A STUDY OF ASPECTS OF HETEROKARYOSIS IN ASPERGILLUS NIDULANS *

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(With 16 tables, 7 plates, 1 figure, 1 diagram and 1 graph)

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CONTENTS

SECTION I — INTRODUCTION

Heterokaryosis is a wide-spread phenomenon among the filamentous fungi. Heterokaryosis doubtless has a role in natural populations of fungi, although the extent and significance of this role are not yet known. In the laboratory, heterokaryosis has been exploited in diverse studies which include the following: crossing in homothallic species, the parasexual cycle, inter-and-intra-genic complementation and factors affecting heterokaryon compatibility.

There remain a number of features of heterokaryosis which are not yet well explored. Heterokaryons of *A. nidulans* (as opposed, for example, to those of *Neurospora crassa*) consist of populations of hyphae with a range of nuclear ratios. The maintenance of a constant overall nuclear ratio in one environment, and the adjustment of this ratio on a change of environment, depend on interhyphal selection CLUTTER-

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BUCK and ROPER, (1966). Adaptation of nuclear ratios of heterokaryons, on environmental change, has been shown already by JINKS (1952) for *Penicillium* and by WARR and ROPER (1965) for *Aspergillus*. The former study relied on genetically undefined, differential growth rates on various media and the latter on selection for a semi-dominant allele which conferred resistance to an inhibitor. One aim of the present investigations was to look for adaptation using various, genetically defined differences between the component haploid strains. This necessitated, as a preliminary, a study of the stability, from culture to culture, of the nuclear ratio in a heterokaryon.

Heterokaryons differ from heterozygotes in two essential respects:

a) the former are flexible in their allele ratios while the latter are fixed at 1 : 1 and

b) the former have the haploid genomes separated by cytoplasm and nuclear membranes.

Comparisons of the phenotypes of heterokaryons and corresponding heterozygotes have been made already in some cases (ROBERTS, 1963; APIRION, 1966) and has been the subject of speculation by ROBERTS (1964) and PONTECORVO (1963). The comparison offers hope of finding situations in which the following factors are important:

1) allele ratio and 2) the inclusion of alleles in a common nucleus. The second of these offers attractive scope for speculation though differences suspected on this basis must be treated with caution. For example, any difference in phenotype between a heterokaryon with a 1 : 1 nuclear ratio and a heterozygote might be due to a function of the nuclear membrane or to cytoplasmic separation of the alleles, including the non-random distribution of nuclei in a heterokaryon. Despite any difficulties in interpretation, comparisons of heterokaryons and heterozygotes offer one approach to what might be called "sub-cellular functional topography".

It was hardly to be expected that definitive results would be achieved within the necessarily limited scope of the present study. The main aim was to investigate a few heterokaryons and heterozygotes so as to define some of the problems involved in this kind of study; at the same time, it was hoped that it might be possible to expose aspects of gene action which could lend themselves to further study along the above lines.

1. EARLY OBSERVATIONS

Heterokaryosis is a condition enjoyed only in fungi and a great number of them display a heterokaryotic phase during their life cycle.

Since the pioneer work by BURGEFF (1912, 1914), a multinucleate conidium or a single hypha is regarded as a heterokaryon if it contains at least two genetically different nuclear types.

HANSEN and SMITH (1932) have studied heterokaryosis of *Botrytis cinerea* taking this criterion and, for the first time, a heterokaryon was synthesized in a laboratory when they were able to rebuild, from the homokaryotic constituents, a heterokaryon similar to the original.

Working with *Neurospora tetrasperma*, DODGE (1942) abandoned the early taxonomic interest devoted to heterokaryosis and showed the ability of two slow-growing mutants to produce heterokaryotic vigour (to complement one another). His paper was not only the first to show complementation but the beginning of physiological studies of heterokaryons.

Two years later, BEADLE and COONRADT (1944) took an important step further: using well-characterized nutritional mutants of *Neurospora crassa*, they synthesized balanced heterokaryons. Since there was complementation in the heterokaryotic condition, it was evident that genes do not need to be in the same nucleus to have a joint action in biosynthesis; a cytoplasmic connection is sufficient.

JINKS (1952) stressed the importance of heterokaryons in providing a flexible system which, through the adjustment of nuclear ratios, could adapt to various and varying environments.

Elucidation of the parasexual cycle opened new possibilities in various fields. Apart from the value of this cycle in genetical analysis, the discovery of a relatively stable diploid phase opened the way to a comparison of heterozygote and heterokaryon. Heterokaryosis, itself, was also seen as an essential step in the parasexual cycle.

2. MECHANISM OF FORMATION

The phenomenon of anastomosis has been known since DE BARRY (1884; English translation DE BARRY 1887), but for a long time it was not associated with heterokaryosis. Hyphal fusion and the ability to form a heterokaryon are determined by the genetic constitution of the parents and also influenced by environmental conditions.

A heterokaryon may originate by anastomosis of adjacent hypha, and migration of nuclei from one hypha into another. A more frequent source of heterokaryosis is that derived by mutation in one or more nuclei within a homokaryotic mycelium. Unless the mutant nuclei enjoy selective advantage, they will not achieve a readily detectable proportion of the total. In fact, any colony of a filamentous fungus is certain to contain at least a small proportion of newly-arisen mutant nuclei. In certain species, such as *Neurospora tetrasperma* and *Podospora anserina*, heterokaryosis is brought about as a consequence of the inclusion of non-identical nuclei within a single cell on the occasion of ascospore formation.

3. ADJUSTMENT OF NUCLEAR RATIOS IN HETEROKARYONS

In Ascomycetes, heterokaryosis has been studied most closely in *Aspergillus nidulans* and *Neurospora crassa*. The two species differ markedly in their behaviour.

The nuclear ratio in a heterokaryon of *N. crassa* can generally be established by the proportions of nuclei used in the initial inoculum (ATWOOD and PITTENGER, 1955). This ratio is maintained during growth even when the ratio is such as to permit less than optimal growth rate. In *Aspergillus nidulans*, a pair of strains establish a ratio which is independent of the conidial ratio used in the inoculum and which is probably a function of the nutritional requirements and other characteristics of the two strains. Apart from certain exceptional situations, the nuclear ratio of a *Neurospora* heterokaryon remains unaltered when the environment is changed. On the other hand, in *Aspergillus* the ratio can be altered readily by appropriate environmental changes.

In establishing the above facts the conidial ratio has been taken as a measure of the nuclear ratio. In fact it may be a very defective measure. At least in *A. nidulans*, a heterokaryon will generally be a dynamic mixture of heterokaryotic hyphae and two types of homokaryotic hyphae. The main exceptions are to be expected when the intermediates involved in heterokaryotic complementation are non-diffusible so that little homokaryotic growth is supported by excretion of nutrients from the heterokaryon. Generally speaking, the conidial ratio will reflect only the overall ratio of the dynamic system. However, even if the conidial ratio gives no exact measure of nuclear ratio, a *change in conidial ratio* will generally reflect a *change in nuclear ratio.*

To overcome the difficulty of indirect determination of nuclear ratios, CLUTTERBUCK and ROPER (1966) used a cytological approach. In heterokaryons with diploid and haploid nuclei they determined ratios on nuclear size difference. This study explained the difference in behaviour between *Aspergillus* and *Neurospora*. The effective growing unit in *Aspergillus* is the hyphal tip which contains 50 to 100 nuclei. The nuclei are not in rapid movement and, in a heterokaryon, there is scope for great variation of nuclear ratio from one tip to another. Selection operates by favouring those tips with the most advantageous ratios. This process forms also the basis of normal heterokaryon maintenance and explains why a particular ratio is established regardless of inoculum ratio. In *Neurospora*, the growing tip is about 1 cm (RYAN, BEADLE and TATUM, 1943) and the nuclei are in rapid circulation. There is little scope for variation of ratio from one "metabolic unit" to another and so little chance of change of nuclear ratio.

Previous work (REES and JINKS, 1952) had suggested differential nuclear division as a mechanism of change of nuclear ratio, on the basis of their studies in *Penicillium*. The known synchrony of division within a hypha makes this an unlikely alternative to the process of hyphal selection.

4. INCOMPATIBILITY

Heterokaryon incompatibility is well established in *Neurospora* (HOLLOWAY, 1955; GARNJOBST and WILSON, 1956) and has been shown also in *Aspergillus* (GRINDLE, 1963). The strains used in this study were all derived from a single original stock and complete incompatibility,

as opposed to occasional poor heterokaryotic growth, has never been observed. Although incompatibility must be born in mind in any study of heterokaryosis it seems unlikely to have immediate relevance to the present work.

SECTION II — MATERIAL AND METHODS

1. Organism and strains

Aspergillus nidulans (Eidam) Winter, the imperfect stage of *Emericella nidulans*, belongs to the Family *Eurotiaceae*, Order *Eurotiales* and Class *Ascomycetidae*, Martin (1961).

In the genus *Emericella* no sex organs are formed but, from a hyphal coil, originates the cleistothecium. (In *Aspergillus* genetical analysis this ascocarp is usually referred to as perithecium by analogy with *Neurospora*). When mature, the cleistothecium is dark and covered with thick-walled cells, "Hulle cells". The asci are evanescent and the ascospores, when fully mature, are red.

In the conidial stage of *Aspergillus* the somatic hyphae produce conidiophores inflated at the tips (vesical) which give rise to the sterigmata. From the secondary layer of sterigmata originate the conidial chains. The conidia are uninucleate (Yuill, 1950).

The characteristics of the life cycle and morphology of this homothalic and saprobe species are described by Thom and Raper (1945).

The strains used were all derivatives of a wild-type *Aspergillus nidulans* isolated by Yuill (1939). The mutant alleles and their locations are given by Pontecorvo, Roper, Hemmons, MacDonald and Bufton (1953), Roper and Käfer (1957) and Käfer (1958). They originated from the Glasgow stocks and are maintained in Sheffield. These strains and their genetic characters are listed in Table 1.

Stocks of strains were preserved at 5°C on complete medium slopes. Samples for immediate use were maintained at room temperature. Before use a monospore colony of each strain was obtained by low density plating and was characterised by auxanography.

Symbols of mutant alleles and the phenotype they determine are shown in Table 2. The linkage groups of the various markers used in this work are shown in Figure 1.

The genetic nomenclature and strain designations used have appeared in previous publications, otherwise they follow the recommendations of Demerec, Adelberg, Clark, and Hartmann (1966).

Convention to express: Heterokarion: + to separate components.
Diploid: // to separate haploids in diploid.
Cross: × to separate the mates in a cross.

TABLE 1

STRAIN LIST

Genotype	Origin and reference
ad8 paba1	Recombinant from cross *paba1 y; co* × *ad8 bi1*
bi1; Acr1	ROPER and KÄFER, 1957
bi1; Act1; nic8	Pedigree unknown
paba1 y; Acr1; co	ROPER and KÄFER 1957
pro1 bi1	U. V. irradiation of *bi1*
pro1 paba6 y; w3	Recombinant from cross *paba6 bi1* × *pro1 y; w3*
pro1 y; Acr1	Recombinant from cross *y; Acr1 ad3* × *an1 pro1 bi1; w3; s12; pyro4*
ribo1 pf21 y; nic8	WARR and ROPER 1965
y; pyro4	Recombinant from cross *y; s12* × *bi1; pyro4*
y; pyro4; ribo5	U. V. irradiation of *y; pyro4*

The genotype of "Master strain" MSE (MCCULLY and FORBES 1965) is:

y su1 ad20; w3; gal1; pyro4; facA; s3; nic8; ribo2

All strains were translocation-free.

TABLE 2

LIST OF MUTANTS

Symbol		Phenotipe	Linkage group
i.	Visible		
	co	compact colony type, reduced growth rate.	VIII R
	sm	small colony type, reduced growth rate.	VI
	fw	yellowish brown conidial pigment, interacts with *y and w3*, recessive.	V
	w3	colourless conidia, epistatic to *y* and *y+*	II L
	y	yellow conidial pigment	I R
ii.	Resistant		
	Acr1	resistant to acriflavine, semi-dominant.	II L
	Act1	resistant to actidione, semi-dominant,	III L
	pf21	resistant to DL-p-fluorophenyl-alanine	I L

iii. Nutritional

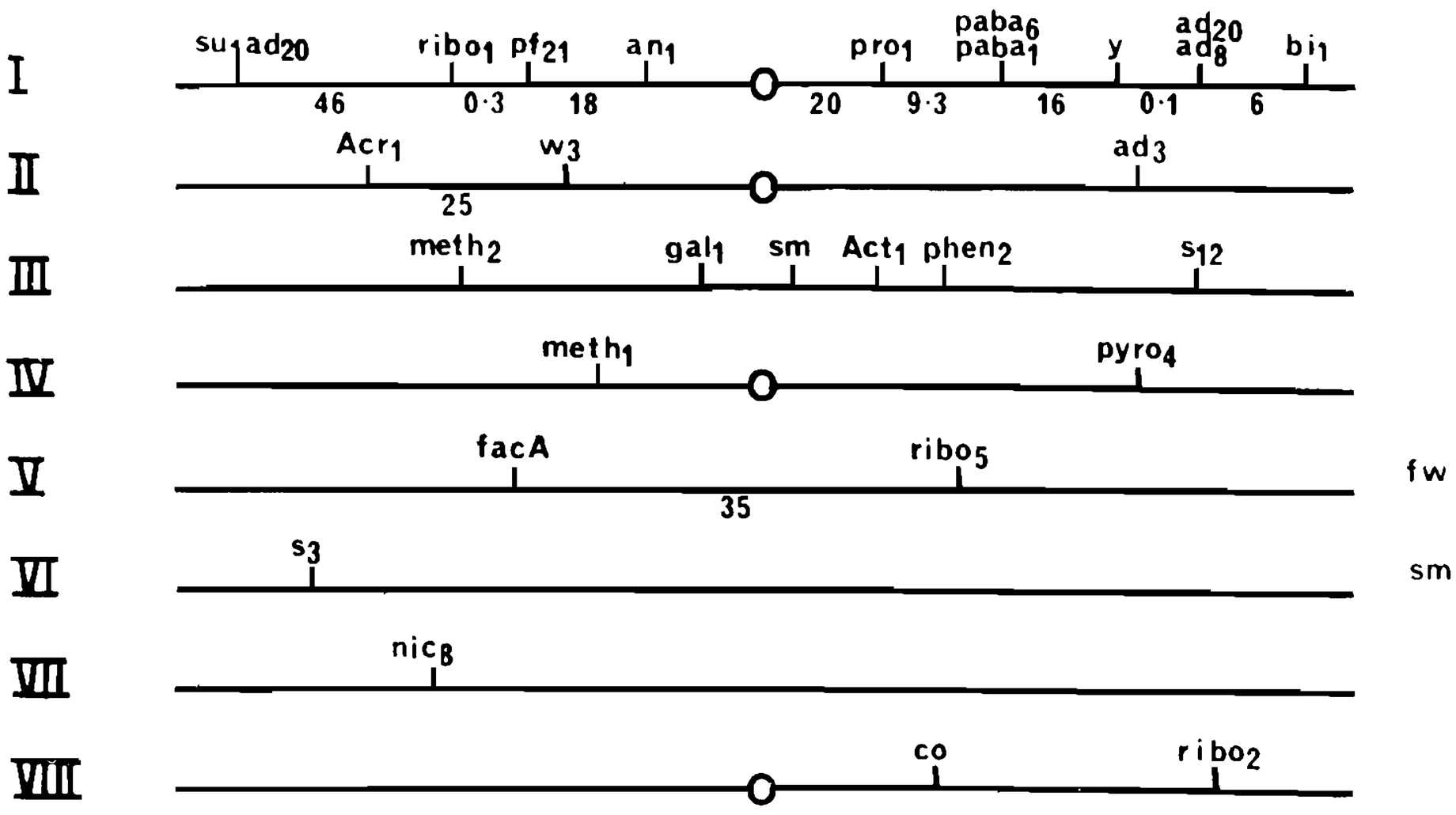
ad3	requirement for adenine	II R
ad8	" " "	I R
ad20	" " "	I R
an1	" " thiamine (aneurin)	I L
bi1	" " biotin	I R
nic8	" " nicotinic acid	VII
paba1	" " p-amino-benzoic acid	I R
paba6	requirement for p-aminobenzoic acid	I R
pro1	requirement for proline	I R
pyro4	" " pyridoxine	IV R
ribo1	" " riboflavin	I L
ribo2	" " "	VIII R
ribo5	" " "	V
s3	growth response to sulfite	VI
s12	" " " "	III R
facA	inability to utilise acetate	V
gal1	inability to utilise galactose	III L

iv. Suppressor

su1 ad20	suppressor of *ad20*	I L

FIGURE 1

Partial Linkage Map of *Aspergillus nidulans*



Map distances are not to scale. The recombination frequencies are approximate. The symbols at the right of a linkage group are the ones located to them during the present work.

