STUDIES ON HETEROCARYONS IN NEUROSPORA

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INTRODUCTION

Heterocaryosis is the association, in a cell or mycelium, of genetically different nuclei in a common cytoplasm. Many of the Fungi Imperfecti are known to be heterocaryotic. This may serve as a mechanism which allows accumulation of a reserve of variability by mutation. Selection of nuclei within the mycelium might serve a similar purpose to reassortment of genes in sexually reproducing fungi. In the higher Basidiomycetes, one phase of the life cycle is passed as a stable dicaryon, the cells of which contain nuclei of both mating types. Naturally occurring heterocaryons have also been found in various Ascomycetes.

Heterocaryons can be formed in two ways; by mutation in one or more nuclei of a previously homocaryotic mycelium or by hyphal fusion and subsequent mixing of nuclei and cytoplasm of two dissimilar strains. The latter process is a complicated one and in Neurospora crassa, for example, is under the control of a number of genes. Strains of the same mating type form heterocaryons more readily than strains of opposite mating type. Garnjobst (1953, 1955) and Holloway (1955) have shown that homocaryons must be similar at a number of other loci before a heterocaryon with wild type growth rate and morphology is obtained. Incompatibility effects range from complete prevention of anastomoses between hyphae to anastomoses with little subsequent growth and from irregular phasic growth to steady but submaximal growth.

The process of hyphal fusion is facilitated by the component strains being auxotrophic, haveing different growth requirement factors, due for instance, to inability to synthesise a particular active enzyme. Inocula of conidia of the two strains are superimposed on minimal medium, which cannot support growth of either strain after germination but upon which the heterocaryotic mycelium formed by hyphal fusion can grow. The accepted explanation for growth of these inter-genic heterocaryons is that one nucleus supplies the enzyme or other growth factor which the other lacks.

Heterocaryons can also be made from auxotrophs, lacking the same enzyme and requiring the same factors for growth, whole mutations in fact map at the same locus. Such complementing mutants are assumed to have changes in functionally different sites within the locus. The fact, however, that a pair of alleles each deficient in the same enzyme, when together in a heterocaryon produce an active enzyme is less easily explainable than the situation in inter-genic heterocaryons.

The original purpose of this thesis was to use inter-genic heterocaryons between mutants of the arginine cycle in such a way that by manipulating nuclear ratios and hence relative levels of pairs of enzymes certain situations might be investigated and questions answered; what is the minimum level of each enzyme which could maintain normal output of the system as measured, for example, by the level of the arginine pool - are all enzymes equally important in this respect or is there a single 'control' step where enzyme level must be

critically maintained? Such a situation as the latter might manifest itself as selection in the heterocaryon to maintain the level of one enzyme at the expense of the other. These and other aims concerning regulation and control of biosynthetic pathways were not, for reasons given below, realised.

In a previous investigation (Diploma thesis, 1961) a heterocaryon had been synthesised from homocaryotic auxotrophs arg.-1$^-$ and arg.-10$^-$, the former lacking argininosuccinic synthetase and the latter lacking argininosuccinase, these enzymes catalysing successive steps in the arginine pathway. This heterocaryon was found to have an extreme ratio in favour of arg.-10$^-$ nuclei. Indeed all heterocaryons of these mutants originating from single heterocaryotic conidia had similarly extreme ratios. At first sight this seemed to point to the fact that the synthetase is a less dispensable enzyme than argininosuccinase, the production of the former being increased in the heterocaryon by selection for arg.-10$^-$ nuclei.

That these extreme ratios were not due to the arginine mutants was established in later experiments, when colonies from single conidia were grown on arginine supplemented medium. On this medium the homocaryons are fully supplemented and grow at wild type growth rates. The heterocaryons, however, still continued to produce extreme nuclear ratios, showing that any possible arginine requirement within the heterocaryon was not the cause of extreme ratios.

Preliminary investigations indicated that, in these and similar heterocaryons originating from single heterocaryotic conidia, there was a definite and regular change in nuclear ratio of the mycelium as growth progressed. It was decided to concentrate upon this problem since it was not only an unusual situation but an opportunity to add to the general knowledge of an organism so widely used in genetical and biochemical investigations. In particular it was also thought that an understanding of this system would allow it to be used as an additional tool in the sort of enzymatic and biochemical studies mentioned previously. It is important too, in many cases, that any interaction between nuclei in a heterocaryon be due solely to the mutants under investigation, and that background complications such as compatibility effects and ratio changes be recognisable and avoidable.

Beadle and Coonradt (1944) first postulated that in a heterocaryon, selection against hyphae at the growing front would result in changes in nuclear ratios. This assumed that the front consisted of a population of hyphae each with a different nuclear ratio and that nuclear ratio was correlated with growth rate. Pittenger and Atwood (1956), however, showed that _Neurospora crassa_ heterocaryons with a wide range of nuclear ratios are of normal growth rate and maintain their ratios over a wide variety of conditions. At very extreme input ratios, 50-100 to 1, heterocaryons frequently had

sub-maximal growth rates. This was probably due to limiting amounts of the enzyme whose synthesis was controlled by the minority class of nuclei since, on the supplement required by the majority nuclear class, growth rates were normal. On minimal medium there were no changes of nuclear ratio to less extreme values at which growth would have been normal. They also showed that when hyphal tips are isolated from a slow growing heterocaryon with a ratio of approximately 500:1 some grow at suboptimal rates, others grow maximally and others fail to grow. They infer from these results that it is the overall nuclear ratio of the mycelium that is important for growth rate, and that a transport system of enzymes or metabolites extending over only one centimeter, would allow a large number of nuclei to contribute to the front. Selection of hyphal tips having, by chance, favourable ratios therefore appears not to operate strongly.

Kiritani (1959) on the other hand, using heterocaryons of _Aspergillus oryzae_ whose component strains were fast and slow growers on casein medium, found that on this medium there was selection within the mycelium of heterocaryons for a high proportion of the fast growing nuclear type. There were significant changes between initial nuclear ratios and ratios measured after a period of growth. Aspergillus differs from Neurospora in being much less stable as heterocaryons and hence perhaps much more sensitive to metabolic conditions.

Non adaptive selection, so called because it results in an increase of one nuclear type in a heterocaryon with no obvious beneficial result to the organism, has been reported on a number of occasions. Ryan and Lederberg (1946) found that hyphal tips, isolated from a Neurospora crassa heterocaryon between a leucineless mutant and a prototrophic revertant of the same mutant, became homocaryotic for leucineless nuclei when grown on leucine supplemented medium. Hyphal tips also isolated at random on to minimal medium continued to grow, so that they must have contained prototrophic nuclei. A similar phenomenon was found by Davis (1960). N. crassa pan-1,m homocaryons, are pantothenate requiring mutants in which a gene,m,modifies uptake of pantothenate with the result that these mutants grow on a concentration of pantothenate upon which pan-1 mutants grow little or not at all. In heterocaryons between the two, pan-1 m nuclei are often selected against on limiting medium. Changes in nuclear ratio in this case were associated with changes in growth rate.

The same sort of behaviour has been found in another fungus Aspergillus glaucus. In an attempt by Jinks (1959) to produce spontaneous vegetative death in unaged material, a homocaryon was irradiated with U.V., and some colonies were recovered which grew for a time and then stopped. These colonies showed all signs of the vegetative death phenomenon.

In this case, however, the effect was reversed by growth on casein medium. Further investigation revealed that a gene mutation had occurred, and that death of the mycelium was due to a non adaptive increase in the auxotrophic nuclei.

Another manifestation of nuclear selection is to be found associated with the Buller Phenomenon, that is the dicaryotization of a homocaryon by a dicaryotic mycelium. [Kimura (1958), and Ellingboe and Raper (1962).] In a mating between a dicaryon and a homocaryon one expects that the derived alternate dicaryons would occur in equal frequencies. Kimura studying _Coprinus macrorhizus_ ascribed the occurrence of inequalities in the numbers of the two types of derived dicaryons to cytoplasmic relationships. Ellingboe and Raper found that in _Schizophyllum commune_ that the primary control of selective behaviour was due to genes of the incompatibility system but also in some cases due to the influence of other genes.

Possibly the most interesting work to date on nuclear selection in _N. crassa_ heterocaryons is that of Pittenger and Brawner (1961). They suggest that non adaptive nuclear selection in heterocaryons composed of various pairs of auxotrophs is controlled by a pair of allelic genes, I and i. I nuclei inhibit the multiplication of nuclei of i genotype and this is independent of any other genes used to force the formation of the heterocaryons. I is only 'weakly dominant' to i and an increase of I nuclei only takes place if

initially they compose 30% or more of the nuclear population.

Operationally this latter system is similar to the one to be described in this thesis. The main conclusion in the present case is that selection is, however, the result of nucleo-cytoplasmic factor interactions, rather than gene-gene effects. A system is suggested to explain the experimental results, namely that selection to extreme ratios is due to interaction in the heterocaryon between a gene in one of the two homocaryon's nuclei and cytoplasmic elements. The result of this interaction is inhibition of division of nuclei of the other homocaryon. The elements replicate in the heterocaryon, and are introduced into the heterocaryon by the homocaryon whose nuclei form the minor component of the selected heterocaryon. There appears to be a threshold value below which these elements do not increase and there is no selection, ratios remaining at intermediate values.

In those heterocaryons where no selection to extreme ratio operates, between the two arginine mutants, the final ratios, after a period of growth, appear to be determined within broad limits by requirements for an optimum ratio of the two enzymes affected by the arginine mutations.

MATERIALS AND METHODS

Strains

The wild type *Neurospora crassa* used was St. Lawrence strain (ST*A*) and also a wild type (3*A*) derived from the third backcross to ST*A* of an arginine mutant B362-3-1*a*. The arginine requiring mutants B362-3-1*a* (arg.-10$^-$) and 46004-1-10*a* (arg.-1$^-$) were obtained by this laboratory from Dr. D. Newmeyer. Their genetic background closely resembles that of ST*A*. B362 was isolated, after gamma irradiation, by Dr. V.W. Woodward, and 46004 is described by Srb and Horowitz (1944).

Arg.-10$^-$ lacks any detectable argininosuccinase, which converts argininosuccinase to arginine and fumarate, and arg.-1$^-$ lacks the enzyme responsible for the conversion of citrulline to argininosuccinate.

To avoid confusion of nomenclature of the above mutants and mutant progeny from backcrosses,the stock name will only be used when identification is important,otherwise mutants and mutant nuclei in heterocaryons will be referred to as arg.-10$^-$ and arg.-1$^-$.

Culture methods

Stocks were carried on agar slants of Vogel's minimal medium, 2% sucrose, 2% Difco agar and 2% Vogel's synthetic medium (1955). This medium was supplemented with 0.05% arginine for growth of the mutants. Stocks were transferred every

three or four months, or when fresh material was required, by massive conidial inocula. The same medium was used in growth tubes and plates. A 6% malt agar medium was used when increased growth rates were required and proved useful in producing abundant conidiation in arg-10^{-} strains which normally have very few conidia. Restricted, colonial growth on plates was achieved by using a 1% sorbose, 0.1% sucrose medium. Crosses were made on Westergaard's synthetic medium using 6% malt agar and also on a medium consisting of 1.5% corn meal agar, and 0.05% sorbose. Except where stated to the contrary, plating and growth rate determinations were carried out at 25°C.

Crosses were made by allowing one mating type to form protoperithecia on a slant and then flooding with a suspension of conidia of the opposite mating type. Ripe ascospores, ejected on to the walls of the tube, were washed off, spread at a low dilution on supplemented agar plates and activated at 57°C. for 2-3 hours. After germination and overnight growth, colonies were picked and wild and mutant types separated by testing for growth on unsupplemented medium.

Construction of heterocaryons

Heterocaryons were generally synthesised by superimposing loops of conidial suspension of the two mutants on a minimal slant, each mutant being inoculated separately as

a control. Growth occurs from the double inoculum presumably as soon as two unlike conidia germinate and anastomose. The possibility must be excluded that growth is the result of cross feeding between hyphae, each mutant supplying the other's nutritional requirements. Conidia from the double inoculum culture were therefore plated on minimal medium where only heterocaryotic conidia can germinate and form colonies. Such colonies were picked, grown up and used for further studies. The concentration of conidia on the plates was low enough to ensure that only colonies arising from single conidia would be picked.

