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A direct determination of nuclear distribution in heterokaryons of *Aspergillus nidulans*

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1. INTRODUCTION

Until now the different types of nuclei in a heterokaryon have not been distinguished by direct observation. In most studies on heterokaryosis nuclear ratios have been determined indirectly through conidial ratios, and nuclear distribution has been inferred through differential production of conidial types in various parts of the colony. Although these indirect methods have had considerable success, two basic problems of heterokaryosis were inaccessible. First, how is heterokaryosis maintained? Second, and this may be related to the first question, what is the mechanism by which heterokaryons may show adaptive adjustment of nuclear ratios in response to environmental change?

This paper describes an attempt to distinguish nuclear types directly through cytological study of heterokaryons whose components differed in ploidy. Such an approach was suggested by M. J. D. White in discussion of Pontecorvo (1946). Acridine fluorescent staining was used to permit unselective staining of all nuclei, an essential for unbiased sampling. The actively growing regions of a heterokaryon were determined in a preliminary experiment and the cytological study confined to these regions.

2. METHODS

General techniques were those of Pontecorvo, Roper, Hemmons, Macdonald & Bufton (1953). Incubation was at 37°C.

(i) *Media*

Minimal medium (MM) Czapek-Dox with 1% glucose. Complete medium (CM), a complex medium with hydrolysed casein, hydrolysed nucleic acid, yeast extract, vitamins, etc. Solid media had 1·5% agar.

(ii) *Organisms*

Strains were taken from laboratory stocks maintained on CM. Mutant alleles are described by Pontecorvo *et al.* (1953) and Käfer (1958). Those of main

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importance in this work were: *w*, white conidia; *y*, yellow conidia; *co*, reduced growth rate; *ad*, *bi*, *lys*, *paba*, *phen*, *pyro*, *s*, requirement, respectively, for adenine, biotin, lysine, *p*-aminobenzoic acid, phenylalanine, pyridoxin, and thiosulphate.

(iii) *Heterokaryons*

These were balanced by complementary nutritional deficiencies; component strains always differed in conidial colour. Heterokaryons were prepared by floating conidia of the two components on liquid MM supplemented with 2% CM. The pad of mycelium which resulted after 2 days' incubation was teased out on dishes of MM (or appropriately supplemented MM when the components had a nutritional mutant allele in common). Heterokaryosis was always confirmed by the presence of 'mixed conidial heads' (Pontecorvo *et al.*, 1953).

(iv) *Diploids*

These were prepared by Roper's (1952) technique. Auxotrophic diploids were derived from components which had a nutritional mutant allele in common.

(v) *Determination of active growth region*

The method of Ryan, Beadle & Tatum (1943) was used. Colonies were grown on a thin, permeable cellophane membrane over solid medium. Cuts were made across the edge of the colony with a sterile razor blade. Growth of individual cut hyphae, as well as undisturbed control hyphae from adjacent regions of the colony, was measured from photographs or camera-lucida drawings made immediately after cutting and after 1 hour further incubation.

(vi) *Slide cultures for cytology*

Nutritionally balanced heterokaryons may break down unless they have a relatively large volume of medium which can dilute excreted diffusible nutrients. Cytology, on the other hand, requires a single layer culture. These two conditions were met as follows. Agar, 0·5% in water, was poured over a tilted well-flamed slide so that only a very thin layer remained on the slide. This was inoculated with a few hyphal tips and then covered with a sheet of sterile cellophane. A retaining ring bent from glass rod was placed over the cellophane and filled with cooled molten agar medium to a depth of 3 to 4 mm. After 36 to 48 hours incubation colonies were about 2 cm. diameter. Before staining, the cellophane and agar over-layer were stripped off.

(vii) *Fixation*

The majority of fixatives suitable for fluorescent staining burst the hyphal tips. Air drying, before fixation, gave elongated nuclei unsuitable for measurement.

The most satisfactory method was to cool slide cultures rapidly to −20°. They were then dried quickly in a current of warm air and treated with Carnoy's fluid (ethanol 6 vol.: chloroform 3: glacial acetic acid 1).