2. Media

2.1. *Liquid minimal medium (MM). Modified Czapek-Dox.*

Sodium nitrate	6 g.
Potassium chloride	0.52 g.
Magnesium sulphate ($7H_2O$)	0.52 g.
Potassium dihydrogen phosphate	1.52 g.
Ferrous sulphate	small crystal
Zinc sulphate	small crystal
D-glucose	10 g.
Distilled water q.s.p.	1000 ml.

The pH was adjusted to 6.5 with sodium hydroxide and hydrochloric acid.

2.2. *Solid minimal medium*

Agar	15 g.
Liquid minimal medium	1000 ml.

2.3. *Solid complete medium (CM)*

Bacteriological peptone (Oxoid)	2 g.
Hydrolysed casein (Oxoid)	1.5 g.
Yeast extract (Oxoid)	0.5 g.
Yeast nucleic acid hydrolysate *	2.5 ml.
Vitamin solution **	1.0 ml.
Liquid minimal medium	1000 ml.
Agar	15 g.

The pH was adjusted to 6.5 as above.

* *Yeast nucleic acid hydrolysate*

2 g of yeast nucleic acid (Oxoid) were hydrolysed in 15 ml of N HCl for 20 minutes, at 100°C. The procedure was repeated with the same amount of yeast nucleic acid (Oxoid) and N NaOH. The two hydrolysates were mixed, the pH adjusted to 6, and then filtered hot. The volume of the solution was adjusted to 40 ml and kept in a dark bottle, with chloroform as a preservative, at 5°C.

** *Vitamin solution*

p-Aminobenzoic acid	10 mg
Pyridoxin HCl	50 mg
Thiamin HCl (aneurin)	50 mg
Nicotinamide	100 mg
Riboflavin	100 mg
Biotin	0.2 mg
Distilled water q.s.p.	100 ml

After it had been sterilised the solution was kept in a dark bottle, at 5°C.

2.4. *Liquid minimal medium plus 2% of complete medium*

2.5. *Acetate medium*

Ammonium acetate	12 g
Liquid minimal medium without glucose	1000 ml
Agar	15 g

2.6. *Galactose medium*

Galactose	10 g
Liquid minimal medium without glucose	1000 ml
Agar	15 g

Autoclaving

Media were sterilised by autoclaving at 10 lb. pressure, for 10 minutes and stored at room temperature. The same applied to saline and tween solution.

2.7. *Saline*

0.89% solution of sodium chloride

2.8. *Tween 80*

0.1% of Tween 80 in water (v/v)

2.9. *Supplement for nutritional mutants*

MM supplemented as follows:

Substance	*Final concentration*
Amino acid	
Proline	4.4×10^{-4}M
Nucleotide	
Adenine	1.1×10^{-3}M

Substance	*Final concentration*
Vitamins	
Nicotinamide	400 µg/l.
Biotin	50 µg/l.
Pyridoxin HCl	200 µg/l.
p-Aminobenzoic acid	200 µg/l.
Riboflavin	400 µg/l.
Thiamin HCl	100 µg/l.
Inorganic salt	
Sodium thiosulphate	10^{-3}M

2.10. *Inhibitors stock solutions*

	(weight for volume)
Actidione (Cycloheximide, Koch Light Lab.)	4 g% w/v
Acriflavine (British Drug Houses) solution I	1 g%
Acriflavine (B.D.H.) solution II	100 mg%
DL-p-fluorophenylalanine (Koch Light Lab.)	1 g%

All drugs used were within the specifications for bacteriological laboratory use.

3. Methods

General cultural conditions and procedures used throughout this work were those of Roper (1952), Jinks (1952), Pontecorvo et al. (1953), Pontecorvo *and* Käfer (1958), Lhoas (1961) and McCully and Forbes (1965).

Incubation was at 37°C. (± 0.5) unless otherwise stated.

3.1. *Spore plating*

Conidia were transferred from a colony to tubes with 2 ml of Tween 80 solution. Conidial chains were broken by repeated agitation. A haemocytometer count was made, then serial dilutions were made by pipetting a known volume of suspensions into 9 ml of sterile saline in universal containers. An appropriate final density of the suspension was chosen as required — about 20 colonies per plate in order to get isolated colonies and 100 colonies per plate for colour score. 0.1 ml samples of this suspension were spread on solid media with a sterile glass rod.

3.2. *Auxanographic technique*

Conidia from a colony derived from a single spore were suspended in saline or Tween 80 solution. Conidial chains were broken by agitation and about 10^5 conidia were suspended in 25 ml of molten MM held at 45°C. The medium was poured into a Petri dish. Small crystals of

growth requirements were spotted at well separated marked points on the surface of the agar. After 24 hr. incubation, the response of the strain to the growth factors was indicated by zones of growth.

3.3. *Ultraviolet*

Conidia were suspended in 10 ml of saline (10^6/ml). The suspension was transferred to a petri dish of 9 cm. diameter. Irradiation treatment was carried out with the lid removed from the petri dish. The source of U. V. was British Thermal Syndicate mercury discharge vapour lamp, T/M5/369 (99% of radiation at 2537 Å, intensity 120 microwatts per square cm). Treatments were from 60 to 90 seconds giving 0.5 to 2.5% survival of conidia. Following treatment and appropriate dilutions, the suspension was plated onto CM. Morphological mutants ("small") were selected visually and limited to those differing from wild-type in colony size but not in, for example, pigmentation or other obvious characteristics.

3.4. *Heterokaryons*

Pairs of strains used to prepare balanced heterokaryons, differed from each other, and from wild-type, in nutritional requirements; members of each pair differed always in conidial colour and sometimes also in morphology and/or drug resistance.

About 10^6 conidia of each of two strains were inoculated together into 2 ml of liquid MM enriched with 2% of CM (the latter provides a small growth essential for hyphal anastomosis). After agitation it was incubated. 3 or 4 days later the mycelial mat was removed and teased out onto MM. After 3 further days of incubation heterokaryotic growth was sought. The ability to grow well on MM, the juxtaposition of the conidial heads of different colour, and furthermore, the presence of "mixed conidial heads" were the characteristics used to indicate heterokaryosis.

When a pair of strains carried the same allele for a particular nutritional requirement, appropriately supplemented MM was used and balance was maintained by other requirements for which the strains differed.

Subculture of heterokaryon was obtained by transferring approximately 0.2 cm. sq. of the heterokaryon, with the minimum of medium possible, onto another MM plate.

3.5. *Isolation of diploids*

Heterozygous diploids were obtained using Roper's technique (Roper, 1952). Up to 10^7 conidia from a balanced heterokaryon were collected and plated onto MM plates, or MM supplemented with the nutrient when necessary. Diploid colonies were recognised after 3 or more days' incubation. Resynthesis of heterokaryon on the MM plates

occurred in some cases but the heterokaryotic colonies were distinguished readily from the heterozygous diploid colonies. Diploids isolated by this technique were purified by plating on MM, and stored on MM slopes, suplemented as necessary.

3.6. *Analysis via the parasexual cycle*

Plating of green diploids, heterozygous for *w3* and *y*, on CM gave rise to white and yellow sectors. Some of these were diploids (as judged by conidial size) formed by mitotic crossing-over. The rest were haploids. These arise by breakdown of diploid nuclei involving random assortment of the members of each pair of homologous chromosomes (PONTECORVO, TARR-GLOOR and FORBES, 1954). Another procedure for haploidization was later used by incorporating the amino-acid analogue DL-p-fluorophenylalanine into CM, to give a final concentration of 1/10000 w/v (LHOAS, 1961). Diploid conidia were plated on this medium at a density of about 8 per plate. After 7 days' incubation the haploid sectors produced were purified by streaking on CM. Ploidy was checked by measuring chains of 5 conidia.

It is known that somatic segregation induced by DL-p-fluorophenylalanine involves only whole chromosome segregation (MORPURGO, 1961) and haploidization almost invariably occurs without mitotic crossing-over so that all the genes on one chromosome segregate as a unit. Consequently mutant genes can be located to a particular linkage group by using diploids synthesized between a mutant strain and a tester strain with mutant markers on all eight linkage groups.

Haploids were then classified for growth requirement, drug response or other markers in the usual manner (see below).

3.7. *Meiotic analysis*

Heterokaryons were established as described previously and, after 3 or 4 days incubation, the dishes were sealed with cellulose tape. This is known to favour perithecial formation, possibly by causing partial anaerobiosis. After 6 further days' incubation, perithecia had usually been formed and were mature. For ascospore plating it was necessary to clean off mycelium, conidia and "Hulle cells" from the perithecial wall, by rolling the ascocarp on a 3% agar. Single perithecia were then transferred to the side of a test tube containing 1.5 ml of saline. The perithecia were crushed with a pipette. After washing the ascospores from the side of the test tube and making the suspension homogeneous, a drop of each was streaked onto CM, and incubated. The remaining portions were stored in a refrigerator. Since crosses were always made between strains carrying different coloured conidia, examination of the dishes after two days' incubation indicated whether the perithecia were hybrid or selfed. After a perithecium was shown to be a hybrid, the density of ascospores in the stored suspension was estimated by haemocytometer count and the appropriate dilution made and plated out on

CM. Within 48 hr. the colonies were conidiated and could be scored for colour segregation.

The nutritional requirements of colonies derived from the ascospores were usually tested by using a multi-point replicator (ROBERTS, 1959). 26 segregants were transferred to dishes of CM in a 5 × 5 + 1 formation (the pattern of the replicator pins) and incubated for 2 days. Conidia were then replicated from the master plate onto suitably supplemented media. Each plate with all the nutrients required by either parental strain, but lacking in turn a single growth factor for which classification was being undertaken.

Growth response was normally observed after 24 hr. for nutritional requirements, and 48 hr. for resistance to drugs.

The list given in 2.9 shows the nutritional requirements and their final concentrations used in MM.

Classification for *Acr1*, *Act1* and *pf21* mutant alleles was by replication in MM supplemented with all nutrients corresponding to every requirement in the cross, plus 20 mg/l of acriflavine, 0.8 g/l of actidione and 0.6 g/l of DL-p-fluorophenylalanine respectively.

SECTION III — RESULTS

1. HETEROKARYONS ON COMPLETE MEDIUM

1.1. JINKS (1952) found, in a *Penicillium* heterokaryon, that partial supplementation altered the rate of growth and the conidial ratio; in *Aspergillus*, CLUTTERBUCK (1965) studied heterokaryons formed on CM between strains carrying non-allelic mutations determining slow growth rate. The heterokaryons showed an excellent balance with a fine mosaic of conidial heads of the two types and a high proportion of mixed heads. Compared with heterokaryons maintained on MM by nutritional balance, there appeared to be little, if any, homokaryon. This may have been due, at least in part, to the non-diffusibility of the gene products involved in the complementation between strains. Such a system would have advantages for the study of changes in nuclear ratio produced, for example, by addition of an inhibitor to which only one component was resistant. The change in ratio would be likely to reflect a change *within* the heterokaryotic hyphae rather than a possible change in the balance of homokaryons to heterokaryon. In an attempt to explore this possibility a series of slow-growing mutants was sought.

1.2. *Isolation of mutants "small"*

Induction of mutants:

The strain *bi1; Act1; nic8*, translocation free, was irradiated as described in 3. METHODS.

Approximately 50,000 conidia, in all, had been treated in four different U.V. irradiation procedures, with 4% survival. Among the survivors only 15 colonies had normal sporulation and reduced growth rate but no other conspicuous abnormality in phenotype. After three consecutive spore platings on CM, the 15 supposed mutants could still be distinguished easily from the wild-type by their colony size, showing that the slow growth rate was stable on subcultures.

1.3. *Heterokaryon test*

Heterokaryons were forced between each of the 15 isolates and MSE, on MM + 2% of CM supplemented with nicotinic acid. The mycelial mats were teased out on MM supplemented with nicotinic acid. From the heterokaryons obtained, samples of conidia were plated on CM. Among the 15 mutants with slow growth rate, only five gave clear evidence that the determinant of "small" was caused by effects associated with the nucleus.

It would be surprising if 10 out of 15 small variants arose as a result of cytoplasmic, rather than genic, change. The results could be explained by instability of the small variants — instability arising by a high rate reversion. There was insufficient time to study the 10 possibly non-genic small variants and their study did not constitute part of the present programme. The heterokaryon test did indicate — and this was the essential point — that they were unsuitable for this work.

1.4. *Genetic analysis of the small variants*

The five small variants selected for further study were then subjected to meiotic analysis.

Each "small" strain was crossed to MSE. Four crosses yielded results which suggested that the genetic determination of the small phenotype could not be ascribed simply to mutation in a single gene. In all four cases a range of morphological types was obtained from the crosses, and attempts to classify into the two parental types, normal and small, were unsuccessful. Translocations in the "small" variants might offer an explanation of these results but no attempt was made to confirm this suggestion.

Only one cross, *bi1; Act1; nic8; sm10* × MSE, gave a sharp meiotic segregation (Plate 1 and Table 3).

