *ap* region of a colony than from a *p* region of the same area. A family, such as number 6, which contains an equal proportion of *p* and *ap* colonies will, therefore, have been derived from a mosaic parental colony having a much greater area with the *p* than with the *ap* phenotype.

The analysis of variance given in table 1 shows that there was significant variation caused by segregation both between and within the

TABLE 1

Analysis of variance illustrating the segregations within and between the 19 asexually produced "families".

The metric used is the number of colony sixths which were covered with perithecia

Item	d.f.	M.S.	Components of M.S.	Estimates
Between families	18	97·4608	$\sigma_E^2+2\sigma_W^2+40\sigma_B^2$	$\sigma_B^2 = 2{\cdot}2814$
Within families	361	6·2050	$\sigma_E^2+2\sigma_W^2$	$\sigma_W^2 = 2{\cdot}4841$
Duplicate error	380	1·2368	σ_E^2	$\sigma_E^2 = 1{\cdot}2368$

19 families. The estimates of the components of the mean squares show that the variation between the families was the same magnitude as the variation within the families. That is, the variation produced in the first asexual generation persists into the subsequent generation under the standard conditions of this experiment.

(iv) *The heterokaryon transfer test*

This test was carried out to determine whether the *p* and *ap* states depend on any extrachromosomally located factor. Heterokaryotic conidial heads between isolates 37 and 37*y* were produced without the use of auxotrophic mutants, the low natural rate of heterokaryosis, being relied upon.

Ten heterokaryotic conidial heads were obtained from each of the combinations, (37*p*+37*y ap*) and (37 *ap*+37*y p*) and plated out. Heterokaryotic heads of the combinations (37 *p*+37*y p*) and (37 *ap*+37 *y ap*) were similarly sampled as controls. A further class of control was carried out to ensure that cross-feeding could not affect the results. Five heads each of 37 *p* and 37*y ap* were mixed together in a suspension and plated. Mixtures of 37 *ap* and 37 *y p* were sampled in a similar way.

The segregation distributions obtained from the spore mixtures showed that there was no effect of simply physically mixing *p* and *ap* spores. The segregation distributions of the progenies derived from the heterokaryotic associations are illustrated in fig. 4. When both parental components were in the *p* state or both in the *ap* state, the majority of the progeny were *p* and *ap* respectively. But when *p* and *ap* parents were mixed in the heterokaryon, although the largest proportion of the reisolates were of the parental type, a considerable