Using this method there is no means of controlling the initial ratio of the heterocaryon. The nuclear ratio is limited by the number of nuclei in the conidium, and the proportions of the different nuclear types in the conidium depends on the nuclear ratio of the mycelium producing the conidia.

A second method of constructing heterocaryons, described by Pittenger and Atwood (1955), can be used to control the initial nuclear ratios between fairly wide limits, the maximum final ratio achieved being 80 : 1. Various proportions of conidial suspension are mixed together and soaked overnight in supplemented liquid medium at 5°C. The mixtures are then incubated for 2-4 hours at 25°C., centrifuged, and the pellets pipetted on to supplemented sorbose plates.

After three days the coalesced pellets are transferred to growth tubes, when after a period of growth on minimal medium, the final nuclear ratios can be measured. It is assumed that this treatment allows multiple anastomoses throughout the pellet, while at the same time restricting growth. When the pellets are transferred to minimal medium, the actively growing mycelium will initially have a nuclear ratio which corresponds closely to the proportions of different nuclei in the original mixed conidial suspensions.

Measurement of nuclear ratios.

Nuclear ratios were measured by a method based on that of Prout et al. (1953). If a heterocaryon has two types of nuclei, A and B, conidia are produced which are homocaryotic for A, homocaryotic for B, or are heterocaryotic with both A and B nuclei. These three types are in the proportion a:b:h where a + b + h = 1. Assuming a random distribution of nuclei into conidia, the frequency of the three types are:

$$a = p\,\emptyset(1) + p^2\emptyset(2) \ldots\ldots\ldots p^n\emptyset(n) = \sum_{n=1}^{k} p^n\emptyset(n)$$

$$b = q\,\emptyset(1) + q^2\emptyset(2) \ldots\ldots\ldots q^n\emptyset(n) = \sum_{n=1}^{k} q^n\emptyset(n)$$

$$h = 1 - \left[\sum_{n=1}^{k} p^n\emptyset(n) + \sum_{n=1}^{k} q^n\emptyset(n)\right]$$

where p is the frequency of type A nuclei in the parent mycelium and q = 1-p and is the frequency of type B nuclei. ϕ is the frequency of conidia with n nuclei.

Atwood and Mukai (1954) tested the hypothesis that nuclei are distributed into conidia at random. Using the same heterocaryon grown on different concentrations of sorbose, which changes nuclear ratios and numbers of nuclei per conidium, they found that in four out of seven cases there was a departure from randomness in that like nuclei tend to occur in the same conidium. An alternative formula to that of Prout's was proposed which took into account this finding. Klein (1958) found no evidence to support Atwood and Mukai and suggested also, that their conclusions were at variance with their data. Since the estimation of p by the two methods differs by less than 1%, Prout's method, more easily applied in this case, was used for all nuclear ratio estimates.

Nuclear ratio determinations from estimations of the frequency of the two types of homocaryotic conidia was ruled out,because both mutants have to be supplemented with arginine,and consequently conidia cannot be distinguished by growth on different media. It might be expected that arg.1^- would be supplementable with argininosuccinate as well as arginine,but for unknown reasons it will only grow on the latter.

For each heterocaryon, the frequency distribution of nuclei in conidia was determined,and the proportions of a

and b conidia were calculated over a range of values for p and q according to Prout's formula. The sum of the proportions of a and b conidia, subtracted from unity, gives the expected proportion of heterocaryotic conidia for a given value of p or q. A graph of the proportion of heterocaryotic conidia corresponding to p was plotted, so that the estimated frequency of heterocaryotic conidia could be used to find the nuclear ratio of a heterocaryotic mycelium.

The proportion of heterocaryotic conidia produced by a heterocaryon was estimated by plating equal concentrations of conidia on minimal and arginine sorbose plates. The number of colonies growing on minimal plates (from heterocaryotic conidia only) divided by the number on arginine (from both homocaryotic and heterocaryotic conidia) gives the required figure. To identify the majority class of nuclei in the heterocaryon, mutant colonies from the arginine plates were picked, grown up, and identified by complementation tests.

A quicker method for estimating the proportions of heterocaryotic conidia was later developed. A loopfull of conidial suspension was streaked across a minimal sorbose plate which was then incubated at 25°C. for 15-20 hours. By this time heterocaryotic conidia had developed into tiny colonies which along with the ungerminated homocaryons were counted using a low power microscope with an eye-piece grid.

Homocaryotic conidia produce at most a short germ tube and are easily distinguishable from those conidia which are growing and forming colonies. This method compares favourably with plating, see Fig. 1, and results in a considerable saving in time and materials. The concentration of the conidia suspension is not critical for streaking and can be judged by eye. The concentration of conidia tends to fall off towards the end of the streak,so that,even when the numbers of growers and non growers are equal,it is possible to find parts of the streak where growing colonies are distinct from each other,and ungerminated conidia not obscured.

Heterocaryons whose nuclear ratios were to be measured were inoculated as single colonies on to growth tubes. Once the front had reached the other end of the tube, after approximately 300 mm. of growth, and conidiation had occurred, the nuclear ratios were measured as described. The growth tubes allowed sufficient growth for the ratios to become stabilised before measurement.

Measurement of nuclear ratio change

Change in nuclear ratio with time,or distance moved by the mycelial front,was determined in two ways. In the first method a growth tube with funnels at 5 cm. intervals along its length was inoculated. Approximately 48 hours after the front had passed beneath, conidia begin to form in and around the funnels and also at both ends of the tube.

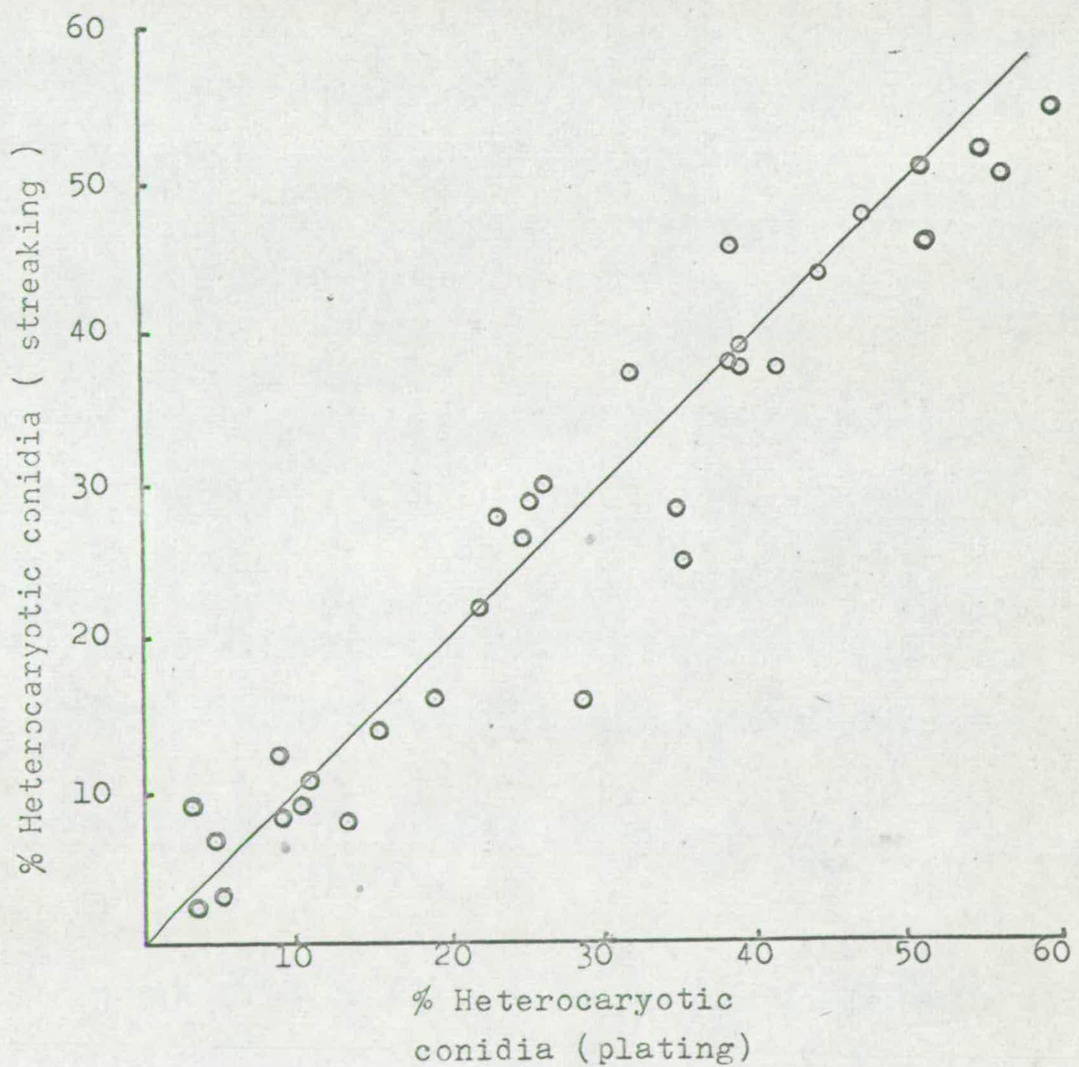


Fig.-1. Comparison of estimated % heterocaryotic conidia by plating and streaking methods

The nuclear ratio of the mycelium forming the conidia can then be determined. The second method allows nuclear ratios to be measured at any desired interval of time, and to measure the ratio of mycelium from conidia whose production starts within 24 hours after sampling. A large petri dish, 15 cm. in diameter, was inoculated and as the front progressed samples of mycelium were removed from it by punching out mycelium and agar with short sterile glass tubes one centimeter in diameter. The tube plus samples was sealed at one end by pressing it into sterile plasticine and at the other end with a cotton wool plug. After 24 hours at 25°C. aerial hyphae had been produced and in two to three days the conidia were ripe enough for nuclear ratios to be measured.

Measurement of growth rate

The type of growth tube used is shown in Fig. 2. Each tube, sterilised and plugged was filled with 10 mls. sterile medium pipetted in via the centre funnel. The tubes were normally inoculated with single colonies previously grown up on non sorbose plates. Progress of the front was marked at regular intervals on the outside of the tube. Approximately 300 mm. of growth was possible in the tube.

Staining and counting of conidial nuclei

The staining of nuclei was carried out using the following procedure. Coverslips were smeared with glycerine

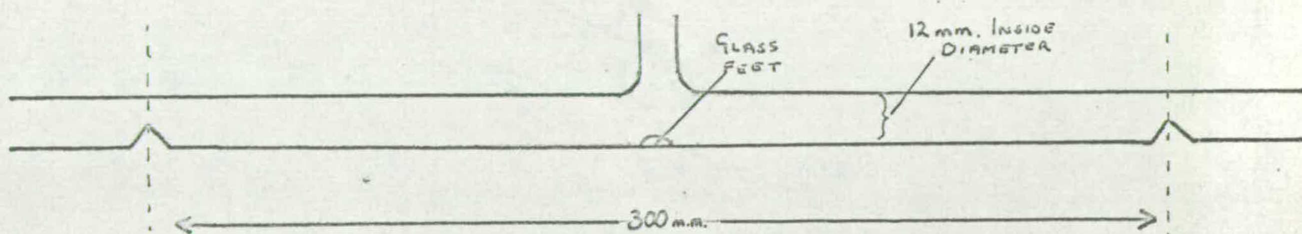


Fig.-2 Growth tube with central filling and sampling funnel

albumin and loopfuls of conidia patted over the surface. The coverslips were then immersed in Carnoy's fixing fluid for 1 hour and then hydrolysed in 1N HCl at 60° C. for 12 - 14 minutes. This was followed by staining for 2 hours in 0.02% aqueous solution of Azure A to which a few drops of thionyl chloride had been added. It was found useful to counter stain for ½ minute in 0.01% aqueous solution of orcein to make the outlines of the conidia easily discernable. After staining the conidia were dehydrated and mounted in Canada balsam.

The nuclei of the conidia were counted using an oil immersion lens.

Relationship between enzyme level and nuclear ratio.

In the interpretation of the following experiments it will be assumed that nuclei are distributed into conidia at random and there is a definite relationship between mycelial nuclear ratio and the proportions of homocaryotic and heterocaryotic conidia which a mycelium produces. Other workers (page 15) have shown that such an assumption is justified and departures from randomness, if any, are negligible.