To assign *sm* to a linkage group, haploids were obtained, on CM supplemented with p-fluorophenylalanine, from the diploil *bi1; Act1; nic8* // MSE. Only morphologically normal haploids were obtained; this was not surprising as the technique favours selection of these as opposed to slow-growing types. A total of 35 haploids were tested for all segregating markers except that determining nicotinic acid requirement; the presence of *nic8* in both parental strains prevented the determination of the segregation of the markers of linkage group VII. Of the 35 putative haploids one, a fluffy, unstable type was discarded as a pro-

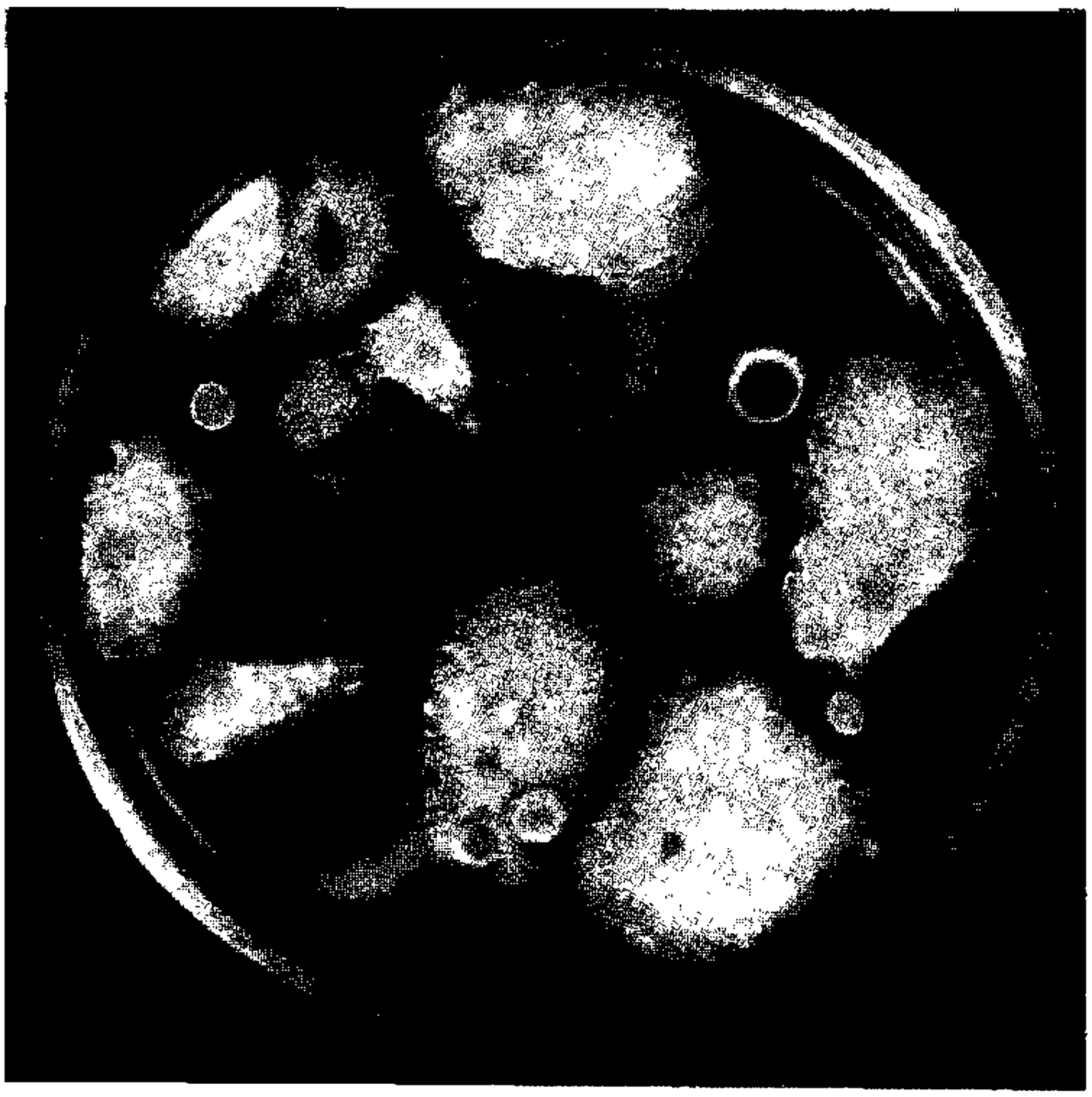


Plate 1. Colonies from a plating of meiotic segregants of the cross: *bi1; Act1; nic8; sm10* x MSE

TABLE 3

MEIOTIC ANALYSIS OF "SMALL"

Total number of ascospores plated: 674					
Small 335			Normal 339		
Green	yellow	white	Green	yellow	white
95	74	166	80	81	178

The colour factors segregated independently showing a proportion of 175 green: 155 yellow: 344 white. Colour and morphological characters segregated independently:

$\chi^2 = 4.6$
$P = 0.5$
} Fit to 1 : 1 : 2 : 1 : 1 : 2 ratio

The ratio of small : normal is 1 : 1.

$\chi^2 = 0.4$
$P = 0.55$
} Fit to 1 : 1 ratio.

bable aneuploid. The other 34 haploids, confirmed as such by conidial size, contained, collectively, the wild type and mutant alleles of all the tested segregating genes except for the *s3* marker; all 34 (14 white, 12 yellow and 8 green) required thiosulphate for growth. The mutant allele *sm* was assigned to linkage group VI. This was confirmed later by analysis of haploids derived from the diploid *bi1; Act1; nic8; sm10 //paba1 y; Acr1; co*.

1.5. *Heterokaryons on CM*

The rationale in the use of non-allelic slow-growing mutants, as heterokaryons on CM or MM, was as follows. It was hoped to synthesize heterokaryons in which the homokaryotic components were present in very limited proportion. It would have been possible, then, to follow changes in nuclear ratio *within* the heterokaryotic hyphae. On CM there would presumably be no nutritional limit on the ultimate ratio though there might have been effects of growth rate determined by disadvantageous ratios of the alleles determining growth rate. Successful production of such heterokaryons could have opened another possibility. It was hoped to use morphological mutants which could be distinguished by their growth form, perhaps in the growth rate of their hyphae or in branching pattern or other characteristic. Microscopic examination of thin-layer heterokaryons might have made it possible to distinguish hyphae which were heterokaryotic from those which were apparently completely or nearly homokaryotic for one or other component. This could then have been confirmed by isolation and growth of individual hyphae and test of the conidia they produced. This was seen as a possible approach to a study of intergenic complementation and the distance over which it can operate for certain classes of genes concerned with growth rate.

Two heterokaryons were prepared on MM:

a) *bi1; Act1; nic8; sm10* + *paba1 y; Acr1; co*

b) *bi1; Act1; nic8* + *paba1 y; Acr1; co*

The two slow growing strains, *bi1; Act1; nic8; sm10* and *paba1 y; Acr1; co*, had linear growth rates on CM of about 15% and 50% respectively compared with wild - type (Plate 2).

Heterokaryon (a) gave a poor balance on CM. This may have been due to the substantial difference in their relative growth rates which precluded a fine balance. Heterokaryotic sectors were seen emerging from the central inoculum which appeared to contain, predominantly, the yellow, compact component. Conidia sampled from the heterokaryotic sectors on CM were scored as 69% green small and 31% yellow compact.

On MM, heterokaryon (a) showed an unusual and very distinctive growth form (Plate 3). It consisted of "islands" of heterokaryotic

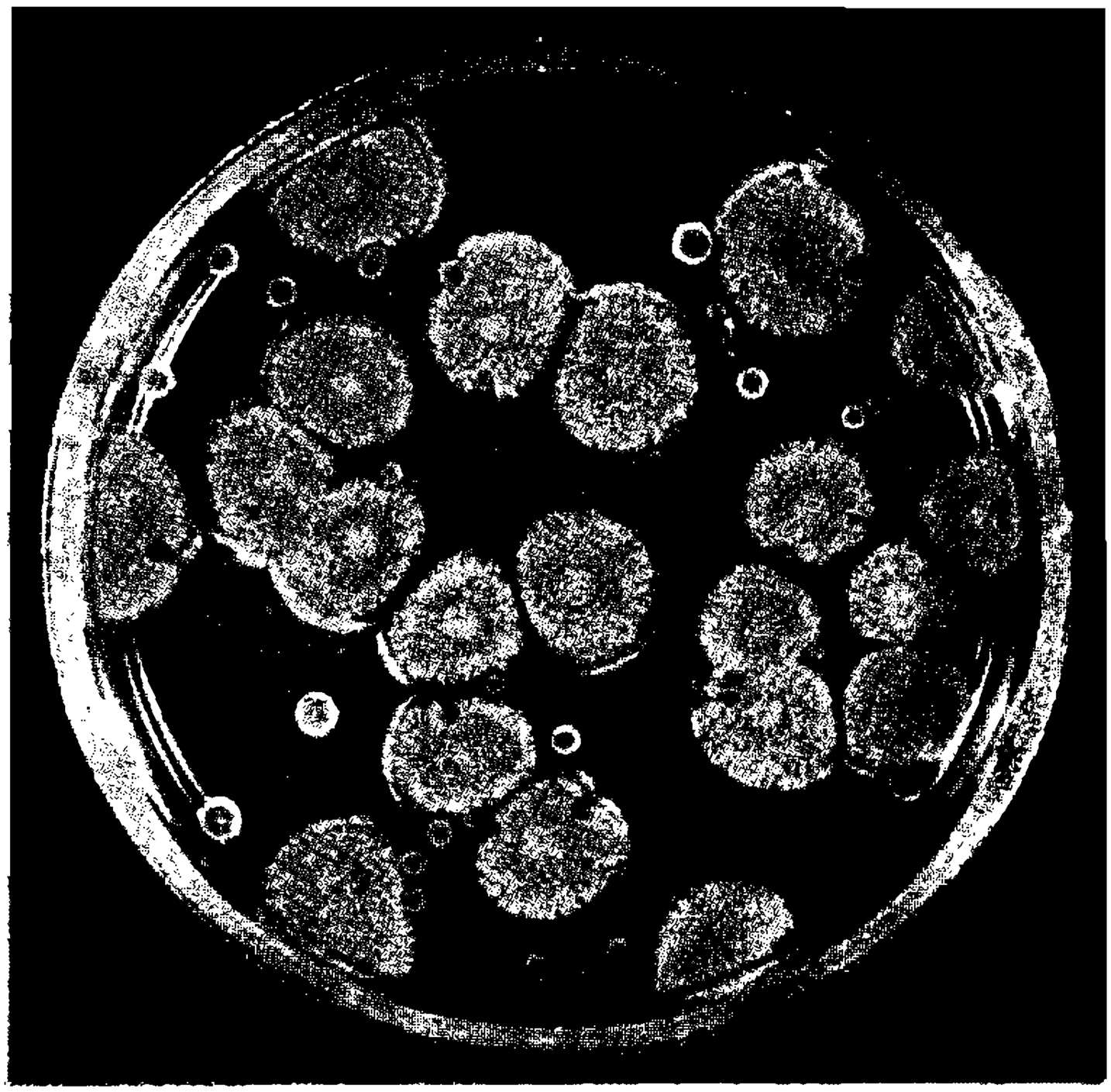


Plate 2. Morphology of small and compact strains on complete medium

growth; microscopically these could be seen to be connected by relatively sparse hyphae. The conidial ratio determined from the heterokaryotic "islands" was 84% green small and 16% yellow compact.

Both on MM, and on CM (where nutritional restraints were presumably removed), the compact strain required a high proportion of *co* $^+$ alleles for its maintenance in vigorous growth. On MM it seems likely that few hyphae had a ratio permitting good growth and this would explain the "islands" connected by sparse hyphae.

Heterokaryon (b) was studied as a control. It showed good balance on MM and carried 95% green and 5% yellow, compact conidia. This confirmed the need of a high proportion of *co* $^+$: *co* nuclei.

In view of the substantial difficulties encountered in the production of genetically defined slow-growers, and the problem of establishing satisfactory heterokaryons, this line of investigation was suspended.

2. Heterokaryon Stability

Since the attempt to use heterokaryons balanced by growth mutants on CM had failed, the interest moved to those balanced on MM.