(viii) *Staining*

Slides were stained in acridine orange, 50 mg./l., in phosphate buffer. pH was not very critical but the optimum was about pH 7. The amount of staining was important; this could be adjusted either by the time of staining and/or the strength of the stain. Understaining gave only green fluorescence from the whole cell. Correct staining gave green DNA fluorescence and red RNA fluorescence. In over-stained preparations the green DNA is no longer distinguishable, especially in younger parts of the colony.

(ix) *Microscopy*

The light source was a Phillips CS 150 W. high-pressure mercury vapour lamp with ammoniacal copper sulphate solution as filter. A dark-ground condenser was used. The secondary filter, in the body of the microscope, was an Ilford 110 minus-blue.

(x) *Measurements*

Slide cultures still with overlay were photographed, incubated 1 hour further, and rephotographed to provide growth rates of individual hyphae. After staining, camera-lucida drawings were made of all hyphae on each photograph. The only omissions were hyphae obscured by others or hyphae bleached by light when adjacent regions were drawn. In each hypha, the outlines of all nuclei up to the first septum were drawn; the resulting projected nuclear areas were measured with a transparent grid designed to measure areas directly in units of 0·25 μ^2.

3. RESULTS

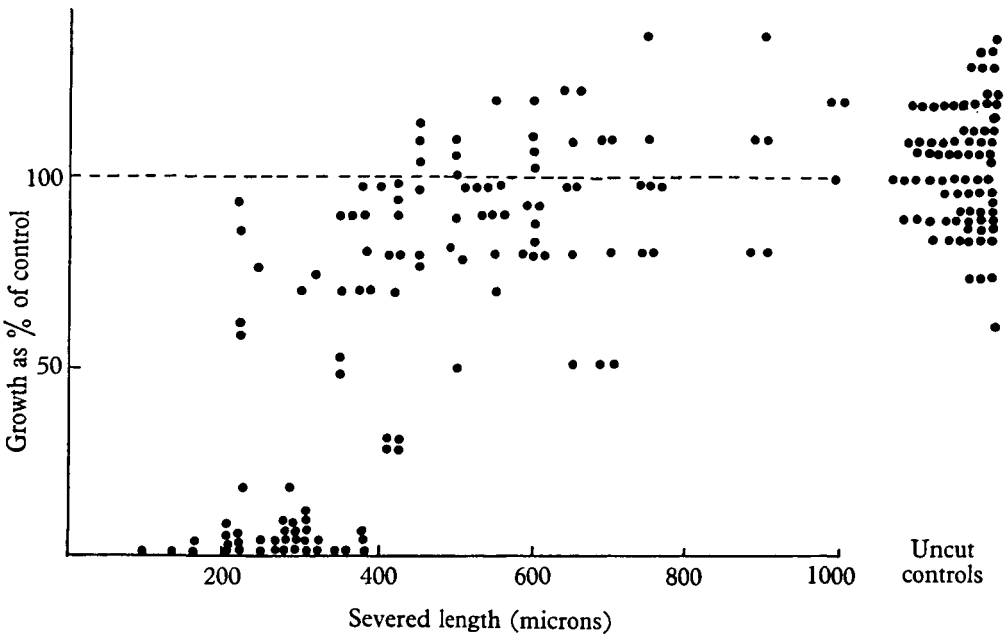
(i) *The region of active growth*

Text-figure 1 shows the relationship between the initial length of severed hyphae and their subsequent growth rates. Growth equal to that of control uncut hyphae was achieved by all severed tips 500 to 600 μ or more in length. In the majority of hyphae, under good growth conditions, the first septum is 300 to 600 μ from the tip. It was concluded that the major contribution to growth comes from the tip cell and that the nuclei in this region should be studied for information on effective nuclear ratio.

(ii) *Cytology*

Examples of the results achieved with fluorescent staining are shown in Plate I. There was considerable variation in the shape of nuclei even with fixation by freezing. The most conspicuous feature of the nuclei is the nucleolus which

fluoresces a brighter red than the cytoplasm. In the photographs it appears as a bite out of the nucleus. Despite the examination of thousands of hyphae, no obvious mitotic figures were seen. However, nuclei were seen quite frequently grouped in pairs (Plate 1, Fig. 3) and this suggested that division had occurred recently. In a very few hyphae a series of dumb-bell figures was observed; these may represent an intra-nuclear spindle.