It might, however, be argued in the case under investigation that the phenomena observed are precisely due to processes resulting in non random distribution of nuclei into conidia. Thus the situation could be envisaged in which, for one reason or an other, mycelial nuclear ratios are intermediate but there is a selective distribution of nuclei into conidia so that the majority of conidia contain only one kind of nucleus.

It is possible to obtain information about this by enzyme investigations. If a mycelium contains a minority of arg.-1$^-$ nuclei, then a correspondingly low level of argininosuccinase, as determined in mycelial extracts, would be consistent with such a situation, since the arg.-10$^-$ cannot synthesise this enzyme. On the other hand, if the nuclear ratio as measured from the proportions of the conidial types does not correspond to the mycelial nuclear ratio, the level of argininosuccinase would be greater than expected.

Wild type and heterocaryotic mycelia were grown on minimal slants and conidia from each mycelium were inoculated

into a number of 12 oz. bottles containing liquid medium. They were incubated at 25°C. and, at intervals, the contents of a bottle were harvested to provide a time series of samples. The mycelia were washed, freeze dried and a crude extract assayed for argininosuccinase activity according to the method given by Fincham (1957). When the heterocaryotic mycelium was harvested a small sample of mycelium from each bottle was removed and placed in an empty sterile tube. After 24 hours, aerial hyphae and conidia were being produced directly from the mycelial sample. Once the conidia were mature they were plated out and measured in the usual way.

In Fig. 2a the enzyme level in specific activity units are shown for each culture at the time of harvest. Nuclear ratios of the heterocaryotic mycelium samples are also given. (In the wild type the equivalent of the nuclear ratio is of course 100%).

It can be seen that the argininosuccinase activity of the heterocaryon is about 1/7th that of wild type and the proportion of argininosuccinase producing nuclei, by the plating method, is about 1/10th of the total number of nuclei in the heterocaryon. Assuming that wild type and the heterocaryon have the same total numbers of nuclei, the enzyme results are consistent with the nuclear ratio estimates in that a reduction in numbers of arg.-1$^-$ nuclei

corresponds to a low enzyme level in the mycelium. The discrepancy between approximately 1/7th and 1/10th may be due to small nuclear ratio change, during slight growth before conidiation, being in the right direction for the trend in the time course.

Donachie (1964) has also reported a high correlation between nuclear ratio and enzyme activity in Neurospora crassa. In Donachie's system, aspartate transcarbamylase activity was assayed in heterocaryons of different stable ratios originating from conidial pellets.

The evidence given above is quite consistent with the idea that low enzyme levels are indicative of low numbers of nuclei involved in the production of the enzyme. There is no reason to suspect that there has been a differential distribution of nuclei into conidia. It is probable that the estimated nuclear ratios, calculated from the different proportions of homocaryotic and heterocaryotic conidia, are a close approximation to the actual mycelial nuclear ratios.

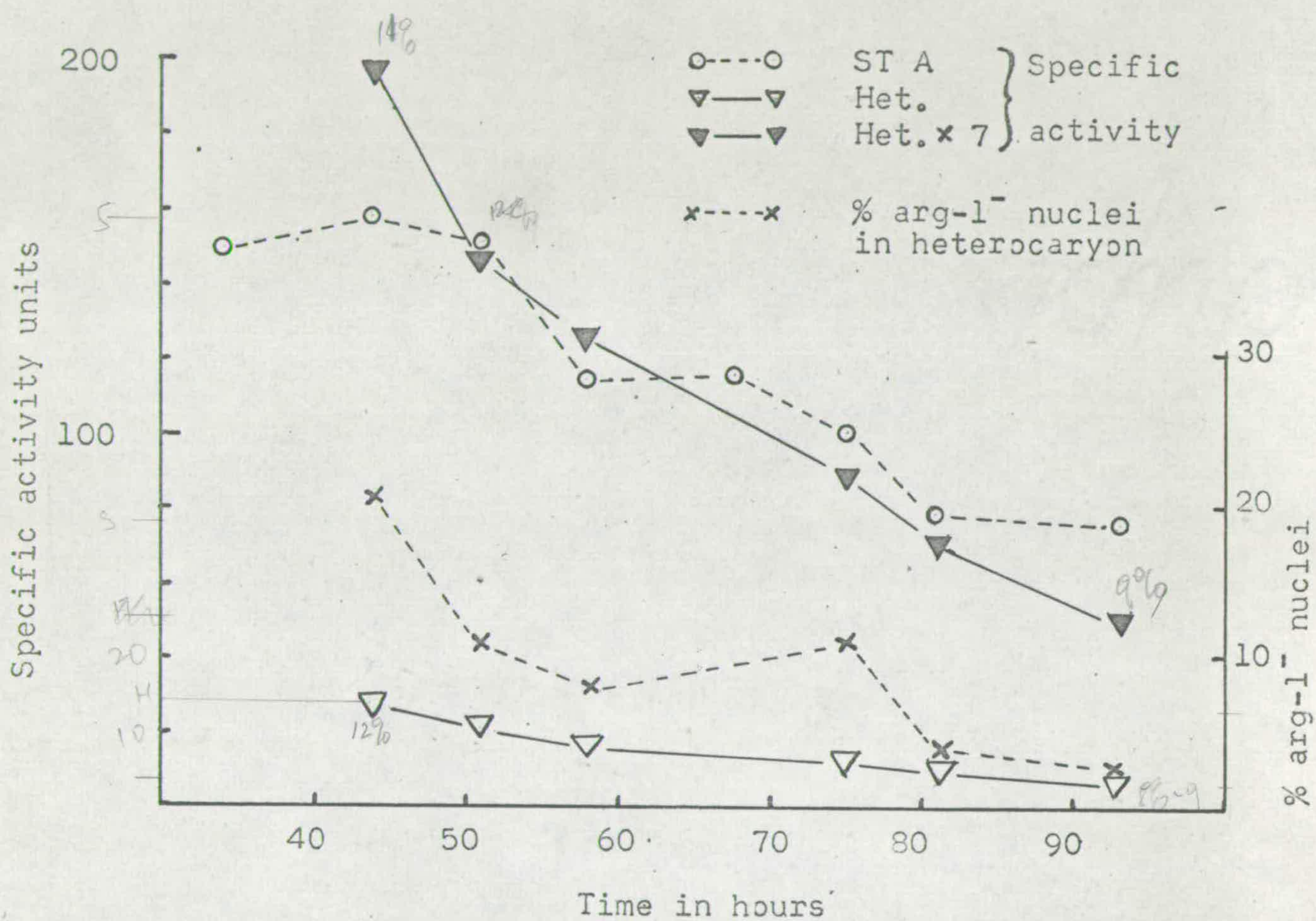


Fig.-2a Specific activity of argininosuccinase in wild type (ST A) and an arg.-10⁻;arg.-1⁻ heterocaryon. % argininosuccinase producing nuclei (arg-1⁻) in the heterocaryon is also shown.

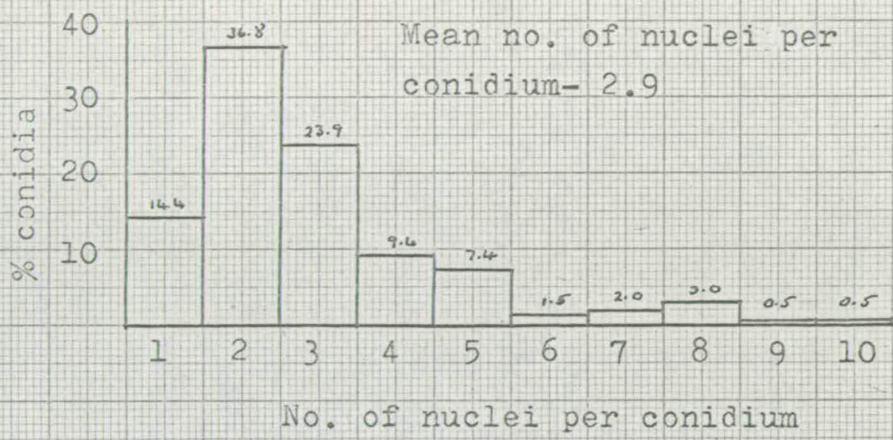
RESULTS

Section I

The experiments to be described later are concerned with situations in which mycelial nuclear ratios changed with time or growth of mycelium. The changes generally took place in the direction of extreme or disproportionate ratios of the two types of nuclei in the mycelium of a heterocaryon. An understanding of the relationship between conidial ratios,(the ratio of the two types of nuclei in the conidium,)and mycelial ratios,(the ratio of the two types of nuclei in mycelium derived from a conidium,)is necessary before the results of such experiments can be interpreted.

As has been outlined in a previous chapter, the nuclear ratio in the mycelium of a heterocaryon was estimated from the frequency of heterocaryotic conidia which the mycelium produced. The frequency value was then referred to a curve relating frequency to the ratio of the two types of nuclei in the mycelium. Figs. 3-6 are a number of such curves for various heterocaryotic mycelia along with the distributions, from which the curves were calculated, of numbers of nuclei in conidia produced by that mycelium.

Once the mycelial ratio has been estimated, it is then necessary to deduce the initial ratio of the mycelium, i.e. the nuclear ratio of the heterocaryotic conidium from



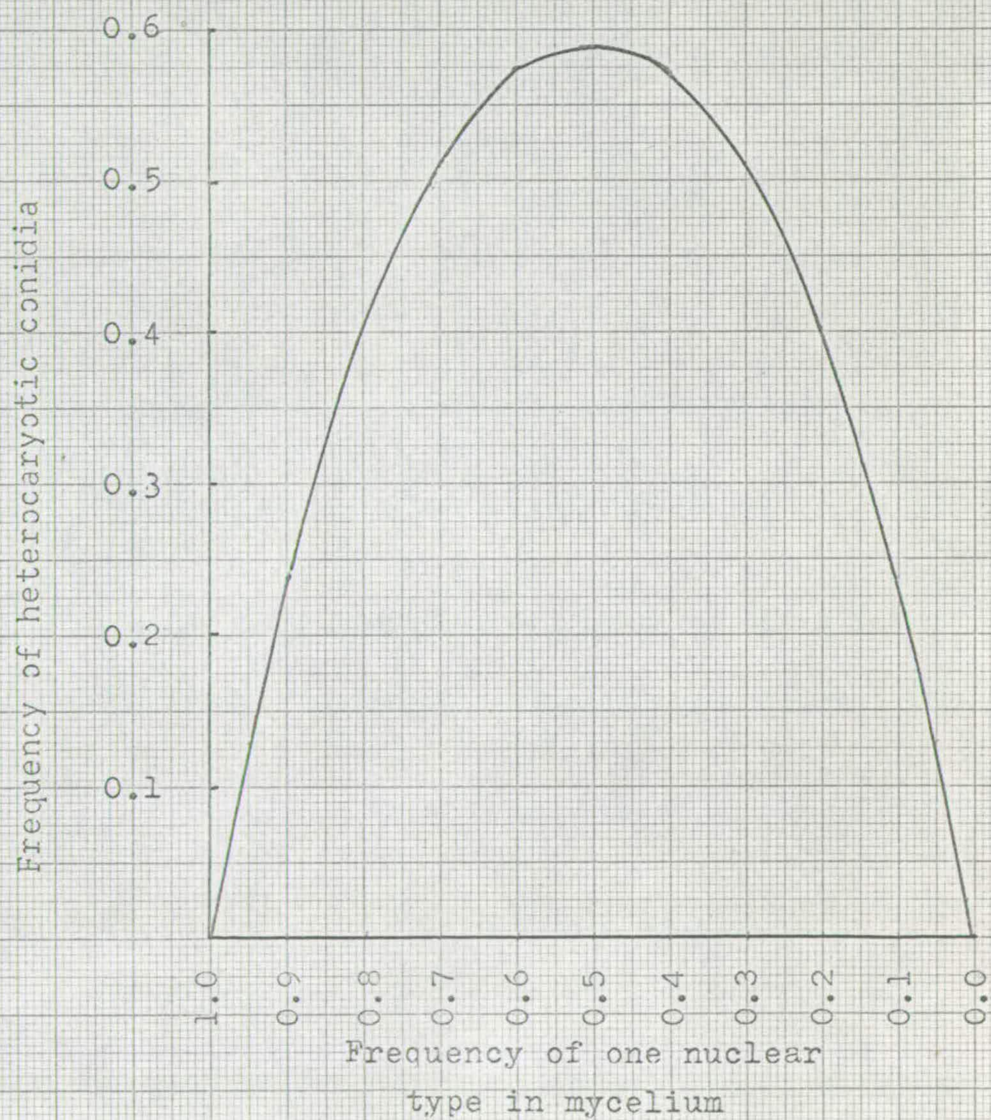


Fig-5 Distribution of nuclei in conidia of B362-18;46004-2 and corresponding frequencies of heterocaryotic conidia expected from mycelium with various proportions of one of the two types of nuclei