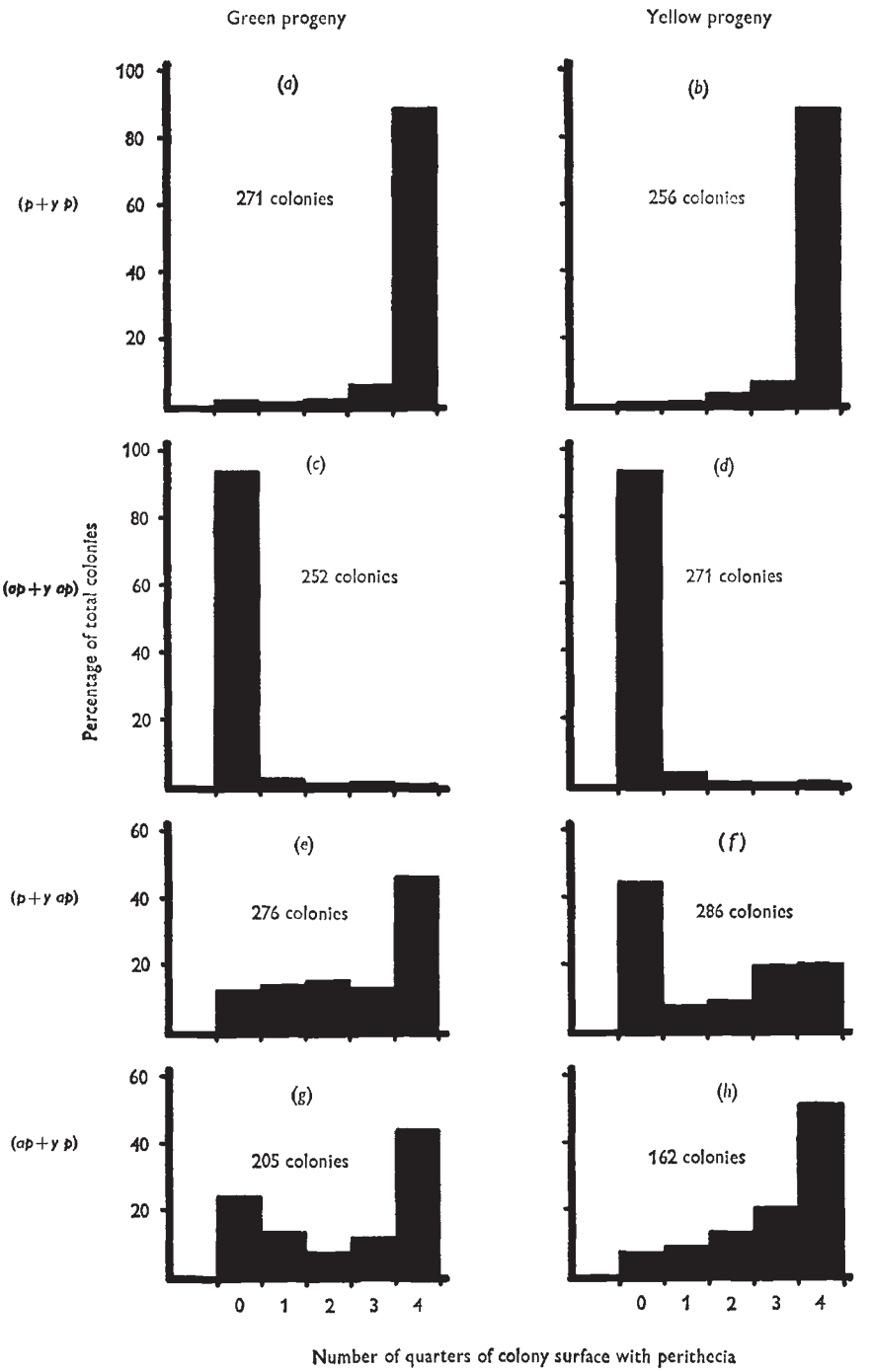


FIG. 4.—Segregation of *p*, *op* and mosaic colonies among the progenies of heterokaryotic conidial heads of isolate 37 *p* and *op* with 37 *y p* and *y op*. The constitutions of the four classes of heterokaryon are given to the left of the diagram.

proportion were in the alternative state with respect to perithecial production. The four separate analyses of variance given in table 2 show that in every case there was a highly significant effect of incorporating *p* and *ap* states in a heterokaryon. Thus it appears that extrachromosomal factors are involved in the difference between the *p* and *ap* states of isolate 37.

TABLE 2

Analyses of variance illustrating the changes which occurred after heterokaryotic association of p *and* ap *mycelia.*

The metric used in the angular transformation of the percentage of the total area (in quarter colonies) which were covered with perithecia

	Item	d.f.	M.S.	P.
37*p i.e.* Histograms (*a*) and (*e*). (See fig. 4)	Control v. Test	1	2700·256	<0·001
	Residual	18	43·706	
37*ap i.e.* Histograms (*c*) and (*g*)	Control v. Test	1	8149·088	<0·001
	Residual	18	64·146	
37*y p i.e.* Histograms (*b*) and (*h*)	Control v. Test	1	1621·981	<0·001
	Residual	18	31·642	
37*y ap i.e.* Histograms (*d*) and (*f*)	Control v. Test	1	4895·633	<0·001
	Residual	18	11·807	

(v) *The stability of the* p *and* ap *states*

Both the *p* and *ap* states are unstable and this instability may be represented as $p \rightleftharpoons ap$. However, this instability is manifest in a number of different ways. In the actively growing hyphal tip the changes $p \rightarrow ap$ and $ap \rightarrow p$ each occur with low and approximately equal frequencies. Both *ap* colonies and *p* colonies, each grown from a single spore, produced only rare sectors of the alternative phenotypic states (see plate 1a). The majority of conidia of ten day old *p* and *ap* colonies germinated to give parental type colonies but with consistent proportion of mosaic colonies and colonies in the alternative state. Again the frequency of change from one state to the other is low and approximately equal in both directions (fig. 1). Therefore both the *p* and *ap* states are relatively stable in young hyphal tips and conidia in this isolate. Ascospores and hülle cells on the other hand always gave rise to a high proportion of *p* colonies irrespective of the phenotype of the parental colony (fig. 2). The probable reason for this will be elaborated in the discussion.