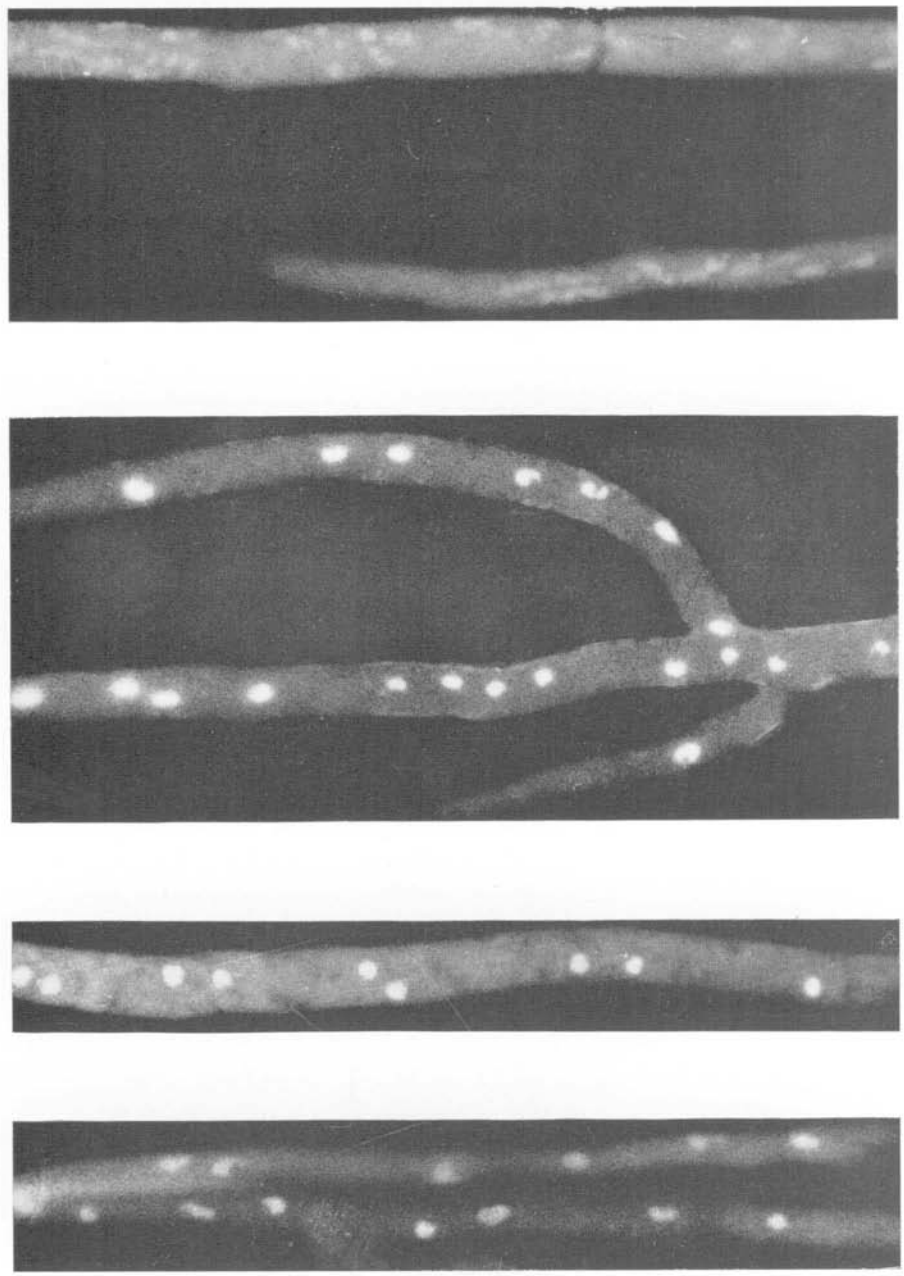


Text-fig. 1. The relationship between initial length of severed hyphae and their subsequent growth rate.