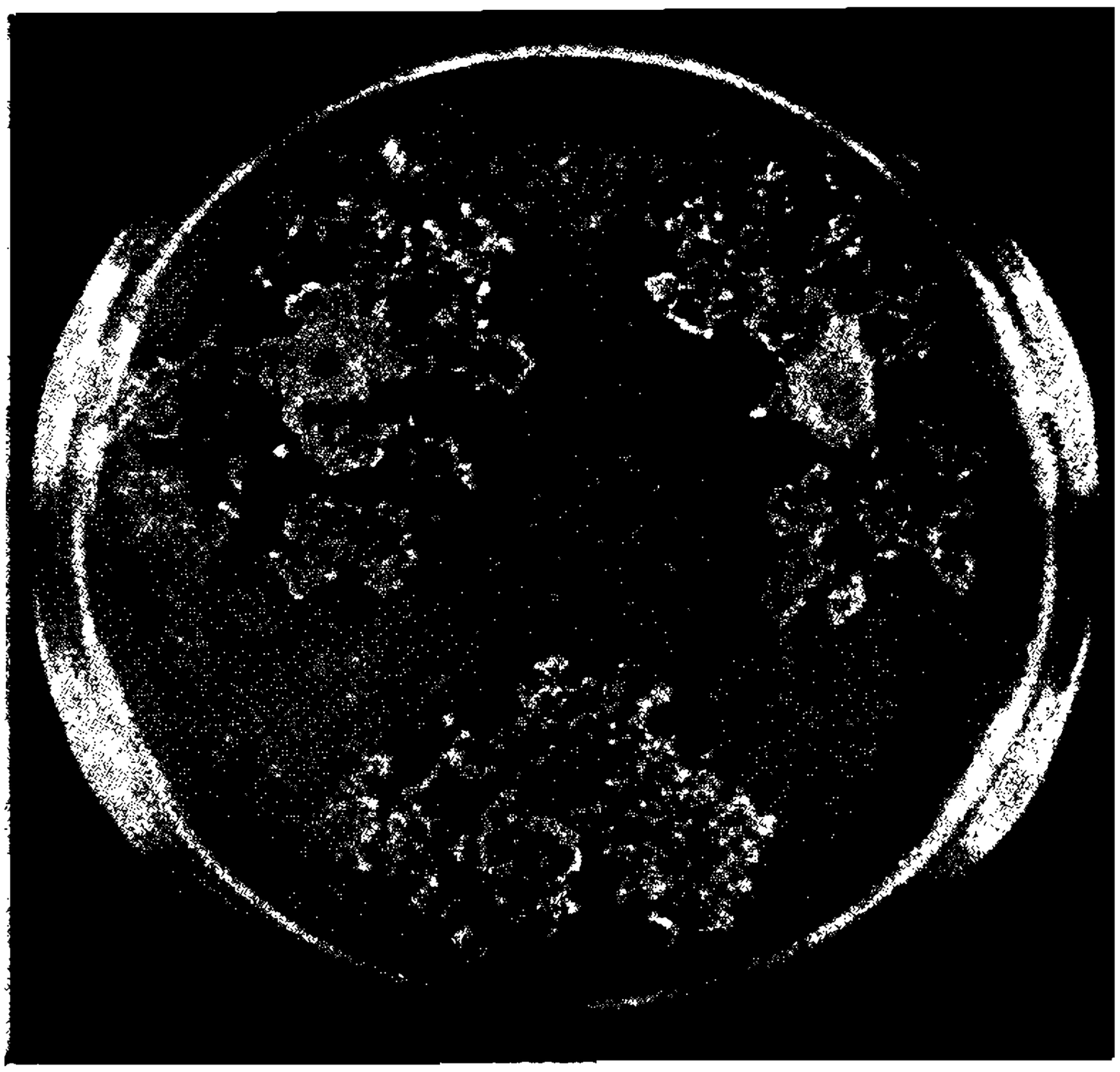


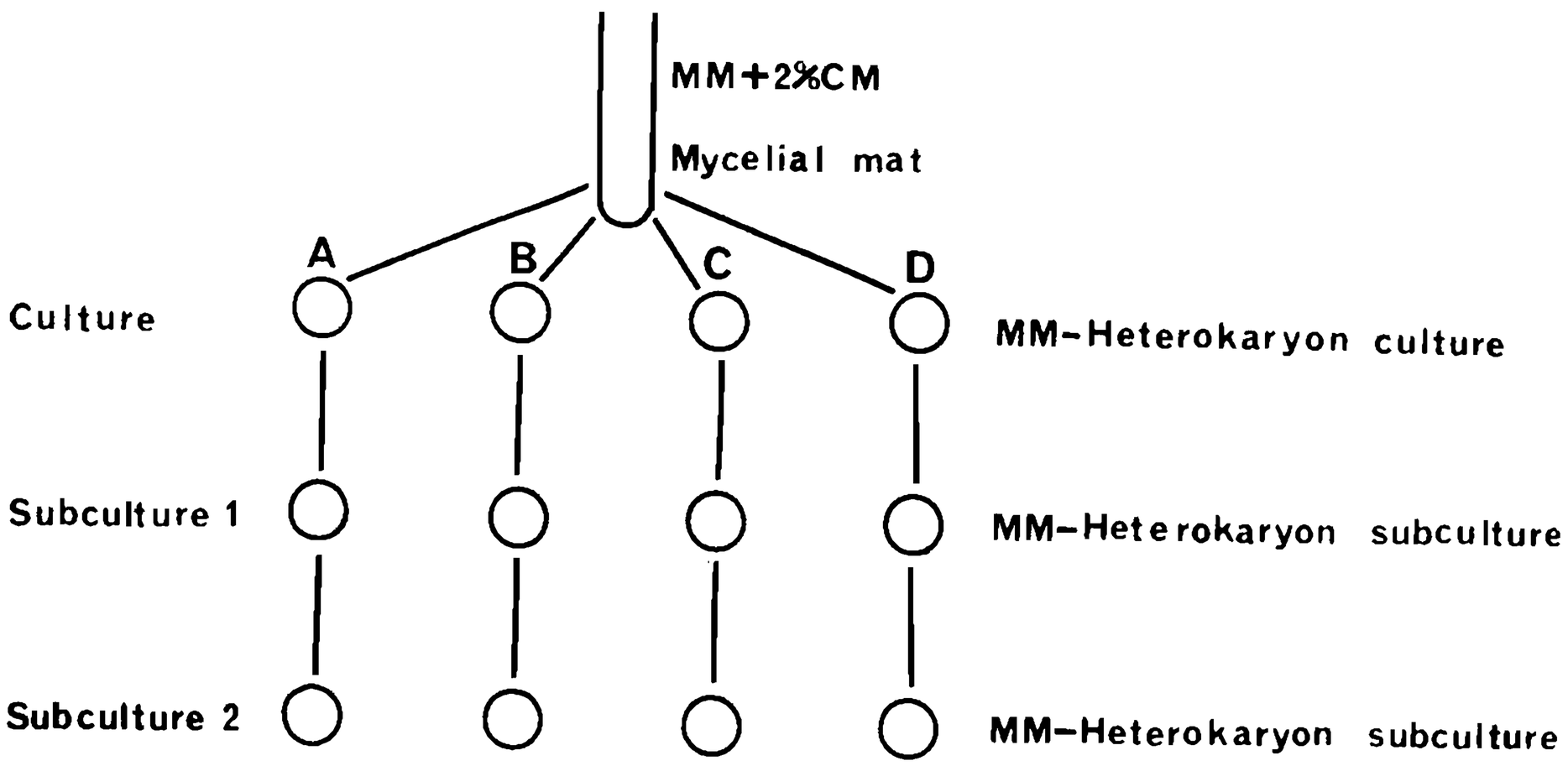
Plate 3. Heterokaryon between *bi1; Act1; nic8; sm10* + *paba1 y; Acr1; co*, on minimal medium.

In *Neurospora* such heterokaryons almost always show stability of nuclear ratio on prolonged culture (PITTENGER and ATWOOD, 1956). However, no detailed study of stability had been undertaken in *Aspergillus* but it was known that the ratio could change as a result of environmental change. Aspects of this work depended on changes of nuclear ratio induced in response to environmental change. It was therefore important to study the stability of at least one heterokaryon under constant growh conditions through a number of subcultures.

The heterokaryon *Act1; pyro4; nic8* + *pro1 y; Acr1* was prepared in the usual fashion. The initial mycelial mat, growth in liquid MM, was teased out onto 4 dishes of MM and subcultures made as shown in Diagram 1.

No inoculum failed to grow at any of the stages shown in Diagram 1. Microscopically the heterokaryons, both within and between series, appeared roughly similar though there were slight variations in the vigour of growth. Conidia were sampled widely, from each plate in each series, and the results of this sampling are shown in Table 4.

DIAGRAM 1



Test tube — Mycelial mat, cross Act1; pyro4; nic8 + pro1 y; Acr1

Culture, Subculture 1, Subculture 2 — Each plate received two inocula, 7 days incubation and then assessment of conidial ratio.

TABLE 4

CONIDIAL RATIOS YIELDED BY SERIES A, B, C AND D, SAMPLED AND SCORED FOR GREEN AND YELLOW CONIDIA, FROM CULTURE AND SUBCULTURES PLATED ONTO FOUR OR FIVE DIFFERENT DISHES

Inoculum / Dishes	Series / Colour	A			B			C			D		
		Green	Yellow	Total	Green	Yellow	Total	Green	Yellow	Total	Green	Yellow	Total
Culture	Dishes												
	1	42	49	91	48	64	112	27	37	64	36	70	106
	2	33	55	88	27	69	96	37	36	73	39	70	109
	3	22	38	60	28	50	78	35	47	82	55	87	142
	4	28	47	75	43	71	114	13	34	47	29	77	106
	5	30	49	79	39	54	93	28	46	74	43	84	127
	Total	155	238	393	185	308	493	140	200	340	202	388	590
	%	39.4	60.6	100.0	37.5	62.5	100.0	41.2	58.8	100.0	34.2	65.8	100.0
Subculture 1	Dishes												
	1	34	81	115	47	100	147	31	95	126	69	140	209
	2	33	60	93	43	100	143	29	80	109	75	130	205
	3	40	63	103	51	128	179	37	105	142	69	145	214
	4	25	68	93	40	95	135	49	108	157	75	145	220
	Total	132	272	404	181	423	604	146	388	534	288	560	848
	%	32.7	67.3	100.0	30.0	70.0	100.0	27.3	72.7	100.0	34.0	66.0	100.0
Subculture 2	Dishes												
	1	23	46	69	41	72	113	11	58	69	19	51	70
	2	29	66	95	42	71	113	13	57	70	20	45	65
	3	23	45	68	50	80	130	17	70	87	20	52	72
	4	21	32	53	31	75	106	15	54	69	30	59	89
	5	21	46	67	36	86	122	13	63	76	28	60	88
	Total	117	235	352	200	384	584	69	302	371	117	267	384
	%	33.2	66.8	100.0	34.2	65.8	100.0	18.6	81.4	100.0	30.5	69.5	100.0

Heterokaryon: *Act1; pyro4; nic8 + pro1; Acr1.*

Statistical analysis of conidial ratios is shown in Table 5. Since any one count of the green and yellow conidia were compiled from four or five (dishes) subcounts, the proportion of green for each (dish) subcount was computed. By this means, using analysis of variance, the variation between series was tested against the variation within series for the culture and for each of the subcultures. As would be expected in the case of the culture there was no significant difference between the four series. In the case of the subcultures, however, the proportion for series C was notably lower than for the other three. In subculture 1 this difference was almost but not quite significant at the 5% level, whereas in subculture 2 the difference was highly significant at the 0.1% level. (Table 5). This suggests that in the case of C, an exception was occurring, only slightly apparent at the first stage of subculture but very marked at the second stage. In the case of the other three, no such difference was in any way apparent, giving evidence of the expected stability of the system in general.

Because of the labour involved, stability was tested for only this one heterokaryon and there is no assurance that the conclusions would

TABLE 5

ANALYSIS OF RESULTS FROM TABLE 4, SHOWING PROPORTION OF GREEN CONIDIA IN SERIES A, B, C AND D FROM CULTURE AND SUBCULTURES AND PLATED ONTO FOUR OR FIVE DISHES

Inoculum / Prop. of green		Proportion of green A	B	C	D	Z
Culture	Dishes					
	1	0.4615	0.4286	0.4219	0.3396	
	2	0.3750	0.2813	0.5068	0.3578	
	3	0.3667	0.4000	0.4268	0.3873	
	4	0.3733	0.3772	0.2766	0.2736	
	5	0.3797	0.4194	0.3784	0.3386	
	Total	1.9562	1.9065	2.0105	1.6969	7.5701
	Mean pop.	0.391	0.381	0.402	0.340	—
Subculture 1	Dishes					
	1	0.2957	0.3197	0.2460	0.3301	
	2	0.3548	0.3007	0.2661	0.3659	
	3	0.3883	0.2849	0.2606	0.3224	
	4	0.2688	0.2963	0.3121	0.3409	
	Total	1.3076	1.2016	1.0848	1.3593	4.9533
	Mean pop.	0.326	0.300	0.271	0.340	—
Subculture 2	Dishes					
	1	0.3333	0.3628	0.1594	0.2714	
	2	0.3053	0.3717	0.1857	0.3077	
	3	0.3382	0.3846	0.1954	0.2778	
	4	0.3962	0.2925	0.2174	0.3371	
	5	0.3134	0.2951	0.1711	0.3182	
	Total	1.6864	1.7067	0.9290	1.5122	5.8343
	Mean pop.	0.337	0.341	0.186	0.302	—

apply to others. However, the results within this one study offered some confidence about stability.

TABLE 5 (Contd.)

ANALYSIS OF VARIANCE: BY THIS MEANS, THE VARIATION BETWEEN SERIES WAS TESTED AGAINST THE VARIATION WITHIN SERIES FOR CULTURE AND SUBCULTURES

Source		SS	DF	MS	MSR	Level of Variance Ratio	Significance
Culture	Between	0.011287	3	0.03762	1.08	5%:$F_{3,16}=3.24$ 5%:$F_{3,12}=3.49$	Not sig.
	Within	0.055551	16	0.03472			
	Total	0.066838	19	—			
Subculture 1	Between	0.011088	3	0.03696	3.40	5:$F_{3,12}=3.49$	Borderline but not quite sig.
	Within	0.013052	12	0.01088			
	Total	0.024140	15	—			
Subculture 2	Between	0.079359	3	0.026453	23.6	0.1%:$F_{3,16}=9.01$	Highly sig.
	Within	0.017909	16	0.0011193			
	Total	0.097268	19	—			

NOTE: For sufficiently large numbers, proportions are approximately normally distributed. In almost all cases the totals exceeded 70 and the lowest was 47. Hence the Analysis of Variance is applied without recourse to a transformation.
SS = Sum Square
DF = Deviation Frequency
MS = Mean Square
MSR = Mean Square Ratio

3. Adaptation to Actidione

3.1. Actidione (cycloheximide) is an antibiotic produced by *Streptomyces griseus*. It has no considerable activity on bacteria and viruses. It was shown to have activity against yeast by Whiffen (1948). Later Middlekauff (1957) located gene-controlled, dominant resistance in *Saccharomyces*. Actidione-resistant mutants have also been isolated in *Neurospora* (Howe and Terry, 1962; Hsu, 1962). Warr and Roper (1965) described a semi-dominant actidione resistant in *Aspergillus nidulans*.

The main evidence to suppose that actidione exerts its fungistatic action by inhibiting protein synthesis is given by its capacity *in vitro* to inhibit protein synthesis and partially to inhibit deoxyribonucleic acid synthesis. Siegel and Sisler (1963) suggested that its antibiotic action might arise by inhibiting the transfer of amino-acids from amino-acyl s- RNA to the ribosomes or by the polymerization of amino-acids into protein.

Walker and Smith (1952) observed that mycelial cells are less sensitive to actidione than are the spores. Since the effects of actidione on

both spore germination and respiration decline with time, these authors suggested an inactivation of actidione with time.

It is worth mention that growth of resistant and sensitive cultures is influenced by the size of the inoculum as well as by actidione concentration.

3.2. *Expression of Act1 in heterokaryons*

Adaptative changes of conidial ratio, in balanced heterokaryons of *Aspergillus nidulans*, was demonstrated by WARR and ROPER (1965).

To repeat and extend the observations of WARR and ROPER a search was made to see whether such adaptation can be found for many different *Act1* heterokaryons.

The following heterokaryons were synthesized on MM:

a) *bi1; Act1; nic8 + pro1 y; Acr1*

b) *bi1; Act1; nic8 + pro1 paba6 y; w3*

c) *bi1; Act1; nic8 + y; pyro4*

After 6 days all colonies grew fairly well, but heterokaryon (c) showed a high proportion of green conidia with a few yellow and mixed conidial heads. It was discarded. The two other heterokaryons (a) and (b) were subcultured by transfer of a mass of hyphal tips in small blocks of agar (about 0.2 sq. cm.) onto MM plates, as control, and MM plates containing a range of concentrations of actidione. All plates received 3 inocula each. Four different concentrations of actidione were used (see Table 6).

TABLE 6

CONIDIAL RATIO OF HETEROKARYON ON VARIOUS CONCENTRATIONS OF ACTIDIONE

Heterokaryon	Concentration of actidione on MM (mg/l)	Total No. of colonies scored	Green colonies %	White or Yellow colonies %
a) *bi1; Act1; nic8* and *pro1 y; Acr1*	0	340	84	16
	50	368	82	18
	100	754	87	13
	200	475	91	9
	400	525	93	7
b) *bi1; Act1; nic8* and *pro1 paba6 y; w3*	0	472	78	22
	50	403	75	25
	100	676	79	21
	200	696	84	16
	400	493	86	14

After 6 further days of incubation, conidia from at least four different areas of heterokaryons on control and actidione containing plates were collected and plated. The conidial ratios were estimated by scoring about 400 colonies from each heterokaryon tested. The results are shown in Table 6.

The range of variation of the conidial ratio on the increasing concentrations of actidione were not as marked as had been expected. Thus it was necessary to seek heterokaryons which would, on MM, achieve a balance with a lower proportion of the *Act1* component.

For this purpose the following cross was made:

bi1; Act1; nic8 × *y; pyro4; ribo5*

From the above cross, a series of actidione-resistant recombinants were obtained and used to synthesize the following heterokaryons:

a) *bi1; Act1; pyro4; ribo5* + *pro1 y; Acr1*

b) *bi1; Act1* + *pro1 y; Acr1*

Heterokaryon (a) on MM, had 92% green conidia and was clearly unsuitable for further study. The second heterokaryon (b) showed many green, apparently homokaryotic, patches; this was doubtless due to cross feeding of the *bi1; Act1,* homokaryotic component by the heterokaryon and any, less obvious, homokaryotic *pro1 y; Acr1.* No further studies were made with this heterokaryon since the presense of substantial homokaryotic patches aggravates the difficulty of satisfactory sampling of conidia.

c) *y; Act1; pyro4* + *bi1; Acr1*

d) *y; Act1; pyro4; ribo5* + *bi1; Acr1*

Combinations (c) and (d) also failed to establish satisfactory heterokaryons, perhaps for the reasons outlined above for (b). Heterokaryon (c) like (a) had an excessive proportion of green conidia for adaptation studies designed to follow increase in proportion of the green component.

A further three heterokaryons were synthesized:

e) *Act1; pyro4; nic8* + *pro1 y; Acr1*

f) *Act1; pyro4; nic8* + *paba1 y; Acr1; co*

g) *Act1; pyro4; nic8* + *ribo1 pf21 y; nic8*

On MM these achieved conidial ratios which, although different for each combination, offered greater scope for study of adaptation. Results of the equilibria reached on increasing levels of actidione are shown in Table 7.