The *p* and *ap* phenotypes vary considerably under different environmental conditions. For example, at 18° C. and 25° C. the *p* and *ap* morphologies were clearly distinguishable although at 18° C. only abnormal perithecia developed and no ascospores were produced. At 35° C. the two phenotypes were indistinguishable, all colonies being highly conidial with a small number of scattered perithecia. However,

the hereditary basis of the two states remained unchanged, because when conidia from colonies grown at 35° C. were germinated and grown at 25° C. the typical segregation distributions were again realised.

A further aspect of the stability of the *p* and *ap* states is seen as the change from the *ap* state to the *p* state within the differentiated structure of the colony with the passage of time. This is clearly illustrated by the following experiment.

An *ap* and a *p* colony were chosen from among the conidial progeny both of an *ap* colony and of a *p* colony. These four colonies were growing on plates each containing about 40 colonies. At this density little growth occurs after the seventh day from inoculation. On the seventh day samples of conidia were removed from both *ap* colonies and stored

TABLE 3

Analysis of variance of the data for perithecial production among the progenies of conidia obtained from p *colonies,* ap *colonies and of detached conidia from* ap *colonies sampled when 10 days, 20 days, and 30-days old.*

The metric used is the angular transformation of the percentage of the total area (in quarter colonies) which were covered with perithecia

Item	d.f.	M.S.	P
Grandparent	1	45·760	...
Parent	2	51536·360	0·01†
Age	2	14353·145	0·1 to 0·05†
Grandparent × Parent	2	100·605	>0·1††
Grandparent × Age	2	117·140	0·1 to 0·05††
Parent × Age	4	2720·062	<0·001††
Residual	256	46·973	

† Tested against parent × age interaction. †† Tested against residual.

in dry Petri dishes in the incubator at 25° C. On the tenth day samples of conidia were taken from both *p* colonies, both *ap* colonies and from both stored samples of conidia from the *ap* colonies, and plated out. The sampling was repeated on the twentieth and thirtieth days and the resultant segregations were scored. When the samples of conidia were taken from the two *ap* colonies on the two later occasions care was taken to remove only conidia which had been produced before the seventh day after inoculation. Any later formed conidial heads, which were few in number, could be recognised by their light green colour and larger size.

The analysis of variance given in table 3 shows that the results obtained were independent of the "grandparental" origin of the colonies, but dependent upon the parental origin. Further, despite the age main-effect being non-significant, the highly significant age × parent interaction shows that there was an effect of age, but that this effect varied according to the class of parental colony from which the conidia were taken. The segregation distributions given in fig. 5 show that, in all three parental classes, the change with time was from

the *ap* to the *p* state. The constant numbers of colonies which grew from the standardised conidial suspensions show that there was no loss of conidial viability throughout the thirty day period and that differential viability could not be the cause of the observed results. Therefore,