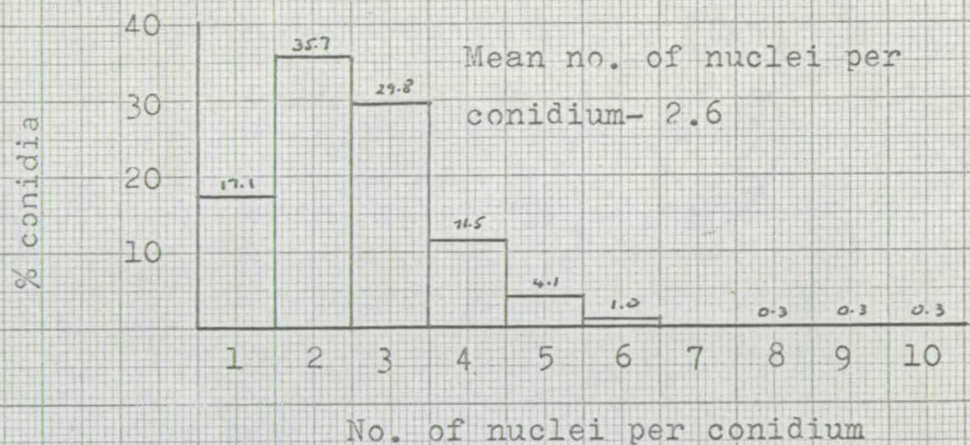
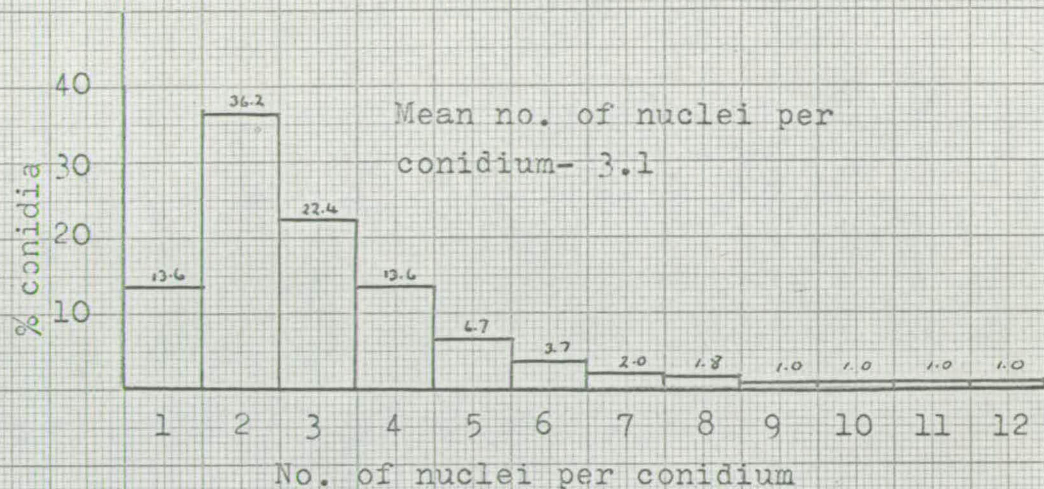


Fig.-6 Distribution of nuclei in conidia of B362-12;46004-10 and corresponding frequencies of heterocaryotic conidia expected from mycelium with various proportions of one of the two types of nuclei



Frequency of heterocaryotic conidia

0.6
0.5
0.4
0.3
0.2
0.1

1.0 0.9 0.8 0.7 0.6 0.5 0.4 0.3 0.2 0.1 0.0

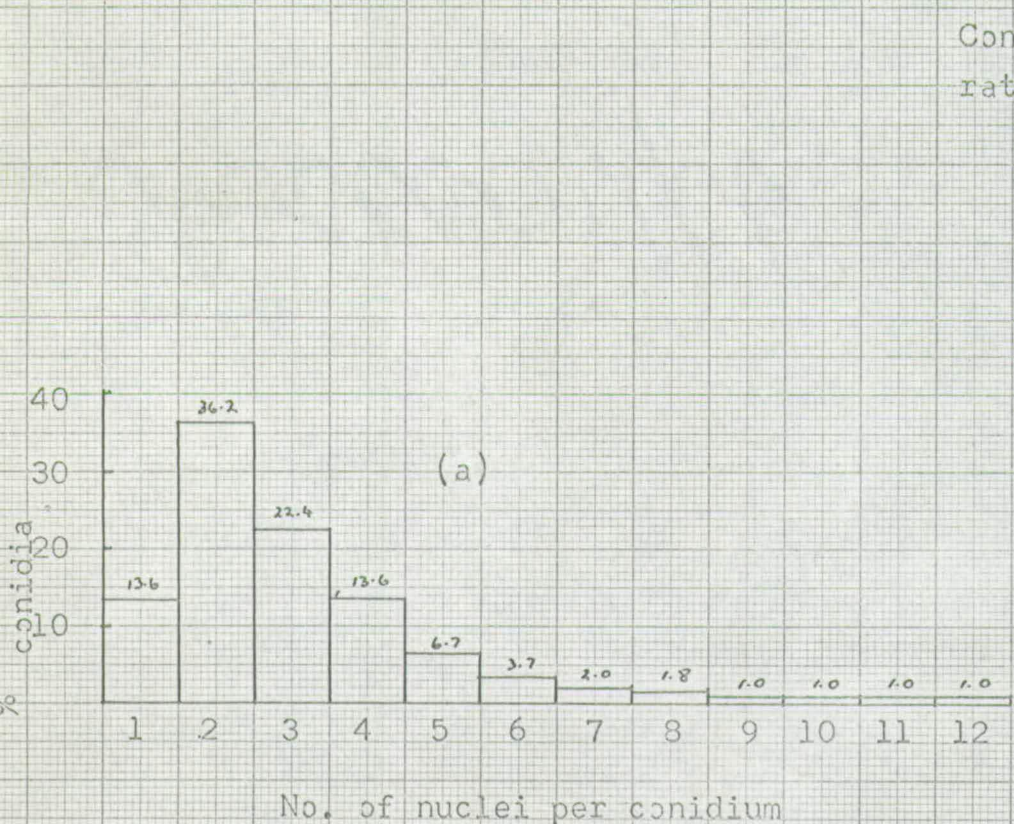
Frequency of one nuclear type in mycelium

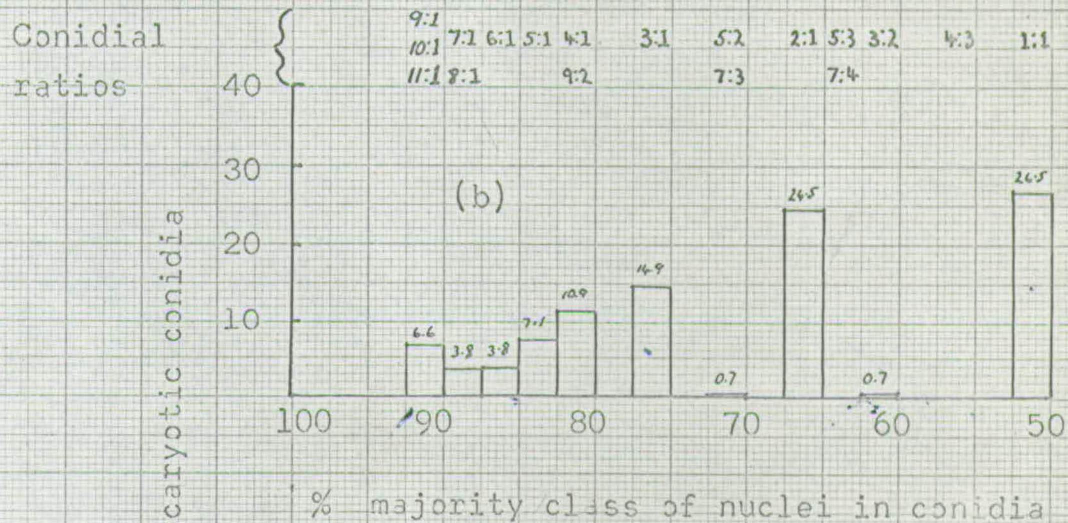
Fig.-4 Distribution of nuclei in conidia of B362-32;46004-2 and corresponding frequencies of heterocaryotic conidia expected from mycelium with various proportions of one of the two types of nuclei

which the mycelium arose, before it can be concluded that a change in mycelial nuclear ratio has occurred. The ratio of the starting conidium cannot be determined directly, since the method of estimation involves a cycle of growth and conidiation, but the probability of it having a particular ratio can be calculated.

Assuming that the hypothesis of random distribution of nuclei into conidia holds, then nuclei will be distributed into conidia according to a binomial distribution. From the expansion of this distribution and the frequencies of conidial types i.e. with 1, 2, 3, ... etc. nuclei, the expected array of conidial nuclear ratios can be found. Fig. 7 gives the calculated distribution of conidial nuclear ratios for a given frequency of conidial types from a mycelium of heterocaryon B362-32;46004-2. The calculation has been done assuming a 50:50 mycelial ratio (7c) and also a 95:5 mycelial ratio (7b). In both cases the predominantly conidial ratios are 1:1, 2:1, and 3:1, but as the ratio of the mycelium becomes more disproportionate the expected numbers of disproportionate ratios in conidia increases.

Using the data in Fig. 7, it is then possible to set limits upon the expected numbers of conidia which would produce extreme ratio heterocaryons if conidial ratios were maintained during germination and growth. At a mycelial nuclear ratio of 50:50, 0.06% of heterocaryotic conidia produced will have





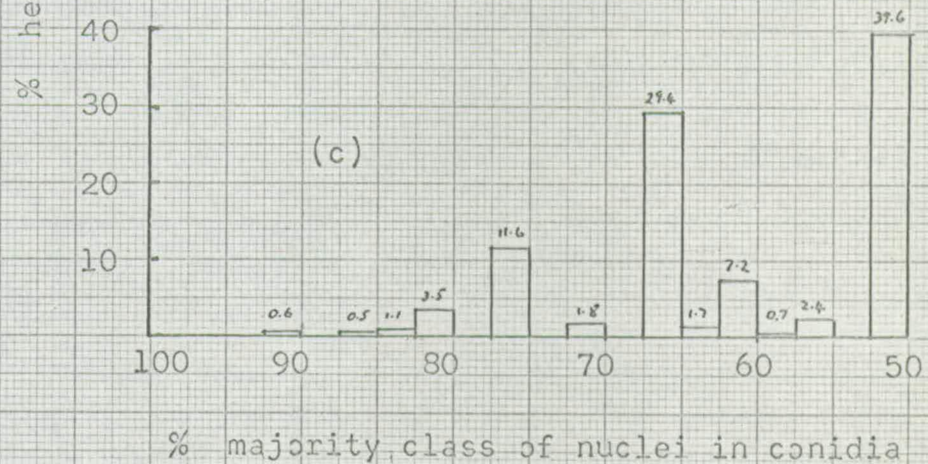


Fig.-7 Distribution of nuclei in conidia of heterocaryon B362-32;46004-2 (a)
Expected distributions of nuclear ratios in conidia produced by this heterocaryon assuming two different mycelial nuclear ratios
(b) 95% : 5%
(c) 50% : 50%

ratios from 9:1 to 11:1, i.e. 90 - 91.7% majority class of nuclei (maximum number of nuclei per conidium in this heterocaryon is 12). At a mycelial ratio of 95:5, 6.7% of conidia will have ratios of 90 - 91.7% majority class of nuclei.

Since in making up heterocaryons by the superimposition of inocula, roughly equal sized inocula are used and no restriction is placed on growth from the mixed inoculum, it is unlikely that, once the first anastomoses occur and growth out of the inoculum begins, extreme mycelial ratios will occur. It was assumed, therefore, that when heterocaryons from single heterocaryotic conidia had ratios of 90% or over, of one class of nuclei, that changes of nuclear ratio had taken place during growth. This limit was assumed for all heterocaryons, since it was calculated for the heterocaryon which had the greatest range in numbers of nuclei per conidium, and hence the greatest proportion of conidia with extreme ratios.