TABLE 7

RESPONSES OF HETEROKARYONS (e), (f) AND (g) TO VARIOUS ACTIDIONE CONCENTRATIONS, SHOWING SOME CONIDIAL RATIO VARIATION IN A SUFFICIENTLY LARGE CONIDIA SAMPLING

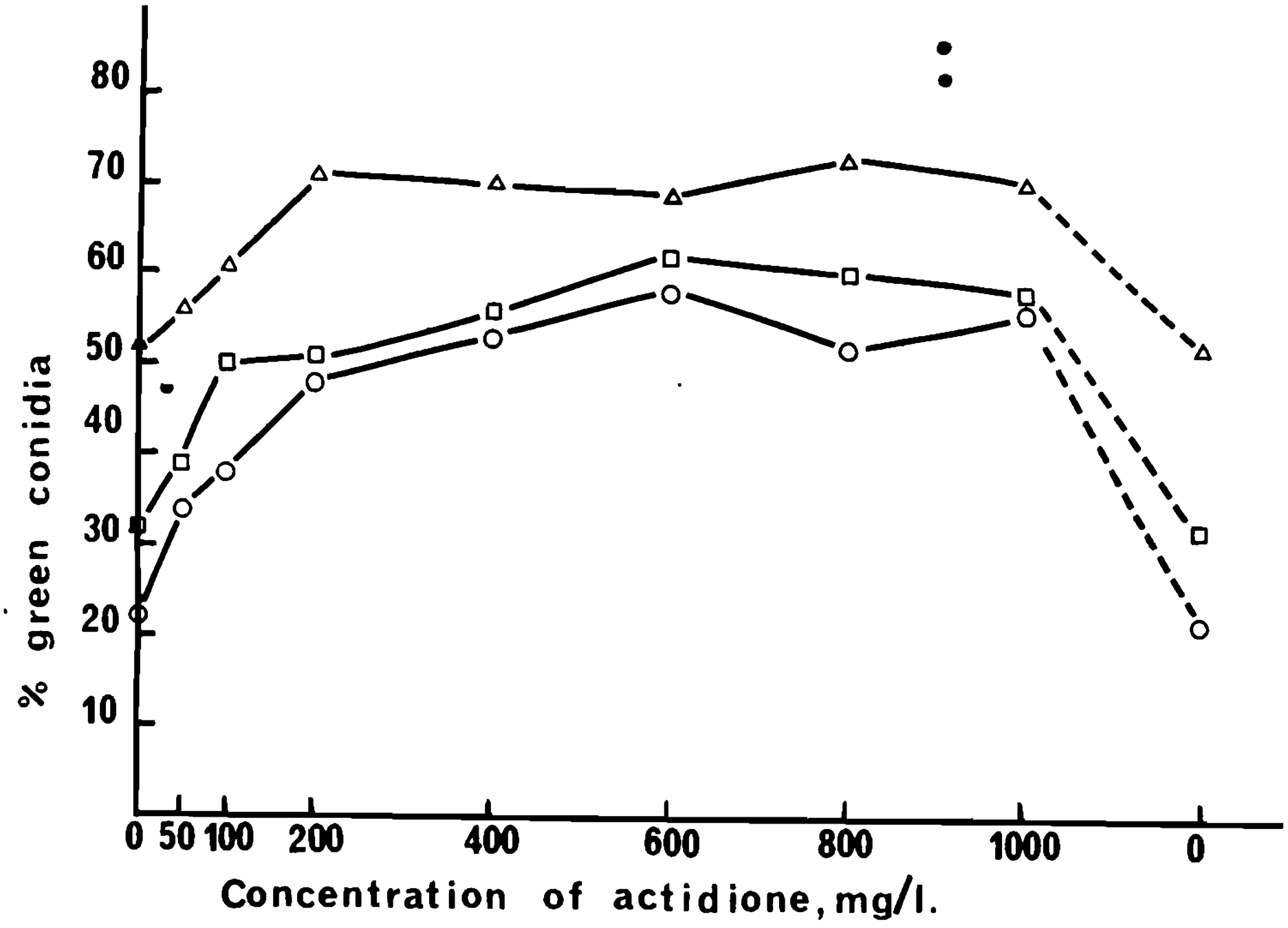
Heterokaryon	Concentration of actidione on MM (mg/l)	Total No. of colonies scored	Green colonies %	Yellow colonies %
(e) *Act1; pyro4; nic8 + pro1 y; Acr1*	0	471	32	68
	50	528	39	61
	100	576	50	50
	200	764	51	49
	400	578	56	44
	600	447	62	38
	800	464	60	40
	1 000	493	58	42
(f) *Act1; pyro4; nic8 + paba1 y; Acr1; co*	0	510	52	48
	50	693	56	44
	100	440	61	39
	200	497	71	29
	400	496	70	30
	600	515	69	31
	800	493	73	27
	1 000	419	70	30
(*g) *Act1; pyro4; nic8 + ribo1 pf21 y; nic8*	0	406	22	78
	50	499	34	66
	100	534	38	62
	200	408	48	52
	400	444	53	47
	600	545	58	42
	800	403	52	48
	1 000	429	56	44

* For heterokaryon (g) the MM was always supplemented with nicotinic acid.

All three heterokaryons showed an increasing proportion of *Act1* conidia on increasing concentrations of actidione, until a plateau was reached (Graph 1). This plateau doubtless represented the nutritional limits of the particular combinations of nutritional requirements. It should be noted that, even at the highest concentration of actidione, 1 g/l, growth was still vigorous. The overall adaptation did not reflect selection of mutant types. This was shown by subculture from actidione back to MM and restoration of the original ratio. However, the results showed only a change in conidial ratio. As mentioned earlier, a heterokaryon is a dynamic system with heterokaryotic and, probably, homokaryotic hyphae. A change in conidial ratio might reflect loss of sensitive homokaryotic hyphae, suppression of conidiation by such hy-

GRAPH 1

Expression of *Act1* in heterokaryons, on various concentrations of actidione



△ = *Act1; pyro4; nic8* + *paba1 y; Acr1;* co

□ = *Act1; pyro4; nic8* + *pro1 y; Acr1*

○ = *Act1; pyro4; nic8* + *ribo1 pf21 y; nic8*

phae and/or a change in nuclear ratio in the heterokaryotic hyphae. These possibilities are discussed later. Nevertheless, treating the heterokaryon as a whole, it seems reasonable to suppose that a change in conidial ratio reflected an overall change in nuclear ratio.

3.3. *Actidione action on diploids*

Aspergillus nidulans and some related species are the only ones that make possible the comparison of complementation, or any other interaction between alleles, within the same nucleus, in a diploid, and within a common cytoplasm, separated by nuclear membranes, in a heterokaryon. Such an important problem is related to the function of the nuclear membrane and cytoplasmic organelles and has been discussed by PONTECORVO (1952), ROPER (1958) and LEWIS (1961). ROBERTS (1963) has explored the efficiency of interallelic complementation of some nutritional mutants, in diploids and their heterokaryons.

Heterokaryons (Table 7) *Act1; pyro4; nic8 + pro1 y; Acr1, Act1; pyro4; nic8 + paba1 y; Acr1; co* and *Act1; pyro4; nic8 + ribo1 pf21 y; nic8* had shown vigorous growth on even 1 g/l actidione. It was of interest to see whether heterozygotes could tolerate such high levels.

A series of diploids were prepared and tested for resistance to actidione. The results are summarised in Table 8.

TABLE 8

SPORULATION AND SURVIVAL OF HETEROZYGOUS DIPLOID ON VARIOUS ACTIDIONE CONCENTRATIONS

Diploid	Concentration of actidione on MM (mg/l)	Colony Diameter size < 0.5 cm.	Sporulated	Viable %	Total No. of colonies scored
Act1; pyro4; nic8 // pro1 y; Acr1	—	—	+	—	606
	50	89	+	73	442
	100	126	+	48	292
	200	49	—	21	128
	400	78	—	14	84
	600	76	—	13	76
	800	57	—	9	57
	1 000	28	—	5	28
bi1; Act1; nic8 // pro1 y; Acr1	—	8	+	—	338
	50	30	+	65	218
	100	74	+	33	113
	200	92	—	27	92
	400	99	—	29	99
	600	87	—	26	87
	800	56	—	17	56
	1 000		—	24	82
paba1 y; Acr1; co // bi1; Act1; nic8	—	2	+	—	334
	50	71	+	85	286
	100	130	+	43	143
	200	120	—	37	123
	400	75	—	22	75
	600	97	—	29	97
	800	75	—	22	75
	1 000	98	—	29	98

+ sporulated

Comparison of Tables 7 and 8 showed that heterokaryons grew vigorously on concentrations of actidione which permitted growth of only a small proportion of conidia of the corresponding heterozygote. The comparison has hazards. Heterokaryons were subcultured by mas-

ses of hyphae, and heterozygotes tested as conidia. There is the known inoculum effect on resistance. But, in the estimation of viability, a note was made of sporulation which is one component of growth vigour. At the higher actidione concentrations the heterozygous diploid conidia which grew failed to sporulate. This showed that, even when the inoculum had become effectively large, vigour was still reduced. It could be concluded that the difference in actidione resistance between heterokaryon and heterozygote was real and was not simply an effect of inoculum size.

3.4. *Heterokaryon adaptation and perithecial formation*

The problem of direct measurement of nuclear ratios has been mentioned earlier. An alternative, though laborious approach to change in nuclear ratio can be made through study of the change in frequency of hybrid: selfed perithecia. It was appreciated that this was, again, an indirect approach subject to some of the reservations made for conidial measurement. Nevertheless, it was of interest to see whether, on increasing concentrations of actidione, the proportions of the three classes of perithecia showed any trends.

Results of the analysis of perithecia from heterokaryons between *Ac1; pyro4; nic8* and *pro1 y; Acr1*, on a range of concentrations of actidione, are given in Table 9.

TABLE 9

FORMATION OF HYBRID AND SELFED PERITHECIA ON VARIOUS ACTIDIONE CONCENTRATIONS

Concentration of actidione mg/l MM	Number of Hybrid Perithecia	% Hybrid Perithecia	Number Selfed Yellow Perithecia	% Selfed Yellow Perithecia	Number Selfed Green Perithecia	% Selfed Green Perithecia	Total No. of Perithecia analysed
—	26	87	4	13	0	0	30
50	19	68	9	32	0	0	28
100	15	48	16	52	0	0	31
200	30	86	4	11	1	3	35
400	26	68	11	29	1	3	38
800	13	37	14	40	8	30	35

Analysis of the results in Table 9 must be made with caution since the sample size was unavoidably small. Nevertheless, some facts stand out. Most significant is the increase in selfed green (*Act1*) perithecia with increase in actidione. The proportion of hybrids shows no clear trend except for a possible reduction at the highest actidione level. It is of interest that the selfed yellow (sensitive) shows no significant decrease at high actidione concentration. This last fact implies that the

selfed sensitives are either "protected" by hyphal-determined resistance or are sufficiently morphologically (and physiologically) isolated from the actidione as to be unaffected by it.

The results are consistent with the view that, on increasing actidione concentration, there was an increase in the overall proportion of *Act1* nuclei.

3.5. *A spontaneous mutation*

Most of the perithecia tested from the cross *Act1; pyro4; nic8* × × *pro1 y; Acr1* (Table 9) gave colonies with just the parental yellow and green colour. However, from plates with 50 and 100 mg/l of actidione yellow, green and fawn colonies were detected. (Plate 4).

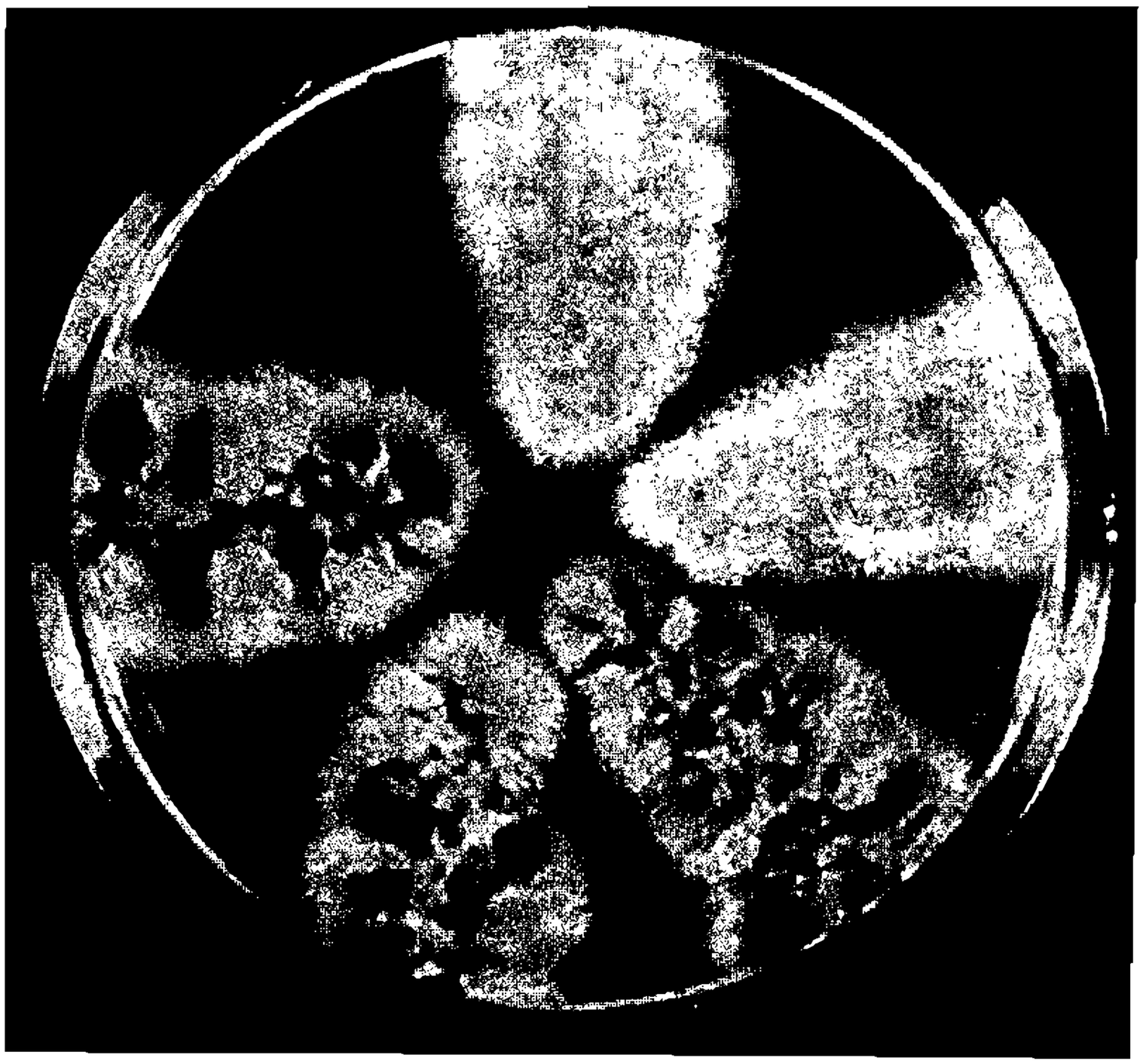


Plate 4. Perithecium analysis.
Cross: *Act1; pyro4; nic8* x *pro1 y; Acr1*

In view of the value of visible markers, attempts were made to analyse the fawn variant.

A perithecium showing just fawn colonies was plated on CM. After classification for the parental nutritional requirements and drug response, all 26 colonies tested were classified as proline requirers and resistant to acriflavine.