(iii) *Size of nuclei in homokaryons*

Text-figure 2 shows histograms of the projected nuclear areas of 3307 haploid nuclei (50 hyphae) of genotype *y*; *w*3; pyro4 and 1642 diploid nuclei (52 hyphae) of genotype *bi*1; *w*3; *pyro*4/*paba*1 *y*; *pyro*4. The larger number of haploid nuclei is due to differences in spacing of nuclei in the two ploidies (Clutterbuck, 1965). No attempt has been made to convert projected areas into volumes. Skewness of distribution can be corrected by plotting the logarithm of projected areas.

The geometric means of the two distributions differ by a factor of 1·75. If the volumes of spherical nuclei differed by a factor of two, their projected areas would differ by 1·59; for non-spherical nuclei the difference in projected area would be greater. The difference in these distributions is clearly sufficient to distinguish populations of haploid and diploid nuclei, but the variance is too great to allow identification of individual nuclei in a mixture of the two ploidies. However,



Fluorescent staining of hyphal nuclei. All × 1760.
Fig. 1. Haploid. No cold treatment.
Fig. 2. Haploid. Cold treated.
Fig. 3. Haploid. Cold treated.
Fig. 4. Diploid. Cold treated.

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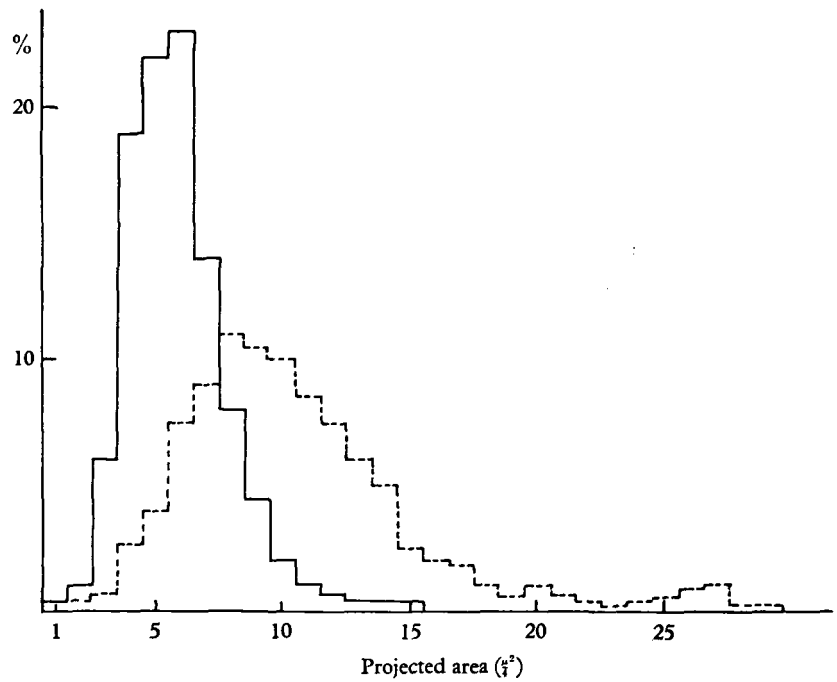
provided there is no substantial interaction between diploid and haploid in the factors determining nuclear size, then it should be possible to estimate the proportion of nuclei of each ploidy from a histogram of a diploid–haploid heterokaryon.

(iv) *Heterokaryons*

Text-figure 3 shows the projected nuclear areas from a diploid–haploid heterokaryon of constitution *w*2; *phen*2; *lys*5; *s*3/*ad*14 *y*; *lys*5 with *y*; *w*3 *ad*3; *co.* 1781 nuclei from 50 hyphae were plotted. The distribution fits closely to a model with