Concerning the accuracy of the estimated distributions of nuclei in conidia for the various heterocaryons in Fig. 3-6, assume that differences between the distributions are due to sampling variance. It can be seen that the error involved in estimating nuclear ratio decreases asthe ratios become more disproportionate. The range in values of heterocaryotic conidia frequencies corresponding to 50% of each nuclear type in the mycelium is 0.55 - 0.625, and the range in values of heterocaryotic conidia frequencies corresponding to 90%

majority of one nucleus in the mycelium is 0.205 - 0.245. Most of the work in this thesis is concerned with estimations of extreme mycelial ratios, and the accuracy of these determinations will be little influenced by any differences in estimated numbers of nuclei per conidium.

Preliminary results

In a previous investigation a heterocaryon between an arg.-1$^-$ mutant and an arg.-10$^-$ mutant was synthesised to study the argininosuccinase produced. In this heterocaryon, isolated from a single heterocaryotic conidium, the proportion of arg-10$^-$ nuclei in the mycelium varied from 99% - 97%. Seven separate determinations of nuclear ratio were made on a number of transfers of this heterocaryon.

To test the hypothesis that this disproportionate ratio was due to a chance extreme ratio in the conidium from which the heterocaryon arose, the following experiment was carried out. A number of heterocaryotic conidia from a double inoculum of the two mutants were isolated and grown up. As can be seen from Table 1 eleven out of twelve heterocaryons had extreme ratios which lay outside the range of ratios corresponding to numbers of nuclei in conidia found for this heterocaryon, Fig. 3. Obviously this is an unlikely result if only random fluctuations in nuclear ratio took place in the mycelium. Six of these heterocaryons were

tested and found to have arg-10$^-$ nuclei as the majority class.

Heterocaryotic conidia from heterocaryon number 7, which did not have an extreme ratio, were grown up. Three out of 13 of these heterocaryons produced conidia of which between 70% and 80% grew on minimal medium. According to the distribution in Fig. 3 the maximum number of heterocaryotic conidia which this combination could produce is 55%. It was concluded that heterocaryon number 7 had been contaminated with wild type Neurospora.

Further heterocaryons were made of the same two homocaryons and again extreme ratios were found.

The conclusion drawn from these results was that a definite change in nuclear ratio was taking place, probably towards a majority of arg-10$^-$ nuclei in the mycelium. It was decided to investigate the process in greater detail, to follow the course of change, and to learn whether or not these changes were due to the arginine mutants themselves.

These heterocaryons were slow growing, (about 2.0 mm. per hour,) compared to a wild type growth rate of 3.8 mm. per hour. The homocaryons, supplemented with 0.05% arginine grew at 3.6 - 3.8 mm. per hour. Fig. 8 gives the growth curves for homocaryons and heterocaryons, all cultures starting from single conidia.

TABLE 1.

Nuclear ratios of B362-3-1/46004-1-10 heterocaryons

Heterocaryon No.	% majority class of nuclei
1	95.0
2	96.5*
3	97.0
4	97.0*
5	97.0
6	97.5*
7	72.5
8	98.0*
9	97.0*
10	99.0
11	99.0*
12	97.5*

* Homocaryotic conidia grown up and tested and majority class found to be arg.-10^{-}.

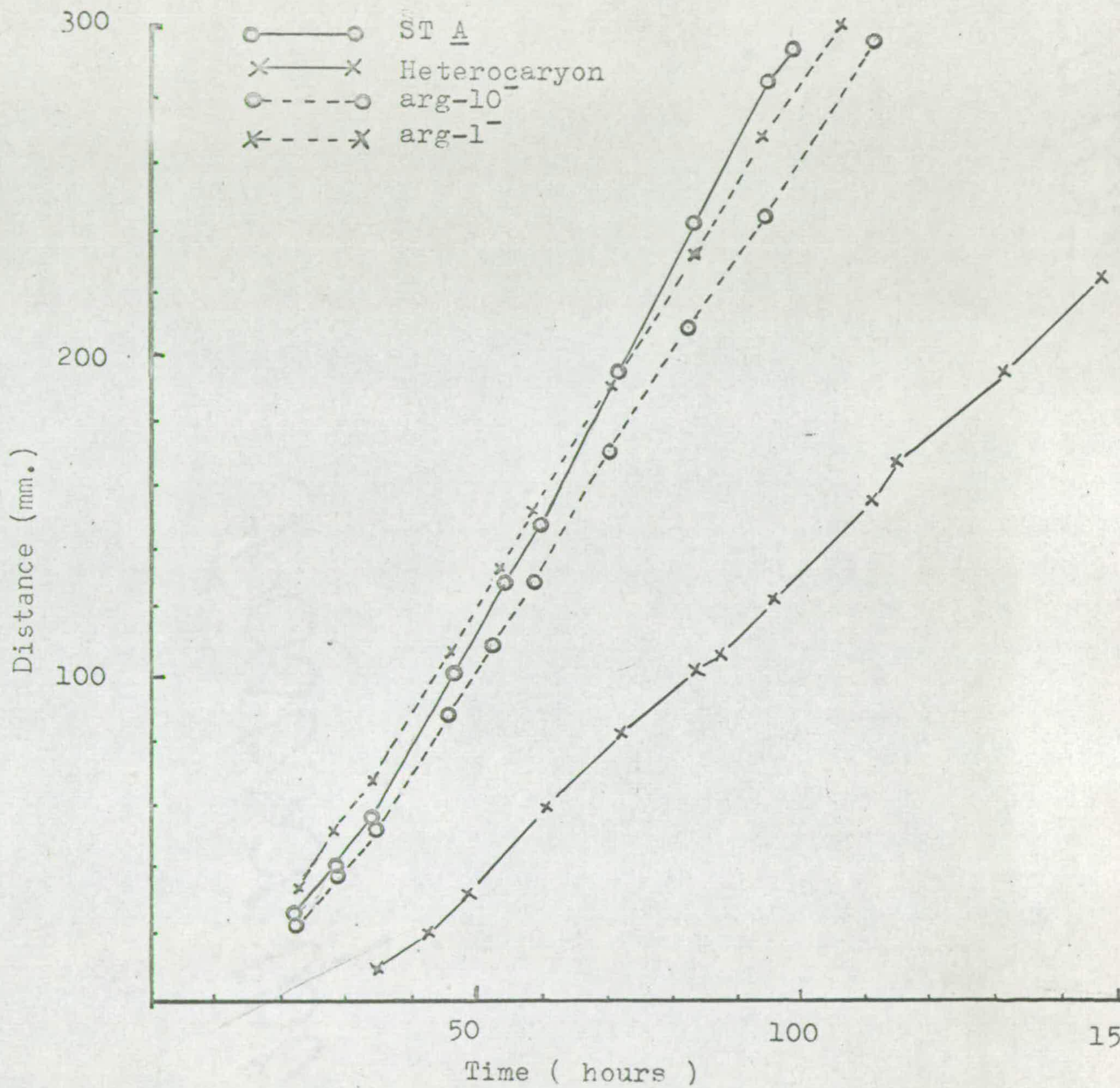


Fig.-8 Growth curves of ST A, an arg-10$^-$;arg-1$^-$ heterocaryon of sub maximal growth rate and the two homocaryons. All cultures started from single conidia

Crosses

A series of back crosses of the two arginine mutants was carried out for two purposes, the first to discover whether the associated slow growth rate of the heterocaryons was correlated with extreme nuclear ratios, and the second to obtain a pair of mutants as isogenic as possible whose heterocaryons were still of extreme ratio. The aim of the latter being to study the phenomenon free from complications of secondary or irrelevant genetic interactions.

After two backcrosses to ST A a pair of mutants were isolated which formed a heterocaryon with wild type growth rate and yielded heterocaryotic conidia which consistently grew into heterocaryons with extreme ratios (four determinations). All other combinations of mutant isolates gave either slow growing heterocaryons with extreme ratios or fast growers with intermediate ratios. At this stage it was decided to select a new standard wild type, isolated from a single ascospore, to replace the laboratory stock. ST A, carried on slants for a number of years, had undoubtedly become heterocaryotic due to spontaneous mutation. The new standard was isolated from the third backcross of arg.-10⁻ to ST A and was designated 3 A. The bulk of the work forming this thesis was done on isolates of a cross to 3 A of the two mutants forming the fast growing, extreme ratio heterocaryons. These isolates will be referred to as B 362 or 46004 followed by an isolation number i.e. B362-24.

Due to the small number of spores isolated at each cross and the equivocal results of the method of scoring ratios of heterocaryons between isolates, to be discussed in a later section, little information on the formal genetics of the situation can be gained. Two crosses were analysed in more detail than most and the results are given in Table 2. The mutants involved in these crosses, themselves form a heterocaryon with a growth rate of 2.0 mm. per hour compared to wild type growth rate of 3.6 - 3.8 mm. per hour. The nuclear ratio of the heterocaryon varies from 91 % - 98% majority class of nuclei in the mycelium.

The progeny of arg.-1⁻ x 3 A in combination with the uncrossed arg.-10⁻ produced only extreme ratio heterocaryons of slow growth rate. The progeny of the cross of arg.-10⁻ x 3 A in combination with uncrossed arg-1⁻ produced both fast and slow growing heterocaryons of which about half had extreme ratios. All extreme ratio heterocaryons were slow growers, but some intermediate ratio heterocaryons grew at wild type growth rates, and there was a tendency towards higher growth rate in the latter class. From these data it would appear that the ability to form extreme ratio heterocaryons segregates in a 1:1 ratio, while there is no regular segregation of growth rate. The obvious interpretation of these results as segregation of a single gene has to be modified as will be seen in the light of results given later.

TABLE 2.

Analysis of cross between 3 A and B362-7, and between 3 A and 46004'-2

46004'-2 x 3 A

Mutant progeny of 46004'-2 x 3 A	Heterocaryons derived from single conidia of double inocula of B362'-7 and mutant progeny	
	Growth rate mm./hr.	% majority class of nuclei
1	1.9	91.0
2	2.0	94.0
3	2.0	94.0
4	2.0	95.0
5	3.0	96.0
6	2.4	96.5
7	1.8	96.5
8	2.0	97.0
9	2.2	97.0
10	2.0	97.5
11	1.6	98.0
12	2.0	98.0
13	2.0	99.0
14	2.0	99.0
15	2.0	99.0
16	2.4	99.0
17	2.4	99.0

continued overleaf

TABLE 2

(continued)

B362'-7 x 3 A

Mutant progeny of B362'-7 x 3 A	Heterocaryons derived from single conidia of double inocula of 46004'-2 and mutant progeny	
	Growth rate mm./hr.	% majority class of nuclei
1	3.8	50.0
2	3.4	50.0
3	1.4	57.0
4	3.6	62.0
5	1.6	63.0
6	3.6	65.0
7	3.4	69.0
8	3.5	70.0
9	3.4	70.0
10	3.6	74.0
11	3.0	76.0
12	3.0	77.0
13	3.4	84.0
14	1.5	87.0
15	1.8	95.5
16	3.4	95.5
17	2.0	96.0
18	2.8	96.0
19	2.5	96.0
20	2.0	96.0
21	2.2	97.0
22	3.0	97.0
23	1.4	98.0
24	2.4	98.0
25	1.8	99.0
26	2.0	99.0
27	2.6	99.0

The main object of the crosses was to provide reasonably isogenic material with which to study the phenomenon of nuclear change.

Nuclear ratio changes

Change in nuclear ratio to more extreme values than that expected in conidia has been followed a number of times, in heterocaryons of the original mutants at a sub-optimal growth rate, and in backcross mutant heterocaryons of wild type growth rate. Two methods, by punch tube samples and funnel race tubes, have been used on two types of material.

Figs. 9,10 & 11 give the ratios of punch tube samples taken over a period of time from the front of colonies, derived from single heterocaryotic conidia. Figs. 12a & 12b show similarly obtained results from colonies which started as pellets, made up according to Atwood and Pittenger's method and containing equal numbers of conidia from the two homocaryons.