On the assumption that fawn was due to a mutation, the mutant allele was provisionally designated *fw*. Conidia from the heterokaryon *pro1 y; Acr1* (*fw*) + MSE, plated on CM, gave only fawn and white conidia. This suggested a nuclear determinant for the fawn phenotype.

Ascospores from the cross *pro1 y; Acr1;* (*fw*) × MSE gave the following colour segregation:

$$\left.\begin{array}{l} \text{fawn} = 460 \\ \text{white} = 390 \end{array}\right\} \text{Parental}$$

$$\left.\begin{array}{l} \text{yellow} = 411 \\ \text{white yellowish} = 452 \end{array}\right\} \text{Recombinant}$$

Total number of colonies scored = 1,713.

$$\left.\begin{array}{l} \chi^2 = 7.7 \\ P = 0.05 \end{array}\right\} \text{Fit to a 1 : 1 : 1 : 1 ratio.}$$

The fawn phenotype was determined by mutation in a single gene which showed free recombination with *y* and *w*.

Segregants were classified for all nutritional markers in an attempt to find linkage of any of these with *fw*. Significant deviation from free recombination was found only for *facA* and fawn. Test of 110 colonies is shown below:

fawn *facA*$^+$	=	41	Parental
fawn *facA*	=	15	Recombinant
non fawn *facA*	=	42	Parental
non fawn *facA*$^+$	=	12	Recombinant

$$\% \text{ recombination} = \frac{27}{110} \times 100 = 24.5\%$$

A diploid synthesized between fawn and MSE was analysed by mitotic haploidisation. The results are shown in Table 10. Clearly fawn can be allocated to chromosome V. The mutant allele fawn (*fw10*) is recessive. Its combination with the alleles *y*, *y*$^+$ or *w3*, in a haploid, gave rise to colonies fawn, greenish and white yellowish respectively showing that it interacts with them in such a way that the genotype could be deduced by inspection.

This fawn mutant retained the normal morphology and size of conidial heads as the strain from which it arose. Thus, fawn seems to be a useful colour marker especially by its location on chromosome V whose nutritional markers *lys5* or *facA* in master strains are not always ideal.

TABLE 10

CLASSIFICATION OF HAPLOIDS FROM THE DIPLOID *PRO 1 Y; ACR 1* FAWN WITH MSE

Chromosome number		I	II	III	IV	V	VI	VII	VIII
MSE markers		*pro1+y*	w3 Acr+1	Gal1	pyro4	facA	s3	nic8	ribo2
Medium		MM + All — proline	CM + acriflavine	Basic Medium + Gal + All	MM + All — pyro	Fluora-cetate Medium + All	MM + All — thiosul-phate	MM + All — nicotinic acid	MM + All — ribo-flavin
Fawn 12	Growth	5	12	5	6	12	4	8	4
	No Growth	7	0	7	6	0	8	4	8
White 8	Growth	4	0	5	4	0	4	3	4
	No Growth	4	8	3	4	8	4	5	4
yellow 8	Growth	3	8	2	6	0	4	3	4
	No Growth	5	0	6	2	8	4	5	4
White yellowish 10	Growth	5	0	4	2	10	5	6	3
	No Growth	5	10	6	8	0	5	4	7

*Haploidization on CM supplemented with p-fluorophenylalanine.

Since the fawn mutation, in the strain *pro1 y; Acr1*, was found in heterokaryons on MM supplemented with 50 and 100 mg of actidione per litre, it could be either a spontaneous mutation in the mycelial mat (synthesized in MM supplemented with 2% CM), or a mutation induced by actidione. The first hypothesis is more plausible since the mutant was detected in two different plates whose heterokaryons originated from the same mycelial mat. Furthermore, mutagenic action of actidione has not been recorded and, in the many tests made on actidione, fawn was the only instance of mutation noted.

A limited test for the possible mutagenic action of actidione was made as follows. Conidia of *pro1 y; Acr1* were plated on MM with proline, with and without actidione (100 mg/l). Resultant colonies were scored for colour at 48 hr. (without actidione) and 120 hr. (with actidione). Viability on actidione was 18.7%. No conidial colour variants were detected among about 1,500 untreated colonies and 1,350 colonies grown on actidione.

4. Adaptation to Acriflavine

Acriflavine is a mixture of 1/3 Proflavine and 2/3 Proflavine methochloride, and it is believed that the effect of the two components is greatly potentiated when acting together.

Wild-type strains of *E. coli* are impermeable to acridine dyes. According to Silver and Wendt (1967), acriflavine uptake reflects more an altered permeability rather than surface binding.

Additional evidence supporting this was given by NAKAMURA (1968) working with *E. coli* K. 12.

LERMAN (1964) has demonstrated that acridine dyes become intercalated between adjacent base pairs in deoxyribonucleic acid (DNA), forming a complex, acridine-DNA. Furthermore, HURWITZ, FURTH, MALAMY and ALEXANDER (1962) have shown its capacity to inhibit enzymatic synthesis of DNA and RNA *in vitro*.

Although, there has been a great deal of research on the effect of acridine dyes, their behaviour in *A. nidulans* and related organisms does not seem to have been explored in detail.

In *A. nidulans*, ROPER and KÄFER (1957) discussed the genetic basis of some acriflavine resistant mutants and BALL and ROPER (1966) have studied various acridine-induced mutation processes in resistant and sensitive strains.

The experiments described here were performed to examine the relationship between expression of acriflavine action in strains of *A. nidulans* resistant to acriflavine, in heterokaryotic and heterozygous combinations.

A number of balanced heterokaryons were synthesized in the usual manner:

a) *bi1; Act1; nic8 + paba1 y; Acr1; co*

b) *bi1; Act1; nic8 + pro1 y; Acr1*

c) *bi1; Act1; pyro4; ribo5 + pro1 y; Acr1*

d) *bi1; Acr1 + pyro1 paba6 y; w3*

e) *Act1; pyro4; nic8 + pro1 y; Acr1*

The heterokaryons were subcultured on MM by transfer of hyphal tips in blocks of agar (about 0.2 cm^2) and onto MM containing a range of concentrations of acriflavine. Five concentrations of acriflavine were used (see Table 11) in addition to a drug-free control.

Following incubation, an inspection with respect to overall resistance gave roughly the same results for all heterokaryons. On drug free MM, MM plus 0.05 and MM plus 0.15 mg./1. of acriflavine, the subcultures gave good heterokaryotic growth. On MM plus 0.45 and MM plus 0.75 mg./l. of acriflavine, an irregular and sometimes submerged kind of growth was observed. On the higher concentration of acriflavine (3 mg./l) and more hardly any growth occurred.

Conidial ratios were estimated as previously for each heterokaryon on every concentration of acriflavine which allowed heterokaryotic growth. The results which were quite homogeneous, are shown in Table 11. All heterokaryons showed an increasing proportion of the acriflavine resistant component on increasing concentrations of acriflavine.

TABLE 11

RESPONSES OF HETEROKARYONS (a), (b), (c), (d) and (e) TO VARIOUS ACRIFLAVINE CONCENTRATIONS. (a) AND (b) FROM 0.75 mg/1 ACR. CONC. HAD SHOWN ABILITY TO REVERT TO INITIAL PROPORTIONS ONTO DRUG -FREE MM

Heterokaryon	Concentration of acriflavine on MM mg/l	Total No. of colonies scored	Green colonies %	White or Yellow colonies %
(a)	—	721	95	5
	0.05	549	87	13
bi1; Act1; nic8	0.15	425	81	19
+	0.45	568	78	22
paba1 y; Acr1; co	0.75	595	63	27
	3.0	—	—	—
From conc. 0.75 mg/l	—	413	92	8
(b)	—	397	84	16
	0.05	418	89	11
bi1; Act1; nic8	0.15	510	88	12
+	0.45	428	80	20
	0.75	434	72	28
pro1 y; Acr1	3.0	—	—	—
From conc. 0.75 mg/l	—	390	88	12
(c)	—	311	92	8
	0.05	428	92	8
bi1; Act1; pyro4; ribo5	0.15	549	89	11
+	0.45	389	89	11
	0.75	462	87	13
pro1 y; Acr1	3.0	—	—	—
(d)	—	715	55	45
	0.05	421	50	50
bi1; Acr1	0.15	762	49	51
+	0.45	646	64	36
	0.75	423	67	33
pro1 paba6 y; w3	3.0	—	—	—
(e)	—	531	32	68
	0.05	665	30	70
Act1; pyro4; nic8	0.15	638	30	70
	0.45	562	22	78
pro1 y: Acr1	0.75	547	25	75
	3.0	—	—	—

Heterokaryons (a) and (b) from MM plus 0.75 mg./l of acriflavine, were subcultured onto drug-free MM. From the resulting heterokaryons the conidial ratios were determined; this showed their ability to revert to the initial proportions (see Table 11). On the basis of the present data the possibility of some adaptative response could not be excluded. Because of the low resistance of these heterokaryons to acriflavine it was presumably possible to detect variations of the conidial ratios below the plateau determined by nutritional limitations of the combinations.

The very limited response of conidial ratios to acriflavine took place at concentrations of the inhibitor which permit growth of sensitive strains. This suggested that the resistant nuclei in the heterokaryon were confering little, if any, resistance to the heterokaryotic hyphae. A comparison with diploid heterozygotes was essential. Furthermore, it was necessary to question whether the observed change in conidial ratio reflected a simple change in nuclear ratio or an alternative of the type discussed earlier.

The following heterozygous diploids were synthesized:

(a) *paba1 y; Acr1; co // bi1; Act1; nic8*

(b) *bi1; Act1; nic8 // pro1 y; Acr1*

(c) *Act1; pyro4; nic8 // pro1 y; Acr1*

The diploids were tested on MM with increasing concentrations of acriflavine. By using MM supplemented with the necessary growth factors, possible formation of complexes between substances from CM and acriflavine was prevented (McIlwain, 1941; Roper and Käfer, 1957) and this also enabled a more valid comparison between heterozygous diploids and their respective heterokaryons. The results are shown in Table 12.

Working with a heterokaryon on a range of acriflavine concentrations the nuclear ratio was assessed tentatively through estimation of the frequencies of hybrid and selfed perithecia. The heterokaryon used was:

Act1; pyro4; nic8 + pro1 y; Acr1

Conclusions based on the necessarily limited numbers of Table 13 must be tentative. The total absence of selfed green (sensitive) perithecia may reflect only a low selfertility of that component. At the highest concentration there was a probably significant increase in selfed (resistant) yellow and correlated reduction in hybrids. This could have arisen from an increased proportion of resistant nuclei in the heterokaryon but, for the following reason, this seemed unlikely. The heterokaryon showed little change of conidial ratio on acriflavine (Table 11). However, let us suppose for the moment that this reflected a real change in nuclear ratio in heterokaryotic hyphae. Application of

TABLE 12

GROWTH TYPE, SPORULATION AND SURVIVAL OF HETEROZYGOUS DIPLOID ON VARIOUS ACRIFLAVINE CONCENTRATIONS

Diploid	Concentration of acriflavine mg/l MM	Colony diameter <0.5 cm	Deep growth irregular borders	Sporulated	% Viable	Total No. of colonies obtained
(a)	—	—	—	+	—	334
	0.05	—	—	+	75	252
bi1; Act1; nic8//	0.15	—	—	+	65	218
	0.45	—	—	+	70	235
paba1 y; Acr1; co	0.75	—	+	+	65	217
	3.0	152	+	+	54	181
	4.0	134	+	+	46	154
	10.0	106	+	+	32	106
(b)	—	—	—	+	—	338
	0.05	20	—	+	70	235
bi1; Acr1; nic8//	0.15	12	—	+	67	227
	0.45	7	—	+	61	206
pro1 y; Acr1	0.75	11	—	+	61	206
	3.0	—	+	+	62	209
	4.0	79	+	+	52	177
	10.0	90	+	+	29	97
(c)	—	—	—	+	100	606
	0.05	—	—	+	99	603
Act1; pyro4; nic8//	0.15	—	—	+	98	598
	0.45	—	—	+	99	603
pro1 y; Acr1	0.75	—	+	+	96	583
	3.0	16	+	+	98	595
	4.0	57	+	+	96	585
	10.0	153	+	+	48	291

The colonies were scored after 48 hr. of incubation.
+ = sporulated or growth into the medium.
Viability without inhibitor was assumed as 100%.

the Hardy-Weinberg Law has limited validity since, in heterokaryons, there will rarely be "random mating". Nevertheless, application of Hardy-Weinberg considerations would not lead to an expectation of substantial changes in zygote (perithecial) proportions on so limited a change of allele (nuclear) ratio. A more acceptable interpretation of the results of Tables 13 and 11 is that, on acriflavine, there was either an increase in the proportion of homokaryotic resistant hyphae and/or a preferential development of resistant conidia and selfed, resistant perithecia.

TABLE 13

FORMATION OF HYBRID AND SELFED PERITHECIA ON VARIOUS ACRIFLAVINE CONCENTRATIONS

Concentration of acriflavine mg/l	Number of Hybrid Perithecia	Hybrid Perithecia %	Number Selfed Yellow Perithecia	Selfed Yellow Perithecia %	Number Selfed Green Perithecia	Selfed Green Perithecia %	Total No. of Perithecia analysed
—	26	87	4	13	0	0	30
0.05	32	94	2	6	0	0	34
0.15	20	91	2	9	0	0	22
0.45	33	94	2	6	0	0	35
0.75	15	68	7	32	0	0	22
3.0	—	—	—	—	—	—	—

Medium = MM
Cross: *Act1; pyro4; nic8* × *pro1 y; Acr1*

5. Adaptation to p-fluorophenylalanine

p-fluorophenylalanine (FPA), an analogue of the amino-acid phenylalanine, has been found, like many other amino-acid analogues, to inhibit the growth of micro-organisms.

The effects of these analogues on protein synthesis and the properties of protein synthesized in their presence are considered by Richmond (1962). Such inhibitors often compete with the natural metabolite at the active centre of an enzyme. However, it is now well established that certain analogues, among them FPA, cause partial growth inhibition but do not have a preferential inhibitory effect of protein synthesis (Cohen, Halvorson and Spiegelman, 1958).

Since the work of Halvorson and Spiegelman (1952) showing the inhibitory action of FPA, it has been used frequently in a variety of studies. Morpurgo (1961a) showed its action as an effective inducer of haploidization in diploid strains of *Aspergillus*. Lhoas (1968) and Nga and Roper (1969) have speculated on its possible mode of action in haploidization. Morpurgo (1961b) was the first to detect a mutant of *A. nidulans* resistant to the analogue. Later, Warr and Roper (1965) described a recessive mutant which conferred resistance to FPA and also suppressed the mutant allele *nic8*. The strain *ribo1 pf21 y; nic8*, was adopted for this study and it was necessary, first, to confirm its genotype by outcrossing.

Ascospores derived from a single hybrid perithecium, of the cross *ad8 paba1* × *ribo1 pf21 y; nic8*, were plated on CM. A random sample of segregants derived from the plating were classified for colour, FPA

resistance and nutritional markers. Classification for conidial colour yielded the following:

Total number = 1148

yellow = 598 $\chi^2 = 2.00$

green = 550 P = 0.10

This is consistent with a 1:1 ratio.

The ratio of resistant to sensitive segregants was not significantly different from 1 : 1, showing that the resistance was controlled by mutation of a single gene.