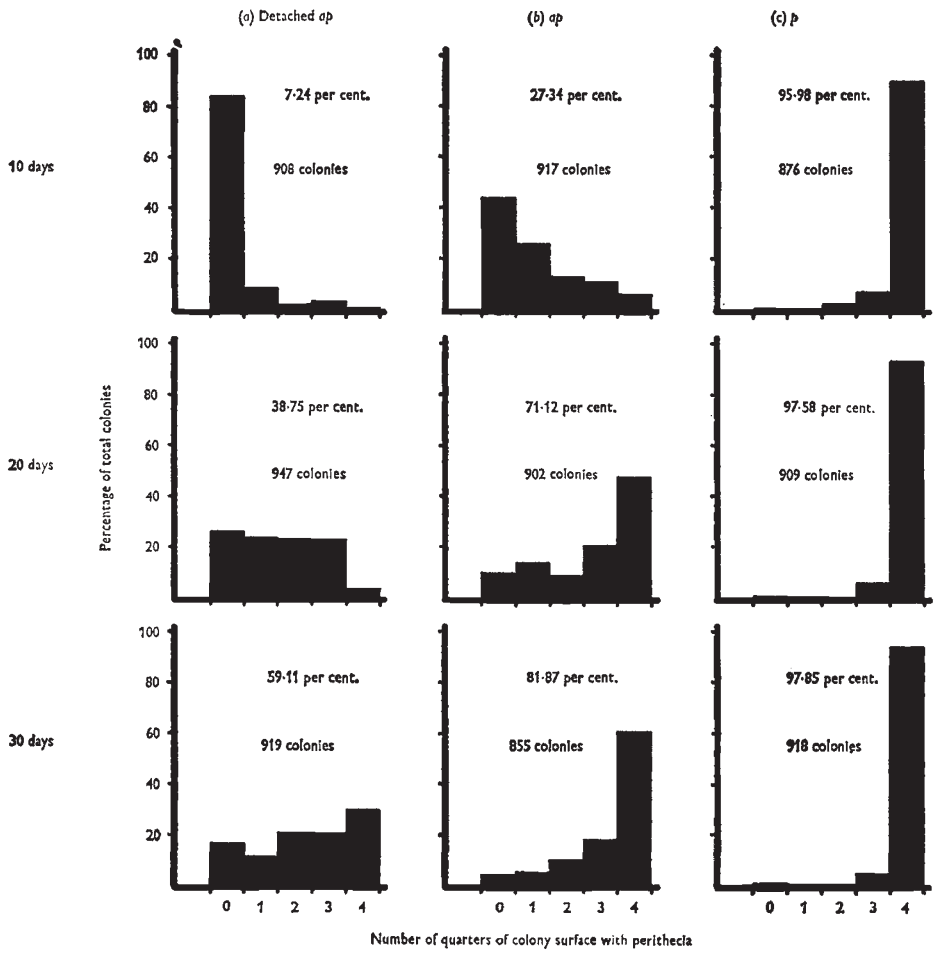


FIG. 5.—Segregation of *p*, *ap* and mosaic colonies among sample progenies grown from conidia stored over a period of 30 days. The conidia were stored (*a*) dry in a Petri dish after removal from a 7-day old *ap* colony, (*b*) on the same *ap* colony and (*c*) on a *p* colony. The percentage of the total colony surface area (in quarter colonies) covered with perithecia is given for each histogram.

the observed change with time from the *ap* state to the *p* state must be occurring within the resting conidia. In another experiment the rate of change was shown to be affected by the storage temperature, 25° C. giving a higher rate than either 5° C. or 35° C. (fig. 6).

There is evidence that this conversion process, from the *ap* state to the *p* state is also occurring within the whole colonial mycelium. During the thirty day period, although growth was limited by the

proximity of other colonies, a few young conidial heads were produced upon the parental *ap* colonies. If these conidia were plated as soon as they matured they always gave rise to a predominance of *p* colonies. At the same time the colony gradually became covered with a small number of scattered solitary perithecia. Therefore any new growth which occurred after a certain period gave rise either to perithecia or to conidia in the *p* state.