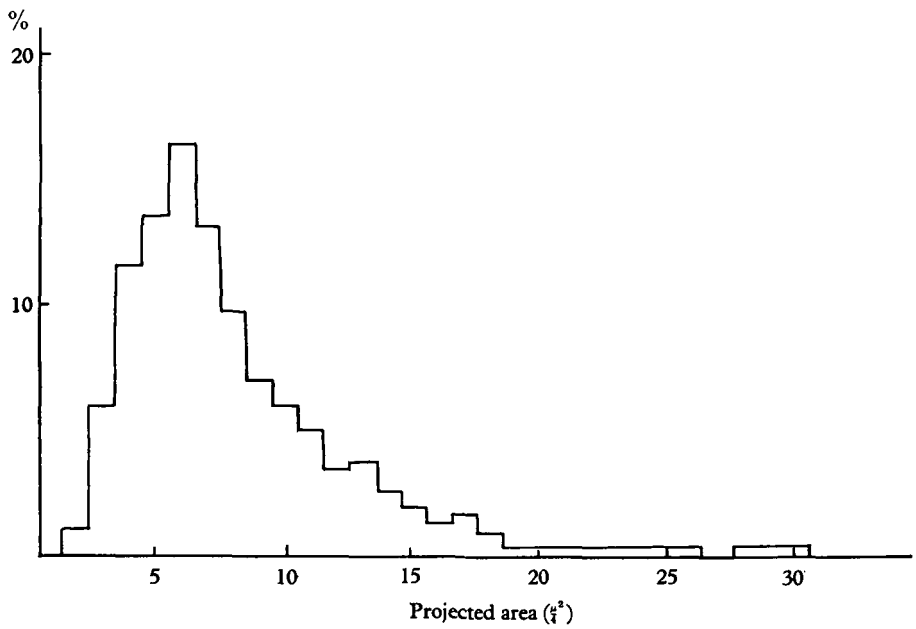
Text-fig. 2. Nuclear area distributions in haploid (unbroken line) and diploid (broken line) homokaryons.

45% diploid and 55% haploid nuclei. In contrast to this, platings of the uninucleate conidia from the heterokaryon gave $19{\cdot}7 \pm 1{\cdot}5\%$ diploid conidia.

Only three slides were used for the sample of 50 hyphae and the cytology was confined to areas photographed. If nuclear distribution is non-random, the discrepancy between nuclear and conidial ratios could be due to sampling error. However, there could be a true difference between these ratios arising from poorer conidiation of the diploid component. Käfer (1961) found that 1 to 2% of conidia from a diploid gave aneuploid colonies. Aneuploids may well be at a disadvantage in producing conidia and certain aneuploid types may produce no conidia at all. The considerable proportion of aneuploids in the hyphae is suggested by two observations. First, the distribution of nuclear areas in a diploid homokaryon is

far broader than the distribution in a haploid, even when a logarithmic scale is used. Secondly, diploid hyphae show a high frequency of very small fragments of green fluorescent material; these are presumably nuclear breakdown products.

Three other heterokaryons were similarly studied. All three had a smaller proportion of diploid nuclei than the one described above. In one case only 0·3% of the conidia were diploid and it was judged, from nuclear measurements, that



Text-fig. 3. Nuclear area distributions in a diploid–haploid heterokaryon.

most hyphal tips had only haploid nuclei. This heterokaryon pointed to a difficulty that occurred, in less extreme form, in the other two. This was an apparent reduction in size of the haploid nuclei compared with those of the haploid homokaryon. In this particular heterokaryon many of the hyphae grew slowly; these were presumed homokaryotic haploid hyphae receiving insufficient nutrition. This led to reduction in both the diameter of the hyphae and the mean volume of cytoplasm per nucleus (Clutterbuck, 1965); it may well be that nuclear volumes are also affected under conditions of semi-starvation.

(v) *The non-random distribution of nuclear types in a heterokaryotic colony*

The possibility of clonal variation in nuclear ratio between sectors of a heterokaryotic colony was mentioned above. Such variation, if it does exist, has importance in relation to the mechanism by which heterokaryosis is maintained.