Results from punch tube samples undoubtedly indicate that a systematic change in ratio does occur during growth of a colony, and that when single conidium colonies are sampled the most extreme ratios are reached by 100 hours growth. In this respect there is little difference between the slow growing colony of Fig. 9 1.4 mm. per hour, and the two faster ones, Figs. 10 & 11, growing at 3.1 mm. per hour. Since pellets contain large numbers of germinated conidia

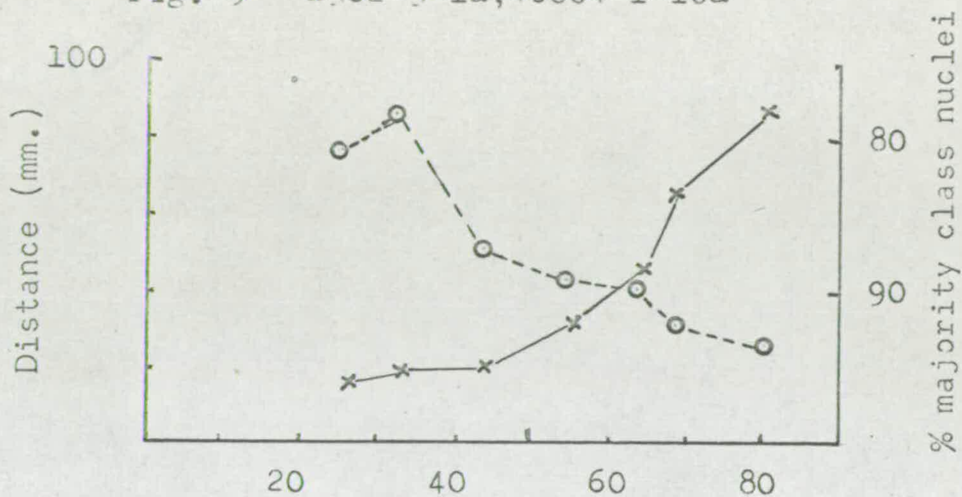


Fig.-10 B362-32;46004-2

Growth rate

Nuclear ratio change

200

100

Distance (mm.)

% majority class of nuclei

70

80

90

20 40 60 80 100

Time (hours)

Figs. 9 and 10 Growth rates and nuclear ratio change, measured by punch tube sampling, in mycelium of colonies from single heterocaryotic conidia.

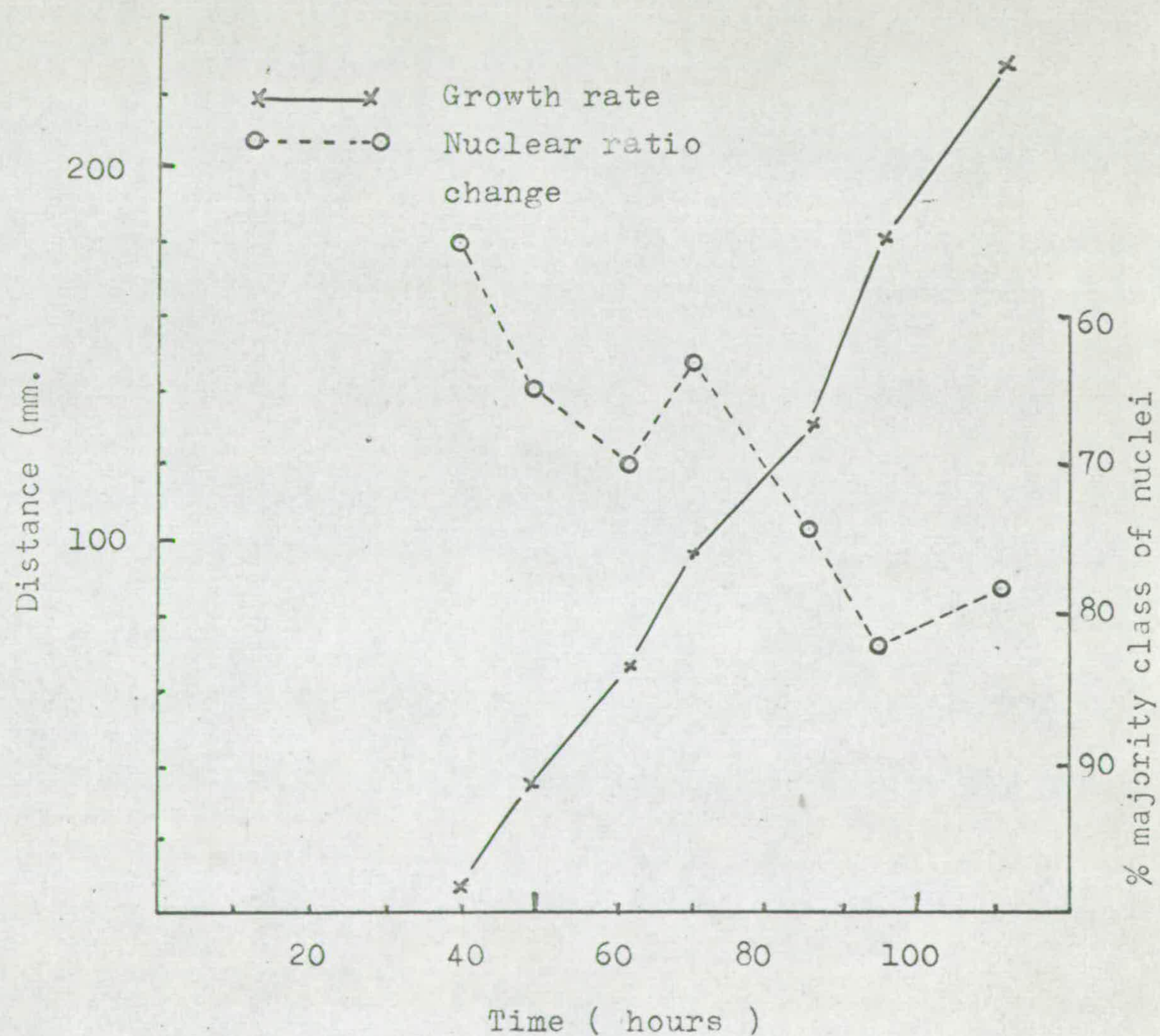


Fig.-11 Growth rate and nuclear ratio change, measured by punch tube sampling, in mycelium of a colony from a single heterocaryotic conidium of heterocaryon B362-32;46004-2

Fig.-12a   Pellet: 40% B362-32;60% 46004-2

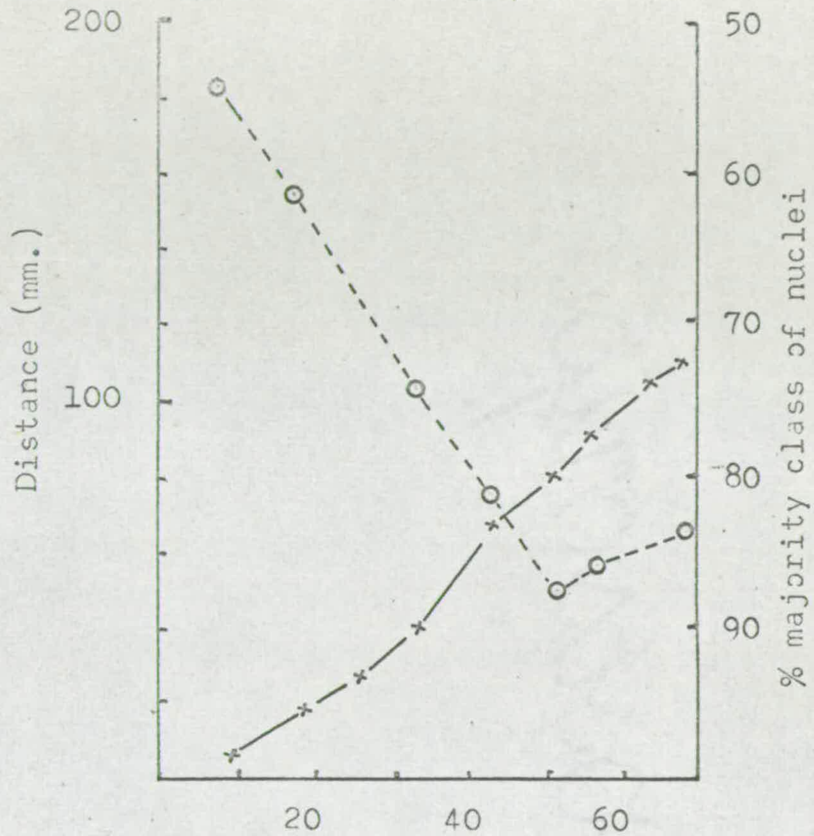


Fig.-12b   Pellet: 40% B362-32;60% 46004-2

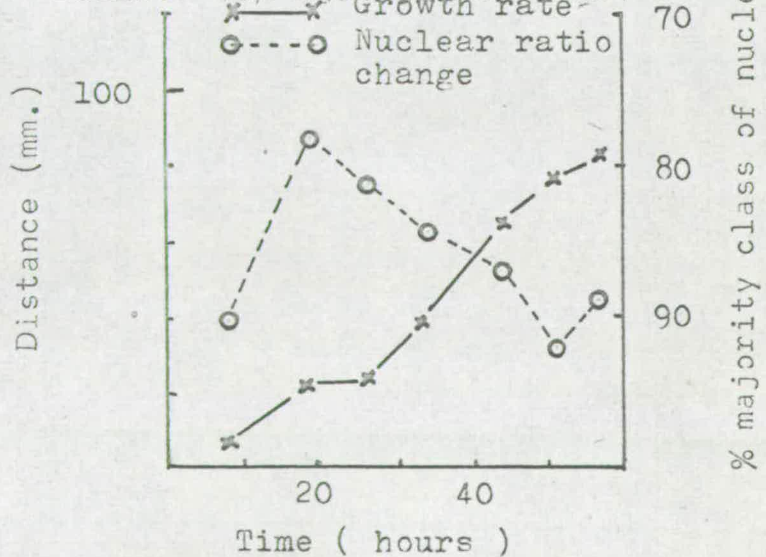


Fig.-12 a and b   Growth rates and nuclear ratio change measured by punch tube sampling, in mycelium from intermediate ratio conidial pellets

the length of time between inoculation and the production of enough mycelium to sample is shorter than that for single colonies. (The total time to reach extreme ratios is therefore less but it appears to be true, that over the same range of ratios found in conidial single colonies, the rate of change of ratio is greater).

That the curves of ratio change are not smooth is due to the fact that successive samples are taken from different parts of the colony. As will be shown in a later section, nuclear ratios of samples taken at the same time around the perimeter of a colony can differ considerably.

Funnel tube results for both single colony inocula and pellet inocula present the same picture of ratio change, but not quite so dramatically. In the graphs of Figs. 13 & 14 nuclear ratios, derived from conidia at the point of inoculation, under the five funnels, and at the end of the tube, are plotted against the time of inoculation, the times when the front passed under the funnels and when the front reached the end of the tube. Conidiation took place some time after these events and the nuclear ratios may or may not represent the ratio of the front at that time. The fact that generally lower initial ratios are obtained by this method would appear to suggest that considerable secondary growth to produce aerial hyphae and conidia allows ratio change to continue.