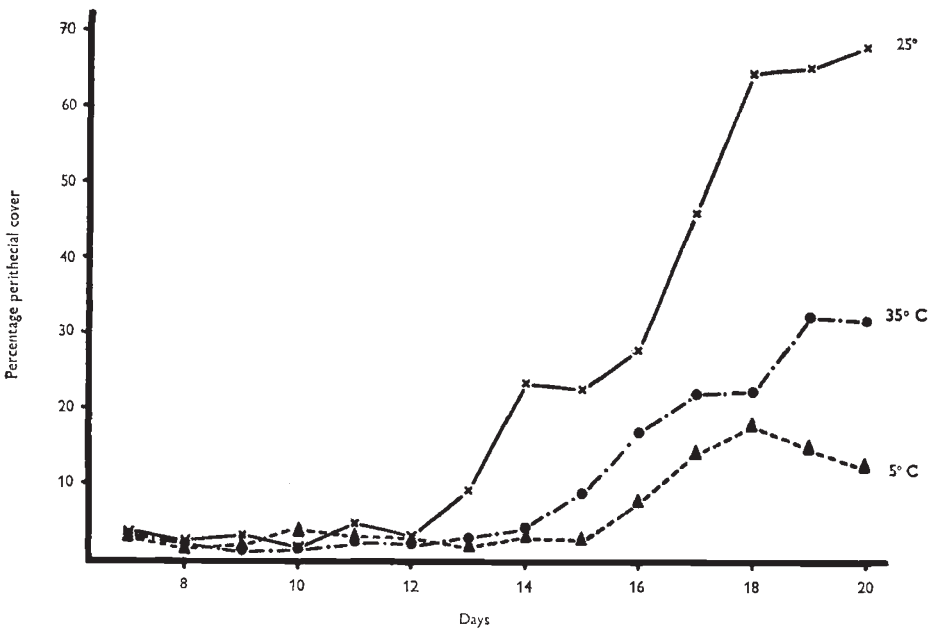


FIG. 6.—The effect of temperature on the rate of conversion of detached, dry stored conidia from the *ap* state to the *p* state. The metric used is the percentage of the total colony surface area (in quarter colonies) covered with perithecia.

4. DISCUSSION

The results of these experiments clearly show that the same haploid genotype can give rise to two alternative phenotypic states, one where the mycelium can and one where it cannot produce perithecia. In the present account this phenomenon has been described in only one isolate, but it is of very common occurrence in *A. nidulans*. Of 175 recently collected wild isolates at least half give the *p* to *ap* segregation. The proportion of *p* to *ap* segregants varies from isolate to isolate and the genetic control of this will be discussed elsewhere (Croft, 1967). Furthermore, similar segregations of *p* and *ap* phenotypes have been observed in homokaryotic wild isolates of *A. glaucus* in this laboratory.

Neither the *p* nor the *ap* state is stable and pure breeding. All *p* and *ap* colonies give rise to both *p* and *ap* progeny, though the proportion of each type can vary. All ascospores and hülle cells, under a given set of conditions, germinate to give constant proportions of *p* to *ap* colonies, but the proportions obtained from conidia under these same conditions

vary from about 95 per cent. *p* to about 95 per cent. *ap* according to the phenotype of the parental colony from which the conidia were obtained.

This situation, with the very variable conidia and the invariable ascospores and hülle cells, supports and in part explains the observations of Jinks (1956) who, when examining a number of cytoplasmically inherited characters in *A. nidulans* and *A. glaucus*, found that the hyphal tips were the most variable, followed by the conidia with the ascospores being the least variable. The present observations may also assist in explaining the phenomenon of cytoplasmic restandardisation (Mather and Jinks, 1958). Restandardisation is shown in the present case by the constant proportion of *p* to *ap* colonies among ascospore progenies both when the ascospores were derived from a highly perithecial colony or from a colony with few perithecia. That, in this case, restandardisation is not a process connected with meiosis or ascospore formation (comparable to the *de-novo* origin of mitochondria from the nuclear membrane at the time of meiosis in *Pteridium* described by Bell and Mühlethaler (1962, 1964)) is shown by the observation that it also occurs in hülle cell progenies. Unpublished work undertaken in this department has shown that hülle cells are asexual structures associated with, but external to and independent in origin of the perithecia. Other experiments carried out in this department also showed that the pre-meiotic contents of perithecia had undergone restandardisation. Therefore, at least in the case of the present character, all the evidence suggests either that any extra-chromosomal change which occurs both precedes sexual reproduction and is shared by cell lineages which are associated with the sexual stage but are not in direct line of descent of the sexual spores, or that the sexual stage develops only in these regions of the colony which, possibly by chance segregation of extra-chromosomal material, are capable of supporting its production. It appears that hülle cells and perithecia require a similar level of cellular organisation for their production, hence the almost invariable association of these two structures in the wild-type colony.

The heterokaryon transfer experiment shows that an extrachromosomal factor is involved in the difference between the *p* and *ap* states, and that this factor can effect a change of state presumably by altering gene expression in some way. Jinks (1956) showed that, in his selection lines, high perithecial density was suppressive over low perithecial density. In the present heterokaryon transfer experiment the results are not so conclusive, but there is evidence that the *p* state is suppressive over the *ap* state. While there has been a reduction in the overall production of perithecia of *p* lines after association with *ap* lines (fig. 4*e* and *h*), there has been a much greater increase together with a very pronounced build up at the *p* end of the distributions of *ap* lines after association with *p* lines (fig. 4*f* and *g*). The short duration of the heterokaryotic association in this experiment may explain why the suppressiveness was not more complete. If the *p* state is suppressive, then the large size and hence the larger cytoplasmic volume of the hülle

cells as compared to the ascospores may explain why some *ap* colonies segregated among the ascospore progenies but none among the hülle cell progenies (see fig. 2).