Table 1 shows the frequency of nuclei of different projected areas in individual hyphae, of two homokaryons and the heterokaryon formed between them. Each group of hyphae is a typical sample. Interhyphal variation within the heterokaryon

Table 1. *Numbers of nuclei with different areas in individual hyphae of a haploid–diploid heterokaryon and its components*

		Nuclear area in $\mu^2/4$																						
	Hypha	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23
Haploid	A	1	11	12	7	11	10	20	5	3	3	2												
	B			14	32	11	3																	
	C				4	10	25	14	16	9	3	3	2		1									
	D			1	6	12	23	15	8	5	2													
	E			2	7	11	8	7	5	5	2													
	F			2	19	18	11	4	1															
	G			2	6	17	22	18	9	3			1											
	H		2	12	64	19	3	1																
	I				4	12	18	9	8	3	1			1	1									
Diploid	A				1		7	1	3	5	3	1	2		2									
	B				1		1		2	2	4		5	2	5		1		1		1			
	C		1	2	3		1	5	1	4	2	3		2	1			1	1		1			
	D				1	1	2	6	13	7	3	2	1	2										
	E				1	1		3	8	4	6	1	2	1	1									
	F									1	3	5	1	1	3	1	2	2						
	G					1	1	1	1	5	5	3	6	2	1		1							
	H					1	1	2	5	2	5	5		3	1	1		1	1					
	I	1			2	1	4	2	4	3	1	3	3	2				1						
Heterokaryon	A					6	4	6	5	2	1	1	3	1	2									1
	B					1	4	3	5		7	3	3	2	4	2	1				1			
	C							4	1	2		1	1	4	1	2	1	2	2		1			
	D							2	1	5	5	5	4	2		4	1	1						
	E							3	2	2	9	2	1	1	2	1	3	2	1					
	F					1	2	3	8	3	5	2			2	1		2						
	G		2	30	25	7	1					1	1											
	H		2	18	10	5		3	1	3	1		1	1		3	1		1					
	I				1			5	7	3	2	1		3	1				1					

is apparent. For example, hypha G has only two diploid nuclei. Distributions in other hyphae, not shown in Table 1, strongly suggest that they are homokaryotic for the haploid component. Homokaryotic diploid hyphae would be less obvious but there is no reason to suppose that they would be less frequent than haploid homokaryotic hyphae.

Some attempt has been made to quantify the overall data in terms of variance of nuclear size within and between hyphae but the analysis is complicated by inter-slide variance (Clutterbuck, 1964). Nevertheless, in the homokaryons the variance between hyphae is much greater than within hyphae; in the heterokaryon, the additional variance, due to mixing of haploid and diploid, appears to go predominantly into the interhyphal component.

4. DISCUSSION

As far as we are aware, acridine fluorescence has not been used previously in extensive studies of fungal cytology. Its advantage for the present work lay in the ease with which it could be used to stain all nuclei. Furthermore, it stains the DNA, while most stains effective in fungi stain only the central bodies of interphase nuclei.

The method of fixation of fungal material has also been seen as an experimental difficulty. Slide cultures have revealed the delicacy of the tip cells which are burst by many commonly used fixatives. Phase contrast observations during fixation showed that even the less violent fixatives, such as osmic acid, cause disturbance of hyphal contents and distortion of nuclei. Some of these difficulties may have contributed to the present controversial state of fungal cytology.

Unfortunately, it has not been possible to make rigorous comparisons of nuclear and conidial ratios of particular heterokaryons. In any event, this would have been really fruitful only if it could have been undertaken as a large-scale survey of many different heterokaryons. However, it is established that nuclear ratios are as variable, between different heterokaryons, as are their conidial ratios, and it is possible that the ratios generally correspond quite well for any particular heterokaryon. Allowances must be made in special cases. In diploid–haploid heterokaryons part of this allowance is probably due to poorer conidiation of the diploid component and its break-down products. In haploid–haploid heterokaryons allowance may also have to be made, on occasion, for poor conidiation of one component. In all heterokaryons there is the problem of 'fill-in' hyphae which develop behind the growing front before conidiation; 'fill-in' hyphae could well have different nuclear ratios from those at the growing front.