Total number = 240

FPA resistant = 117 χ^2 = 0.15

FPA sensitive = 123 P = 0.70

Classification of the segregants with respect to nutritional markers confirmed the finding of WARR and ROPER (1965) that the expression of the *nic8* allele (nicotinic acid requirement) was suppressed by *pf21* (Plate 5). Classification for nicotinic acid requirement and resistance to FPA yielded the following:

FPA sensitive, nicotinic acid-independent = 67

FPA sensitive, nicotinic acid-dependent = 56

FPA resistant, nicotinic acid-independent = 117

FPA resistant, nicotinic acid-requiring = 0

Nicotinic acid requirement is suppressed by *pf21* giving a ratio of approximately 1 : 1 : 2 : 0 for the four expected classes.

χ^2 = 2,24 P = 0.50 Fit to 1:1:2:0

In fact, careful inspection after 48 hr' incubation, permitted differentiation between *nic8* + and *pf21; nic8* since growth of the latter was normally reduced in density.

5.1. *Expression of pf21 in heterokaryons and heterozygous diploids*

To study the expression of *pf21* in heterokaryons it was likely to be necessary to seek heterokaryons, as mentioned previously, showing certain characteristics of growth and nuclear ratio. The following heterokaryons were established on MM, in the usual way:

a) *bi1; Acr1* + *ribo1 pf21 y; nic8*

b) *pro1 pabo6 y; w3* + *ribo1 pf21 y; nic8*

c) *pro1 bi1* + *ribo1 pf21 y; nic8*

Combination (a) failed to establish a satisfactory heterokaryon. Heterokaryons (b) and (c) produced an excessive proportion of white (85%) and green (89%) conidia respectively and were clearly unsuitable for further study.

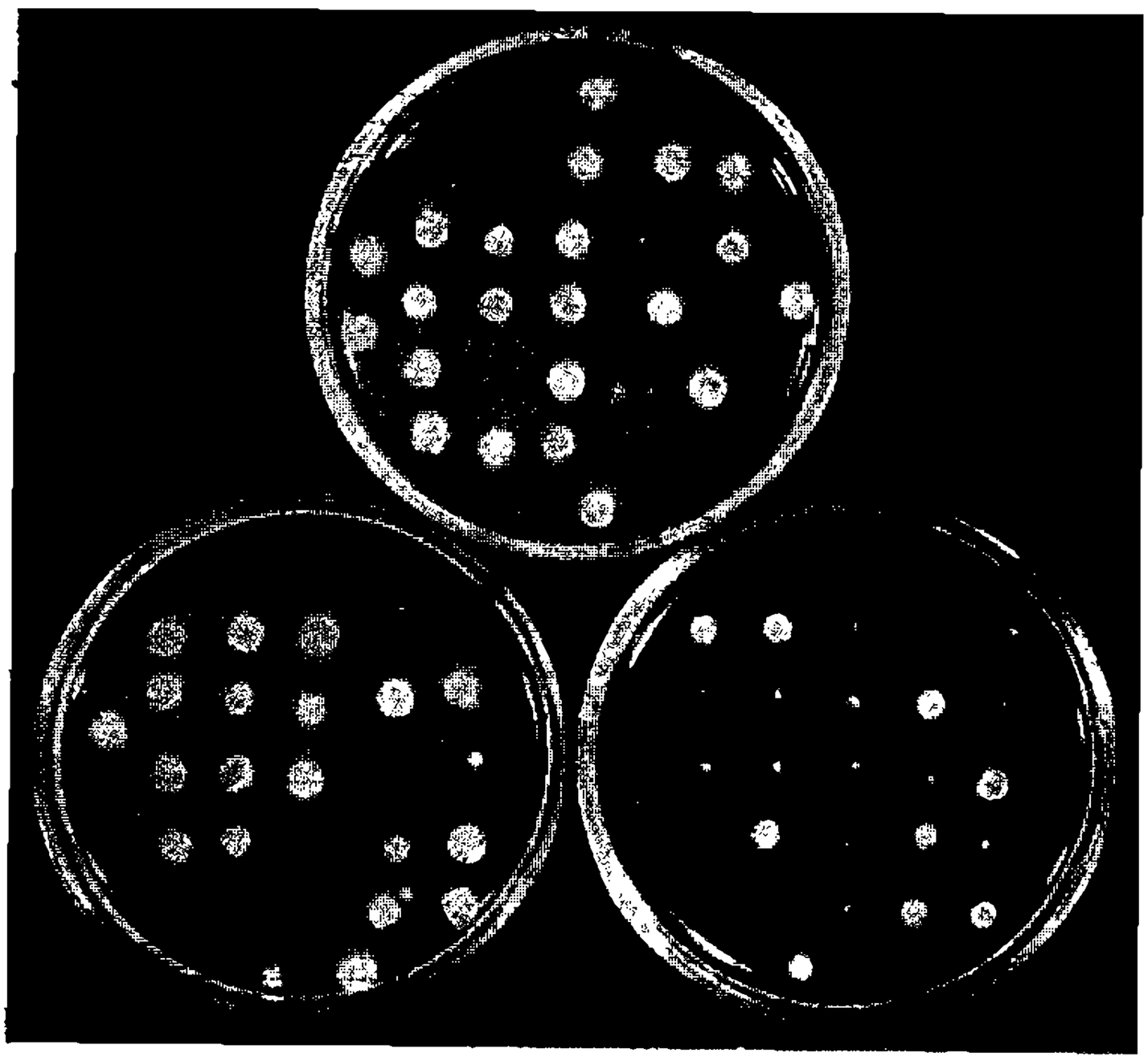


Plate 5. Classification for: Top dish-riboflavin requirement.
Bottom right — resistance on p-fluorophenylalanime.
Bottom left — nicotinic acid requirement.
Cross: *ad8 paba1 x ribo1 pf21 y; nic8*

The heterokaryon *Act1; pyro4; nic8* + *ribo1 pf21 y; nic8* gave good growth on MM + nicotinic acid but failed to grow in subcultures at even the lowest tested concentration of FPA (50 mg/l), on MM supplemented with nicotinic acid.

Several points of interest arose from this combination of strains and they are discussed later.

A further heterokaryon was established satisfactorily on MM: *ad8 paba1* + *ribo1 pf21 y; nic8*

The results of the expression of *pf21* allele in this heterokaryon, on increasing concentrations of FPA, are shown in Table 14.

Throughout this test a peculiar behaviour was observed and no explanation can be offered. On MM and also on MM supplemented with FPA, this heterokaryon yielded from time to time, a cyclical pattern of growth. The heterokaryon grew normally to a certain size and then yielded a green, apparently homokaryotic area; at other times the heterokaryon would produce such a green homokaryon which was in turn surrounded by a yellow homokaryon.

TABLE 14

HETEROKARYON RESPONSE TO VARIOUS ACRIFLAVINE CONCENTRATIONS

Heterokaryon	Concentration of FPA mg/l on MM	Total No. of colonies scored	Green colonies %	Yellow colonies %
ad8 paba1 + *ribo1 pf21 y; nic8*	—	493	36	64
	50	448	18	82
	100	572	14	86
	200	514	4	96
	400	397	4	96
	600	312	3	97
	800	—	—	—

Despite the occasional abnormal growth, wide sampling of conidia from heterokaryotic areas showed that in the above heterokaryon, adaptation on increasing concentrations of FPA was marked (Table 14).

Adaptative response, as opposed to selection of new mutants, was shown by the ability of the heterokaryon to restore the original conidial ratio after subculture from FPA back to MM without inhibition.

The following heterozygous diploids were isolated:

a) *Act1; pyro4; nic8 // ribo1 pf21 y; nic8*

b) *ad8 paba1 // ribo1 pf21 y; nic8*

c) *ad8 paba1 // ribo1 pf21 y; Act1; nic8*

Conidia of these diploids were plated onto MM (MM supplemented with nicotinic acid for (a) with various concentrations of FPA. The results are shown in Table 15. However, these results must be taken with extreme caution. It is very well known that the action of the amino acid analogue, FPA, takes place in an early stage of the development of diploids leading, by successive losses, to the formation of haploid nuclei. Therefore, apart from the possible action of FPA on the many

TABLE 15

COLONY DIAMETER, SPORULATION AND SURVIVAL OF HETEROZYGOUS DIPLOID ON VARIOUS FPA CONCENTRATIONS

Diploid	Concentration of FPA mg/l MM*	Colony diameter <0.5 cm	Sporulated	% Viable	Total No. of colonies scored
(a)	—	6	+	—	452
	50	87	+	34	152
Act1; pyro4; nic8	100	96	—	21	96
	200	16	—	4	16
ribo1 pf21 y; nic8	400	20	—	4	20
	600	6	—	1	6
	800	2	—	<1	2
	1000	2	—	<1	2
(b)	—	2	+	—	412
	50	262	+	82	336
ad8 paba1//	100	87	—	23	94
	200	95	—	23	95
ribo1 pf21 y; nic8	400	60	—	15	60
	600	68	—	16	68
	800	5	—	1	5
	1000	1	—	<1	1
(c)	—	5	+	—	544
	50	173	+	48	260
ad8 paba1//	100	48	—	12	64
	200	56	—	10	56
ribo1 pf21 y; Act1; nic8	400	29	—	5	29
	600	36	—	7	36
	800	19	—	3	19
	1000	16	—	3	16

* MM supplemented with nicotinic acid for diploid (a) + Partially sporulated colonies.

Results read with 48hr. of incubation.

processes which regulate the viability and growth rate, there is its action in causing haploidization. A proportion of colonies scored as survivors might have been complex mixtures including aneuploid and haploid resistant types. Care was taken in scoring so that, despite this reservation, the results of viability tests can be accepted with reasonable confidence.

Comparison of the results in Table 14 and 15 showed that a heterokaryon was able to display vigorous growth on a concentration of FPA which permitted only poor growth of a small percentage of conidia of the corresponding heterozygote.

The heterokaryon *Act1; pyro4; nic8* + *ribo1 pf21 y; nic8*, was of special interest for two reasons. First, it failed to grow on MM (plus

nicotinic acid) with only 50 mg/l of FPA. Second, both component nuclei carried *nic8* which is suppressed by *pf21*.

5.2. *Interactions of Act1 allele*

The second point of interest, mentioned above, was investigated as follows. In its determination of resistance, *pf 21* is recessive (or nearly so) in heterozygotes (WARR and ROPER, 1965). (This fact does not show up in the present results since haploid and homozygous diploid resistants were not tested). We may ask, then, whether *pf21* is recessive in its ability to suppress *nic8*. The diploid, *Act1; pyro4; nic8 // ribo1; pf21 y; nic8*, showed only very slight, non-conidiating growth on MM without nicotinic acid. The growth was comparable with that of the slightly leaky *nic8* on MM, *pf21* is, therefore, recessive in its suppressive action. It could then be asked whether ,in the heterokaryon *Act1; pyro4; nic8* + *ribo1 pf21 y; nic8*, there was requirement for nicotinic acid. The results are summarised in Table 16 and Plate 6. MM could support this heterokaryon, though the growth was poor. However, growth continued on subculture from one MM dish to another of MM, showing that the growth was significant and not due to carry over of nutrients from the initial inoculum. Comparison of conidial ratios on MM, and on MM plus nicotinic acid, showed that the former gave, as might have been expected, a higher proportion of *pf21* conidia.

TABLE 16

POSSIBLE INTERACTION OF MARKERS IN *ACT1; PYRO4; NIC8* + *RIBO1* *pf21 Y; NIC8* HETEROKARYON

Mycelial Mating	Heterokaryon		Conidial ratio		
Medium	Medium	Appearance	Green %	Yellow %	Total
MM+ 2% CM+ Nicotinic acid	MM	Poor heterokaryon. Poorly sporulated Few mixed conidial heads	17	83	767
	MM+ Nicotinic acid	Good heterokaryon. Well sporulated Plenty mixed conidial heads	28	72	641
MM+ 2% CM	MM+ Nicotinic acid	Good heterokaryon Well sporulated Plenty mixed conidial heads	21	79	366
	MM	Poor heterokaryon. Poorly sporulated Few mixed conidial heads	12	88	383

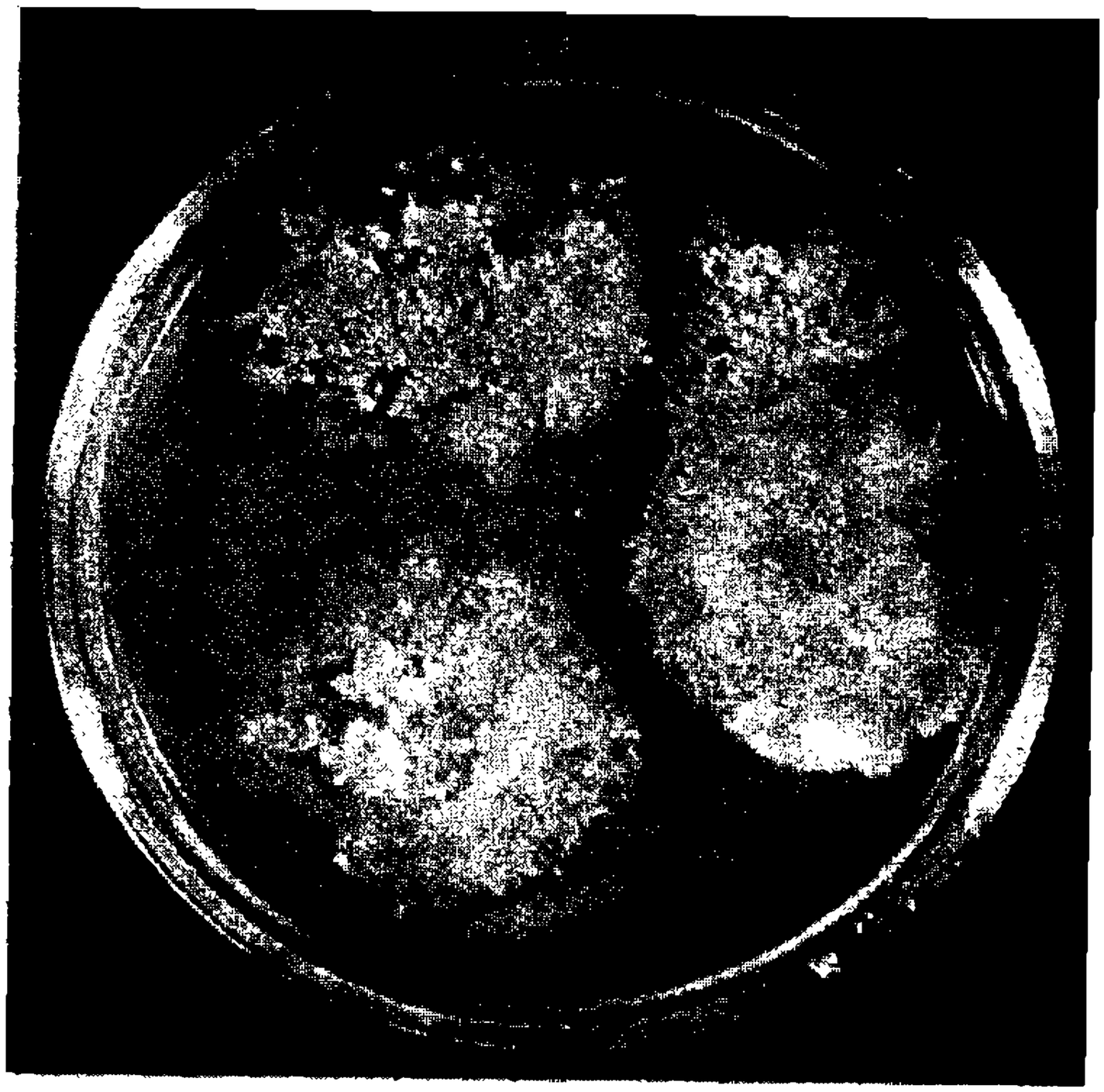


Plate 6. Growth of heterokaryon:
Act1; pyro4; nic8 + *ribo1 pf21 y; nic8,* on minimal medium.