From the present results it is not possible to decide whether the cytoplasmic component may be considered as an autonomous self-replicating particle, or as part of a self-perpetuating metabolic system. These two general hypotheses have been discussed elsewhere (see *e.g.* Jinks, 1963, 1964; Delbrück, 1949; Pollock, 1953; Wilkie, 1964), and they will not be considered at length here. However, the change with time from the *ap* state to the *p* state within the resting conidium is worthy of further discussion.

This change can be explained on either general hypothesis. The self-replicating particle model requires that the segregation is not for the presence or absence of the particle but for the segregation of fully operational particles from, for example, undeveloped particles. In time these undeveloped particles become fully operational. The segregation of a low number of particles below a certain threshold to give the *ap* state and the gradual build up of the number of particles above this threshold in the resting spore would also explain the results. But this would require the additional postulate that the expected aparticulate spores were inviable, because no pure breeding *ap* colony has ever been recovered. The evidence against this is that conidia from an extreme *ap* colony, which would be expected to include many with no particles, have an equal and consistently high viability as the conidia from an extreme *p* colony. This difficulty would be overcome however if a very large number of particles are involved, the probability of obtaining an aparticulate spore then being very low. Nevertheless, there is evidence that a threshold system may be operating. Examination of any of the segregation distributions obtained throughout these experiments shows there to be an excess of the two extreme classes of segregants, with very few intermediate mosaic colonies. This may be explained by assuming that upper and lower threshold values, in terms of particle numbers, are present and that the difference between these values is small. That is, the change from the *p* state to the *ap* state in terms of particle number is small and only those segregant spores with particle numbers between these threshold values would give rise to the intermediate mosaic colonies.

On the self-perpetuating metabolite model the initial segregation can be explained as the presence in or absence from the cytoplasm of a substance involved in perithecial production (*e.g.* an inducer or repressor acting upon the genes concerned with perithecial production), itself being a product of nuclear gene action. In the *ap* spores the inducer would be accumulated (or the repressor broken down) so that the spores change to the *p* state.

The experiment described in the results section (iii) shows that at a particular moment (in this case 10 days) the conidia present on a colony display hereditary differences which are revealed in the resultant

colonies as mosaics with different proportions of *p* to *ap* regions and in later conidial generations as segregation distributions varying from 95 per cent. *p* to about 95 per cent. *ap* with the same low percentage of intermediate mosaics in all cases (fig. 3). It was also shown that selected extreme *ap* parental colonies produce mainly *ap* conidia but, with time, these conidia gradually change to the *p* state. Therefore, although conidia may arise in any of a wide range of states, it appears that a population of conidia is undergoing a dynamic change from the *ap* state to the *p* state. At any one moment the population would contain all stages in this conversion process and the intermediate colonies obtained from this population would arise from those spores which, at that time, were in an unstable transitory stage between the *p* and *ap* states. The process will presumably continue to the final position of dynamic equilibrium corresponding to the spontaneous rate of origin of *ap* spores from a population of conidia in the *p* state.

Many examples of phenotypic instability have now been described and the present case has many similarities to some of these. In various *Salmonella* species it has been observed that, within a strain, the antigens associated with the flagella may be present in one or the other of two alternate phases. The spontaneous change from one phase to the other occurs with a low frequency and is reversible. The antigens of the two phases are genetically determined by two loci, *H1* and *H2* (Lederberg and Edwards, 1953) and which phase is expressed is controlled either by *H2* or by a gene closely linked to *H2* (Lederberg and Iino, 1956). There is also evidence that the phase controlling gene exerts its control over *H1* by the production of a cytoplasmic repressor. (Pearce and Stocker, personal communication). The cilia of *Paramecium aurelia* are also able to express more than one antigenic state (Beale, 1954). In this case the change from one state to another occurs as a response to changes in the external temperature but once more it is thought to be mediated by the cytoplasm. Finally, the change from the *ap* to the *p* state within the differentiated structures of the *Aspergillus* colony invites comparison to the *mi-1* (poky) maternally inherited mutant of *Neurospora crassa* (Mitchell and Mitchell, 1952) where a gradual "recovery" from the mutant state to one approaching wild-type has been shown to occur (Silagi, 1965).