One of the main aims of the present work was to probe the mechanisms of heterokaryon maintenance and adaptive response. Pontecorvo (1946) described heterokaryons in terms of the populations of nuclei in each active cell; nuclear ratios might be affected by mutation, migration, selection and drift. He posed the question whether selection could alter nuclear ratios within hyphae, or whether selection operated only between hyphae whose individual ratios differed as a result

of drift. Rees & Jinks (1952) considered that intrahyphal selection must occur to establish a ratio which would then be maintained automatically. Their conclusions were based on the observation that mitosis within each tip cell of *Penicillium cyclopium* is synchronized. Clutterbuck (1964), from phase contrast observations, also has evidence for such synchrony in *A. nidulans*. Furthermore, the relatively low intrahyphal variation in nuclear areas of homokaryons indirectly supports intrahyphal synchrony. But the phase contrast observations showed little circulation of nuclei and, in a linear structure, even one with up to 100 nuclei, drift towards homokaryosis would occur rapidly (Buxton, 1954). Branching of the hyphal tip occurs approximately once per nuclear generation. This would break up some clones but it would provide material for selection rather than uniformly adapted hyphae. Further anastomoses would also assist in nuclear mixing but, in *A. nidulans*, these are very rarely seen in established colonies. The present studies show substantial interhyphal variation; if intrahyphal adjustment occurs at all it is insufficient to suppress such variation. Selection based on interhyphal variation seems very likely, then, to be the mechanism of heterokaryon maintenance in *A. nidulans*. Furthermore, Clutterbuck (1964) has shown, in diploid–haploid heterokaryons, a differential growth rate of individual hyphae correlated with intrahyphal variance of nuclear areas; optimal heterokarotic hyphae are presumably selected in preference to homokaryotic hyphae or hyphae with less advantageous nuclear ratios.

The above considerations are clearly relevant to the adaptive change of nuclear ratios in response to environmental change. This has been shown in *Penicillium* (Jinks, 1952) and *Aspergillus* (Warr & Roper, 1964). Again it is easier to visualize such adaptations at the level of interhyphal selection. Intrahyphal selection would require a breakdown of synchrony; it is difficult to advance plausible biochemical explanations of this for heterokaryons whose components are closely related. In fact, one of the difficulties in the present work probably stems from a problem of synchrony. Clutterbuck (1965) has shown that the mean volume of cytoplasm per nucleus, in diploid strains, is twice that for haploids. If the mean volume of cytoplasm per nucleus is related to the mitotic 'trigger', hyphae with diploid and haploid nuclei may have difficulty in maintaining synchrony. In diploid–haploid heterokaryons there appears to be a high rate of diploid breakdown, and diploidization of the haploid component has been found on several occasions. These events may well reflect an attempt to restore a common ploidy and maintain synchrony.

It would be hazardous to attempt to apply our conclusions outside *A. nidulans* and certain closely related species. For example, the situation in *Neurospora* is different. Heterokaryons of *Neurospora* do not show adaptive change; they remain constant in nuclear ratio even when a change would lead to faster growth rate (Pittenger & Atwood, 1956). This can be interpreted in the light of the findings of Ryan, Beadle & Tatum (1943) who showed that the population of active nuclei extends for 1 cm. behind the growing tips. Since the nuclei are in rapid circulation drift and interhyphal variation are insignificant. The absence of adaptive response in *Neurospora* does, however, support one of our main contentions for *Aspergillus*,

which is that intrahyphal selection does not occur. It is quite clear that in *Neurospora* there is no effective mechanism for the preferential selection of individual nuclei.

SUMMARY

A cytological study has been made, in *Aspergillus nidulans*, of haploid and diploid homokaryons and of heterokaryons formed between them. The effective region of growth was shown to extend about 600 μ behind the growing edge of the colony and to correspond largely with the tip cells. Acridine fluorescence gave unselective staining of all nuclei; projected nuclear areas, and their distributions in individual hyphae, were determined for the homokaryons and heterokaryons. Diploid and haploid nuclei differ in mean projected area but the overlap of their size distributions precluded unequivocal classification of individual nuclei in diploid–haploid heterokaryons. However, it was shown that the hyphae of a heterokaryon differ in nuclear ratios. This, and other evidence, strongly support the suggestion that heterokaryon maintenance and adaptation occur through interhyphal rather than intrahyphal selection.

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