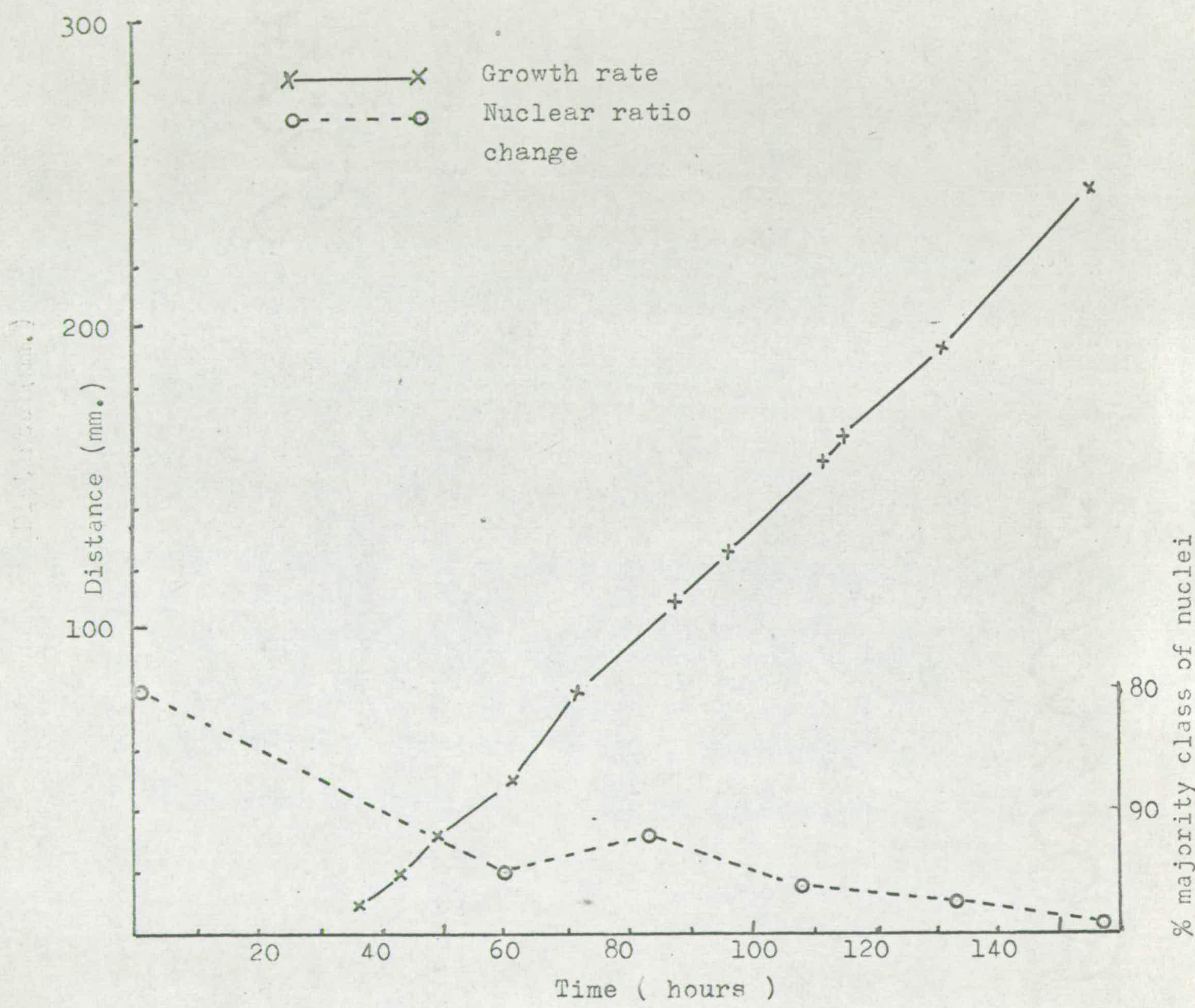


Fig.-13 Growth rate and nuclear ratio change in mycelium from a single heterocaryotic conidium of heterocaryon <u>B362-3-1a;46004-1-10a</u> measured in a funnel growth tube

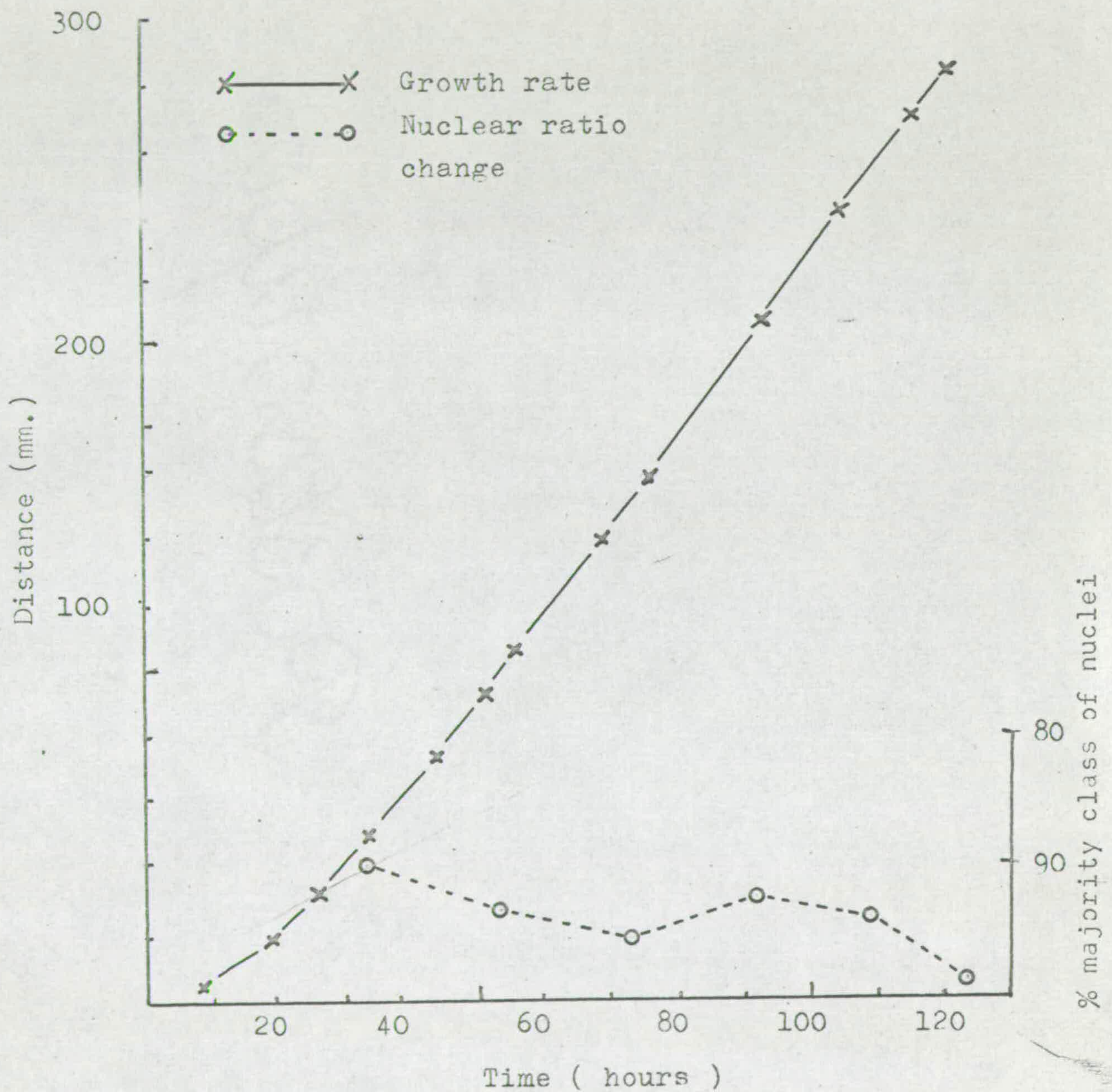


Fig.-14 Growth rate and nuclear ratio change in mycelium from conidial pellet containing 40% B362-32: 60% 46004-2. Measured in a funnel growth tube

In one of the single colony series and all pellet experiments the majority nuclear type was determined and found to be arg.-10⁻.

This process of change in nuclear ratio has been termed selection for descriptive purposes and use of this term does not presuppose any special mechanism bringing about the changes. Ratios similar to conidial ratios of 1:1, 2:1, 3:1 etc. (representing 50%, 66% and 70% majority class of nuclei) will be referred to as intermediate ratios. Ratios of 9:1 or more (representing 90% or more of the majority class of nuclei) will be referred to as extreme ratios.

Stability of nuclear ratios

The results of previous experiments in which the course of selection to extreme ratios was followed, showed no indication that this was other than a unidirectional process, and that once extreme ratios were established, fluctuations were small. Nevertheless, the following experiment was carried out to determine the size of changes in ratio after and extreme ratio had been established. Mass conidial transfers from B362-18;46004-2 of nuclear ratio 99%,: 1% were made on to four slants. After twenty-four hours, when the mycelium had almost reached the ends of the slants, small samples of mycelium were cut from the front and used to inoculate four fresh slants. In this way 18 consecutive transfers of the four lines were made and nuclear ratios

determined on each. Conidia formed near the point from which the sample for transfer was removed.

The results of the four series of transfers are given in Fig. 15, nuclear ratio per transfer being given as percentage majority class of nuclei in the mycelium (composition of the classes was not determined). It can be seen from the data that none of the ratios lay outside the limits for selecting heterocaryons.

Supplementation of selecting heterocaryons

The first question that arose from the observations of nuclear selection in the mycelium of arg.-10$^-$; arg.-1$^-$ heterocaryons was whether or not the selection was due to the metabolic consequences of the arginine mutations themselves. In all heterocaryons tested, the majority class of nuclear type was arg.-10$^-$. The hypothesis that selection resulted in optimum relative proportions of the two arginine cycle enzymes, and that the existence of an optimum was itself the cause of selection, seemed to be a likely explanation. The arginine mutant homocaryons when supplemented with arginine grow at wild type growth rates, Fig. 8. Since the deleterious effects of the mutations are overcome by an exogenous arginine supply, one should expect that, if selection were due to requirements for arginine, single heterocaryotic colonies would not select on supplementation, and the nuclear ratios in any one

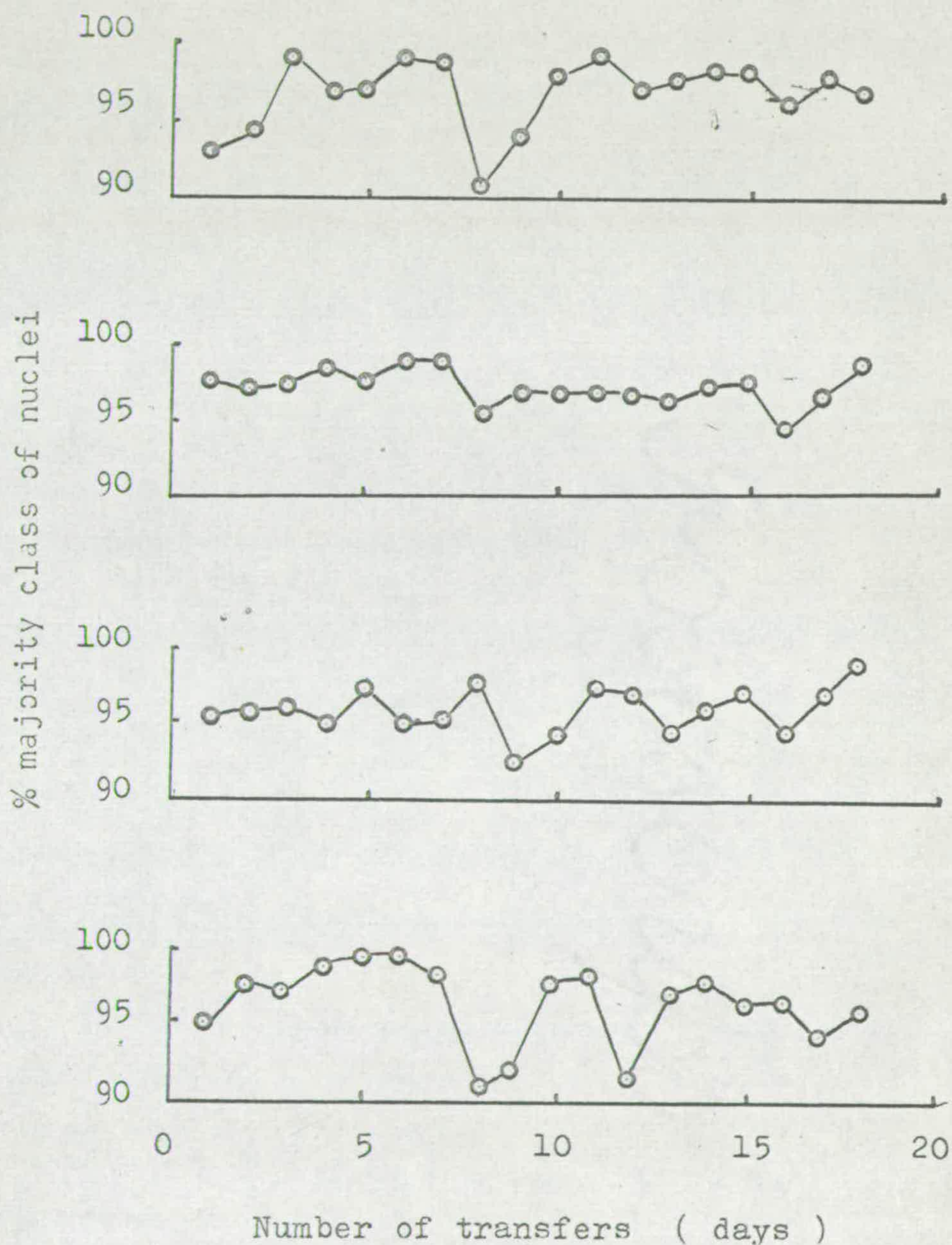


Fig.-15 Nuclear ratios (% majority class of nuclei) of successive mycelial transfers of four cultures of heterocaryon <u>B362-18;46004-2</u>

case would be from the distribution of expected ratios given in Fig. 7.