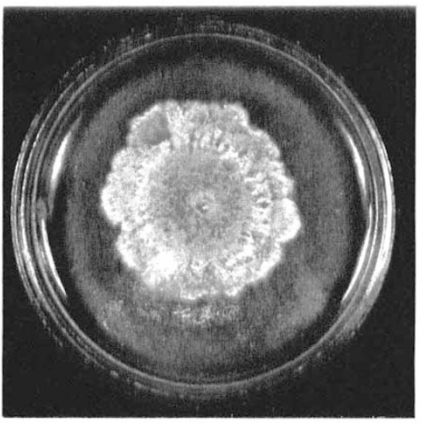
5. SUMMARY

1. A haploid homokaryotic isolate of *Aspergillus nidulans* shows an alternation between two phenotypic forms.

2. One form (the *p* state) produces abundant perithecia; the other (the *ap* state) has no perithecia.

3. In this isolate each state reverts to the other with a low frequency, about 5 to 10 per cent. of the conidia of a 10-day old colony being in the alternative state in each case.

4. Resting conidia in the *ap* state gradually become converted to the *p* state, the 10 per cent. or less reversion in 10-day old conidia becoming 60 per cent. or more in 30-day old conidia.



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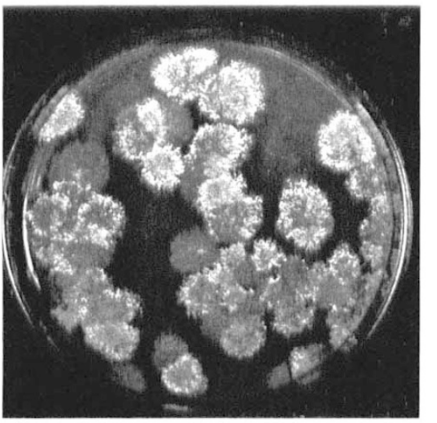

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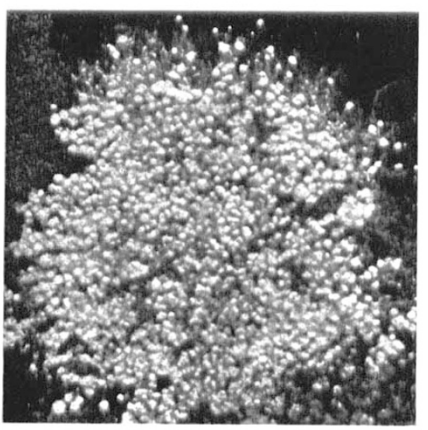
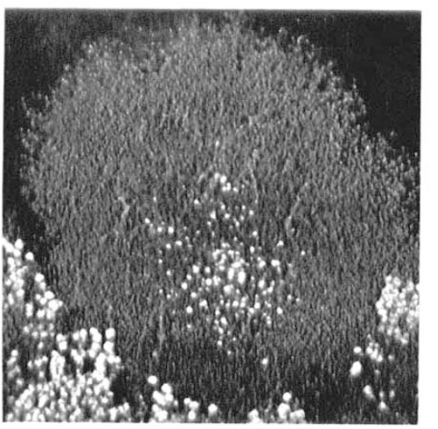


(*c*)



(*d*)

Plate 1

(*a*) Colony of isolate 37 in *p* state. Note *ap* sector arising near edge of colony.
(*b*) Sample plate showing the segregation of *p* and *ap* colonies among conidial progeny.
(*c*) and (*d*) *p* and *ap* colonies, both from the same plate as illustrated in (*b*). Each colony was grown from a single conidium both of which were derived from one colony, itself grown from a single conidium.

5. A heterokaryon transfer test showed that a cytoplasmic component is involved in the inheritance of the two states.

6. The evidence does not allow us to decide between considering the cytoplasmic component as an autonomous self-replicating particle or as part of a self-perpetuating metabolic system.

Acknowledgments.—I wish to thank Professor J. L. Jinks for continued advice and criticism, Dr Ursula B. Pearce for her critical reading of the typsecript and Miss Lynn Williams and Mrs F. E. Robinson for their technical assistance. Part of this work was carried out while the author was in receipt of an Agricultural Research Council Studentship.

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