The failure of heterokaryon *Act1; pyro4; nic8* + *ribo1 pf21 y; nic8,* to grow on low concentration of FPA posed a particular difficulty. To investigate the possibility of complex gene interactions, ascospores from a perithecium of this heterokaryon were plated on CM. Segregation for colour was consistent with a 1:1 ratio. 62 yellow and 62 green segregants, picked at random, were classified for resistance to actidione and FPA and for all segregating nutritional markers. The summarised results of significance were as follows:

1) Of the 124 segregants, 59 were actidione resistant, 65 sensitive. This is consistent with a 1:1 ratio. However, it was clear from the test plates that *Act1* confers a low but significant degree of resistance to FPA. This accentuates the problem of the viability of the heterokaryon to grow on FPA. In fact, WARR and ROPER (1965) noted that *pf21* confers some cross-resistance to actidione but had not observed the converse.

2) Of the 124 segregants, 69 were FPA sensitive, 55 resistant. This is consistent with a 1 : 1 segregation. Slight cross-resistance to actidione was confirmed. Segregation for actidione resistance and FPA re-

sistance was, as expected, independent though (because of cross-resistance) careful tests were needed for unequivocal classification. The 55 FPA resistant segregants, including those which were also actidione resistant, were nicotinic acid independent. The action of *pf21* in suppressing *nic8* appeared unaltered by the *Act1* allele. However, among the 69 FPA sensitives there were two distinct classes with respect to nicotinic acid requirement. There was absolute correlation between the degree of requirement and actidione resistance. 42 of these FPA sensitives, which were also actidione sensitives, were "leaky" and showed "spidery" growth in the absence of nicotinic acid. The remaining 27, actidione resistant, showed no trace of growth without nicotinic acid. *Act1* enhances the effect of *nic8* and makes the requirement for nicotinic acid absolute. These gene interactions are shown in Plate 7.

Further studies on the action of FPA, actidione, and the genes which confer resistance to them, is needed before any plausible interpretation of these results could be advanced. By chance the chosen

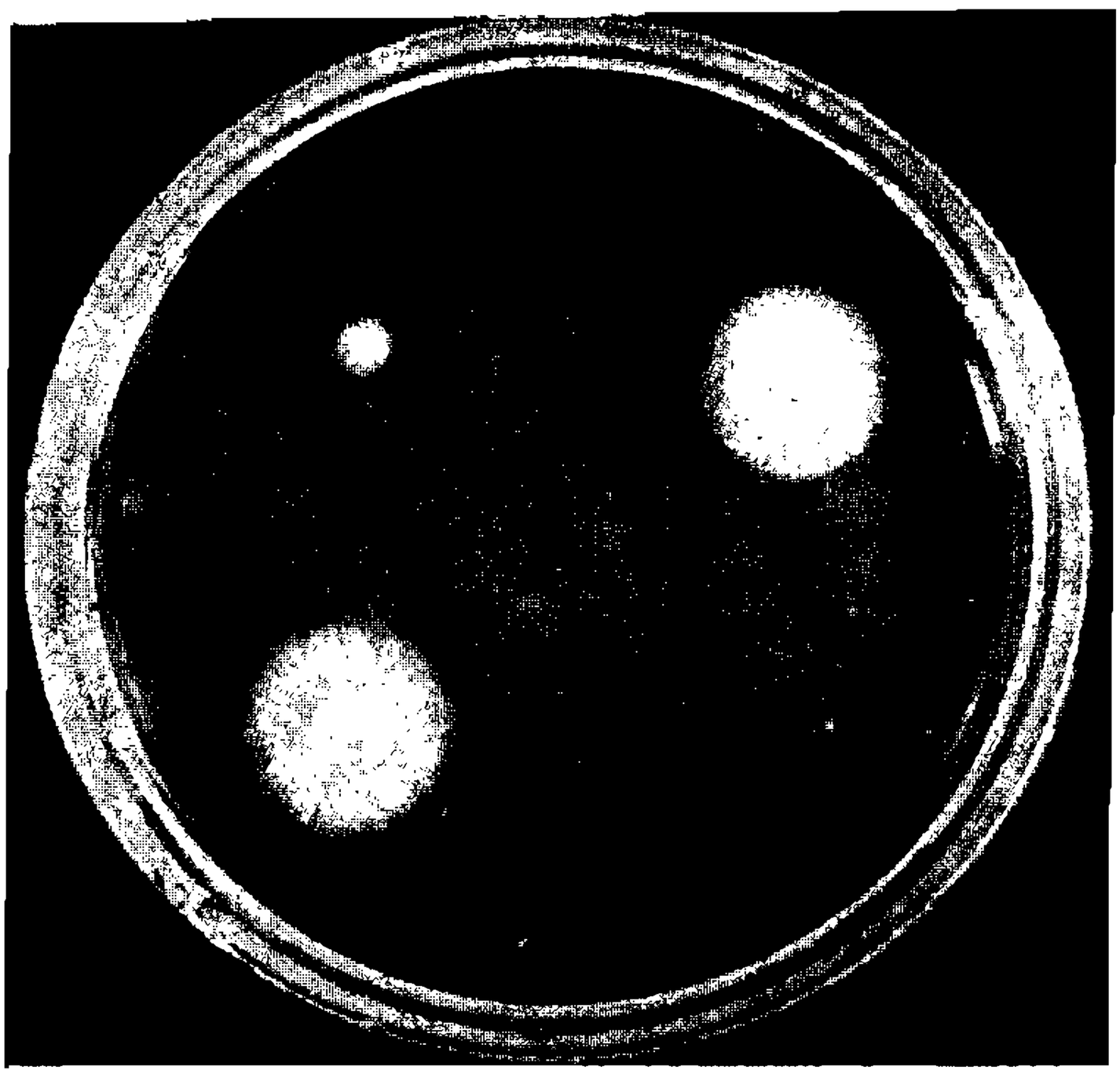


Plate 7. Test for nicotinic acid requirement on MM supplemented with riboflavin and pyridoxine. Strains:

Top left — *ribo1 y; nic8*
Top right — *ribo1 pf21 y; nic8*
Bottom left — *ribo1 pf21 y; Act1; nic8*
Bottom right — *Act1; pyro4; nic8*

heterokaryon, *Act1; pyro4; nic8 + ribo1 pf21 y; nic8*, was one which was likely to involve complex interactions. A solution to such interactions must sought, ultimately, in biochemical terms. But some preliminary steps could be taken by studying other heterokaryons with different combinations of the *Act1*, *pf21* and *nic8* markers.

SECTION IV DISCUSSION

The aim of this work was to study aspects of gene action in heterokaryons and in the corresponding heterozygotes. It was planned as a study in extension, rather than depth, to probe the potentialities of the general approach rather than to give detailed study to one instance.

The purpose of using heterokaryons on CM, balanced by slow-growing mutants, was outlined earlier. CLUTTERBUCK (1965) was successful in preparing such heterokaryons. At the start of this study only one slow-growing mutant, compact (*co*), was available in the laboratory. Other slow-growers were sought and one, *sm10*, was characterised genetically.

Unfortunately *co* and *sm* produced an entirely unsatisfactory heterokaryon on CM. Growth on MM was unusual and interesting in having heterokaryotic "islands" connected by sparse hyphae. Although the "islands" enjoyed some vigour, the heterokaryon as a whole appeared to be in a state of "distress" and liable to breakdown. Perhaps this resulted from inability to find a nuclear ratio which would satisfy the nutritional requirements of the components and their requirements in respect of *co* and *sm*. It would be interesting to know whether the heterokaryotic patches represented just heterokaryotic hyphae or, as is more likely, an unstable mixture of heterokaryons and homokaryons which could accommodate the mutual needs but not perpetuate itself in vigorous, stable form. To pursue the initial aims, it would be necessary to isolate more non-allelic slow-growers and test them in combination with various nutritional markers.

Before work was undertaken with heterokaryons balanced by nutritional markers on MM, heterokaryon stability was studied in a series of cultures on one pair of strains. The results offered some confidence in heterokaryon stability under constant environmental conditions. There was other, indirect evidence of stability from experiments which could well have accentuated any tendency to instability. In those cases where a heterokaryon showed adaptative response to environmental change, the original conidial ratio was restored after subculture back to the initial environment.

Heterokaryons and heterozygotes involving acriflavine resistance provided some of the most striking and definitive results. Heterokaryons with the *Acr1* allele in one component showed apparent adaptation on acriflavine, but only in the concentration range tolerated by sensitive strains. At higher concentrations there was no growth. A study of the relative frequencies of perithecial types, at various acriflavine

concentrations, reinforced the view that relatively little change of nuclear ratio occurred in the heterokaryon; it was almost certain that growth ceased as a result of acriflavine concentration and below the plateau set by nutritional requirements. The heterokaryon failed to grow on 3 mg./l of acriflavine, even when the conidial ratio indicated a fairly high proportion of *Acr1* nuclei. On the other hand, heterozygous diploid conidia, with their fixed 50% ratio of the *Acr1* allele, were able to grow on far higher concentrations of acriflavine. In genetical terms, *Acr1* is almost recessive in heterokaryons and semi-dominant in heterozygotes. In simple physiological terms, it appears that the range of action of the *Acr1* allele is confined entirely, or nearly so, to the nucleus which carries it. Further pursuit of this would require definition of the action of *Acr1*, and of acridines, in biochemical terms; this would be a formidable problem in view of the multiple affinities shown by acridines for biologically important macromolecules (BALL and ROPER, 1966).

Adaptation to actidione was shown by heterokaryons with appropriate starting ratio, that is, those which were not too near the nutritional limits in containing a high proportion of *Act1* nuclei. Adaptation was observed as a change of conidial ratio; a cross-check with the proportions of perithecial types supported the idea of a true change in overall nuclear ratio. With actidione, the comparison with heterozygotes was the converse of that found for acriflavine. Heterozygous conidia showed relatively poor growth and low viability at concentrations of actidione tolerated readily by the corresponding heterokaryons. However, the conidial ratios suggested that, at high actidione concentrations, the proportion of *Act1* nuclei in heterokaryons exceeded 50%. The difference in resistance, between heterokaryon and heterozygote, may have reflected a dosage effect of the *Act1* allele. Nevertheless, the clear implication is that *Act1* is non-autonomous in its action; it has the ability to protect actidione-sensitive nuclei separated from the resistant nuclei by both cytoplasm and nuclear membranes. Actidione is known to interfere in protein synthesis (see Section III-3) and the system sensitive to actidione is clearly one which can be shared between nuclei; if this were not so, *Act1* could offer no protection to sensitive nuclei.

During the work with actidione, a new conidial colour mutant, fawn (*fw*),was isolated and analysed. This new mutant, probably spontaneous in origin should prove a useful marker in itself and in its interactions with other conidial colour mutants.

The mutant allele, *pf21*, was the only recessive used in attempts at adaptation. Although the allele is recessive in heterozygous diploids, heterokaryons showed marked adaptative response to the inhibitor. There was a degree of analogy with the actidione situation, in that heterokaryons displayed vigorous growth on levels of FPA which permitted only poor growth and low viability of heterozygous conidia. But this did not imply that *pf21* was dominant in heterokaryons and recessive in heterozygotes. Adapted heterokaryons had a high proportion ($> 90\%$) of resistant conidia and, presumably, a correspondingly high proportion

of resistant nuclei in the hyphae. The heterokaryon: heterozygote difference doubtless arose as a result of dosage effects. The terms "recessive" and "dominant" can be applied strictly only in heterozygotes and, perhaps, in heterokaryons with a 1 : 1 nuclear ratio. The strains used in these studies carried the alleles *nic8*, *pf21* and *Act1*. *pf21* had been shown previously to suppress *nic8*. It was shown that *pf21* is recessive in its suppression of *nic8* in diploids homozygous for *nic8* and heterozygous for *pf21*. However, in corresponding heterokaryons, with a high proportion of *pf21* nuclei, *nic8* was partially suppressed. This was doubtless, again, an effect of dosage of the *pf21* allele. Strains carrying *nic8* are leaky but the double mutant, *Act1; nic8*, showed no growth in the absence of nicotinic acid. The complex interactions of *nic8*, *pf21* and *Act1* in single strains, heterokaryons, and heterozygotes, require biochemical studies for their proper elucidation. But further comparisons, along the lines of those above, may cast indirect light on the interactions.

In the introduction the term "sub-cellular functional topography" was used. It was intended to convey the undoubted importance of the spatial organization of sub-cellular structures in relation to their functions. There are various approaches to this problem of cellular architecture and function. The present studies, inevitably incomplete, suggest that the heterokaryon/heterozygote comparison may offer a useful approach. Its value would be enhanced in any study which permitted simultaneous biochemical analysis.

SUMMARY

A series of studies have been undertaken to find cases in which heterokaryons show adaptative response to environmental change. Comparisons have also been made between the phenotypes of heterokaryons and corresponding heterozygotes.

Unsuccessful attempts were made to produce heterokaryons, on complete medium, balanced by one previously-existing mutant and one newly-obtained slow-growing mutant.

Adaptation was achieved in heterokaryons carrying different mutant alleles conferring resistance to : (a) acriflavine, (b) actidione and (c) p-fluorophenylalanine. Comparison with the heterozygote in case (a) suggested a highly localised action of the allele determining resistance. A similar comparison for (b) suggested a non-localised action. In cases (b) and (c) dosage effects were observed in the degree of resistance that the heterokaryons, compared with the corresponding heterozygotes, could achieve. In case (c) interaction of the resistance marker with a nutritional marker (*nic8*) has been investigated further and a new interaction between *nic8* and *Act1* detected.

During this work a new conidial colour mutant, fawn, was isolated and characterised. It is likely to be a valuable visual marker, especially in view of its interaction with other colour mutants.

SUMÁRIO

Estudos foram realizados à procura de combinações cujos heterocárions apresentassem adaptação com a variação do meio. Comparações também foram feitas entre os fenótipos de heterocárions e dos heterozigotos correspondentes.

Não foram bem sucedidas as tentativas de obtenção, em meio completo, de heterocárions balanceados por um mutante de crescimento reduzido, já existente, e outro mutante do mesmo tipo obtido pelo autor.

Foi evidenciada adaptação em heterocárions com diferentes alelos mutantes conferindo resistência a: (a) acriflavina, (b) actidione e (c) p-fluorfenilalanina. No caso (a) a comparação com o heterozigoto sugere uma ação altamente localizada do alelo responsável pela resistência. Uma comparação similar para (b) sugere uma ação não localizada. Nos casos (b) e (c) os efeitos de dosagem foram observados nos gráus de resistência dos heterocárions comparados com os heterozigotos correspondentes. No caso (c) a interação da marca responsável pela resistência com u'a marca nutricional (*nic8*) foi investigada e uma desconhecida interação entre *nic8* e *Act1* foi evidenciada.

No decurso dêste trabalho um nôvo mutante de coloração de conidios, "fawn", foi isolado e caracterizado. É provável que seja u'a marca visual de valor, especialmente devido à sua interação com outros mutantes de coloração de conidios.

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