Colonies arising from heterocaryotic conidia of a selecting heterocaryon were inoculated on to growth tubes containing minimal medium and medium with 0.05% arginine added. Nuclear ratios measured at the end of the growth tubes are given below, Table 3.

T A B L E 3

	Colony No.	Nuclear ratio (% majority class)
Minimal medium	1	97.0
	2	98.5
	3	98.5
	4	99.0
Arginine medium	5	91.0
	6	99.5
	7	99.0
	8	97.5

Colony number 5 was grown in a funnel tube, Fig. 16 shows nuclear ratios at the beginning, end, and at the five funnels between. The majority class of nuclei, determined at each point was found to be arg.-10$^-$.

Assuming that the heterocaryons do not break down on supplementation,then it would appear from these results that there is not a causal relation between selection and the arginine mutations. Results such as these could also be produced by partial breakdown of the heterocaryotic mycelium, plus differential growth of the homocaryons to produce an

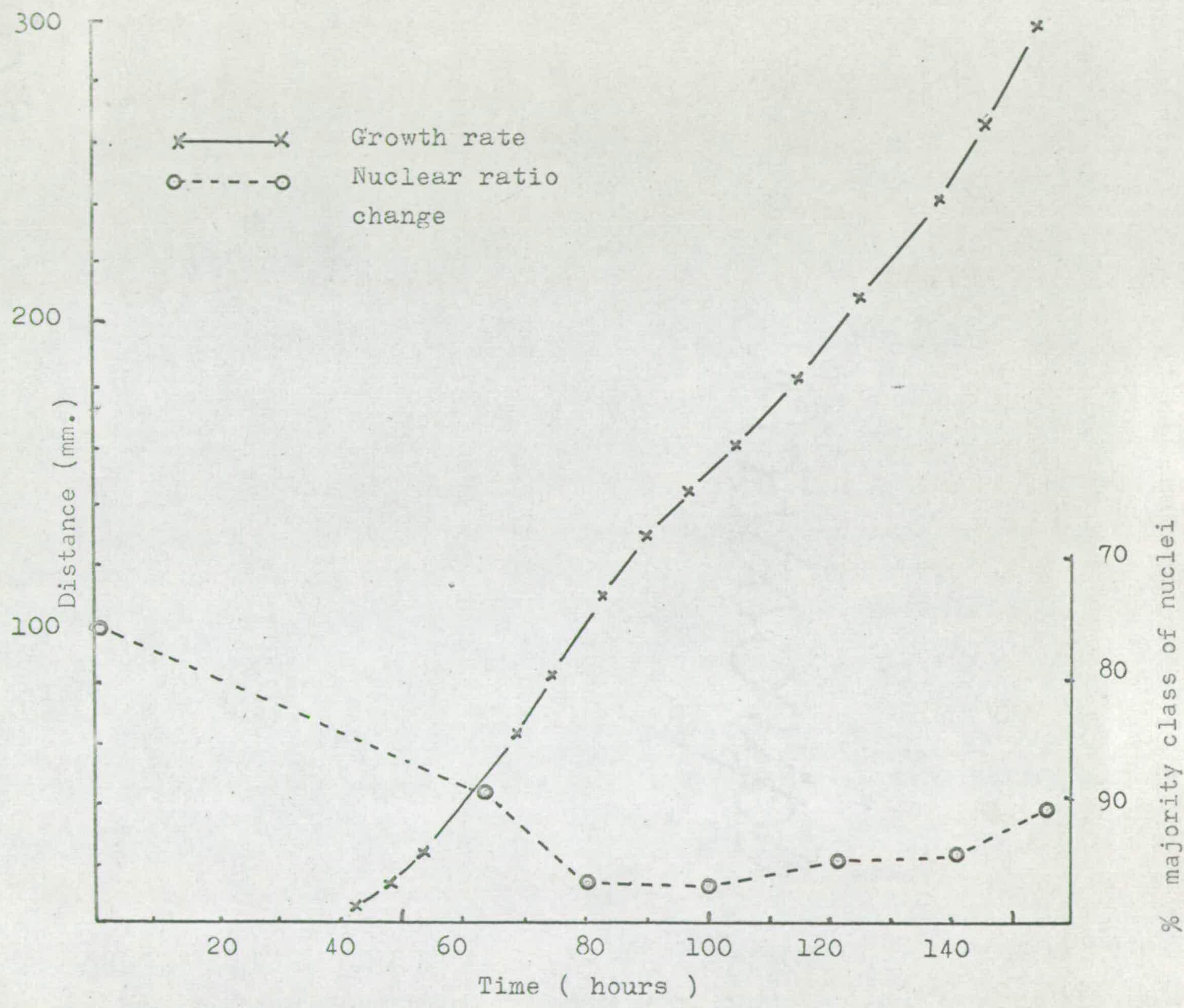


Fig.-16 Growth rate and nuclear ratio change in mycelium from a single heterocaryotic conidium growing on medium supplemented with 0.05% arginine

excess of arg.-10^{-} conidia at each sampling point. Both arginine mutants grow at wild type rate, 3.6 mm. per hour, on arginine medium which in fact contains an excess of arginine. There seems to be no reason why competition for arginine should result in a gain of one over the other. Differential conidiation could also be put forward as an explanation,but since the arg.-10^{-} mutants are normally far less prolific with regard to conidiation,this idea can be discarded.

Heterocaryotic colonies were also grown on race tubes containing complete medium, and on medium containing separately hydrolysed casein, nucleic acid derivatives, and vitamins. For the four heterocaryons tested on each medium only extreme ratios were obtained. At no time did the proportion of the majority nuclear type fall below 95%.

Summary

Conidia obtained from a double inoculum culture of arg.-10^{-} and arg.-1^{-} mutants consistently produce heterocaryotic mycelia whose nuclear ratios were more extreme than their initial starting ratios i.e. the ratios in the conidia. The conidial ratios are limited by the numbers of nuclei in the conidia and the nuclear ratio of the mycelium producing the conidium. Although these heterocaryons were of suboptimal growth rates, crosses to wild type showed that extreme ratios and slow growth rate were dissociable. Intermediate ratio heterocaryons

were also obtained from combinations of the progeny. Mutant progeny from an arg.-10$^-$ x 3 A cross segregated in an apparent 1:1 ratio with regard to ability to form extreme ratio and intermediate ratio heterocaryons with one particular arg.-1$^-$.

The course of change in nuclear ratio was followed in a number of heterocaryons and found to be a continuous 'selection' process which was completed after about 100 hours growth. Once extreme ratios have been reached they remain at that level over many transfers of mycelium.

The selecting heterocaryons continued to produce extreme ratios on arginine supplemented medium, indicating that selection was not due to a requirement for arginine. Similar extreme ratios were produced on other supplements also.

Of all heterocaryons tested arg.-10$^-$ nuclei formed the majority class.

Section II

The expected array of nuclear ratios of conidia from the same 'parent' mycelium is given in Fig. 7. On the assumption that conidial ratios are maintained during growth of the mycelium, this is also the array of the mycelia generated by the conidia. An array of expected nuclear ratios has been calculated for two parent mycelial nuclear ratios of 98:2 and 50:50.

"Non selectors"

Conidia from a mycelium of intermediate ratio would be expected, on the assumption that no nuclear ratio change occurs during growth, to produce mycelia whose ratios were distributed according to the expected distribution in Fig. 7c.

Twenty conidia from an intermediate ratio mycelium, 57% majority class of nuclei, were grown up in growth tubes and nuclear ratios measured. The distribution of ratios among the twenty tubes is given in Fig. 17c. It can be seen that, although there is the expected broad array, in comparison with the expected distribution, there is a deficiency of mycelial nuclear ratios of 70% or less of majority nuclear type. There is also a corresponding excess of more disproportionate ratios. Approximately 17% of conidia produced extreme ratio mycelia having ratios of

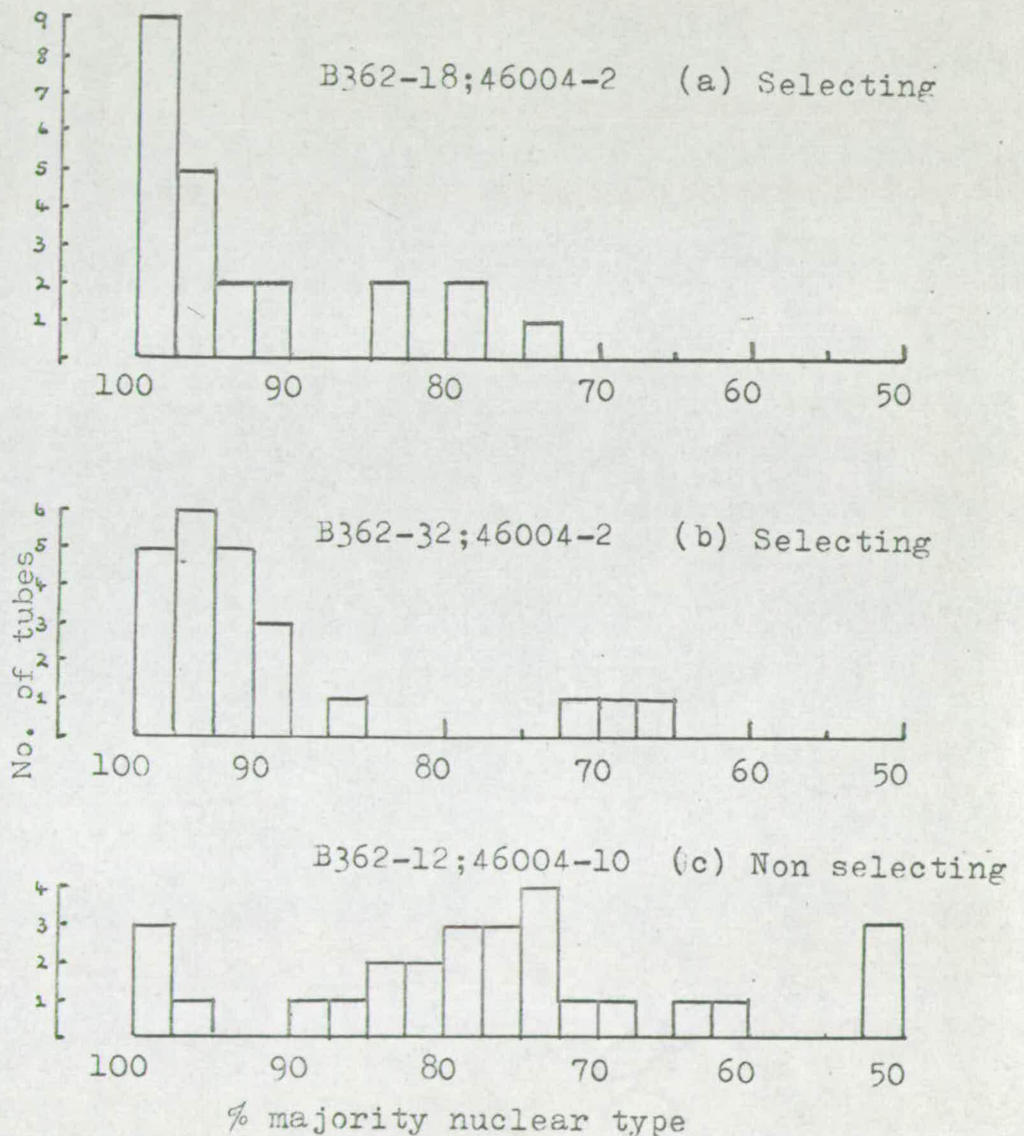


Fig.-17 Distribution of nuclear ratios (% majority nuclear type in 2.5% class intervals) of further heterocaryons produced by conidia grown up in growth tubes, from each of three heterocaryotic mycelia

(a) From mycelium with ratio 99.5% majority type
(b) " " " " 96.0% " "
(c) " " " " 75.0% " "

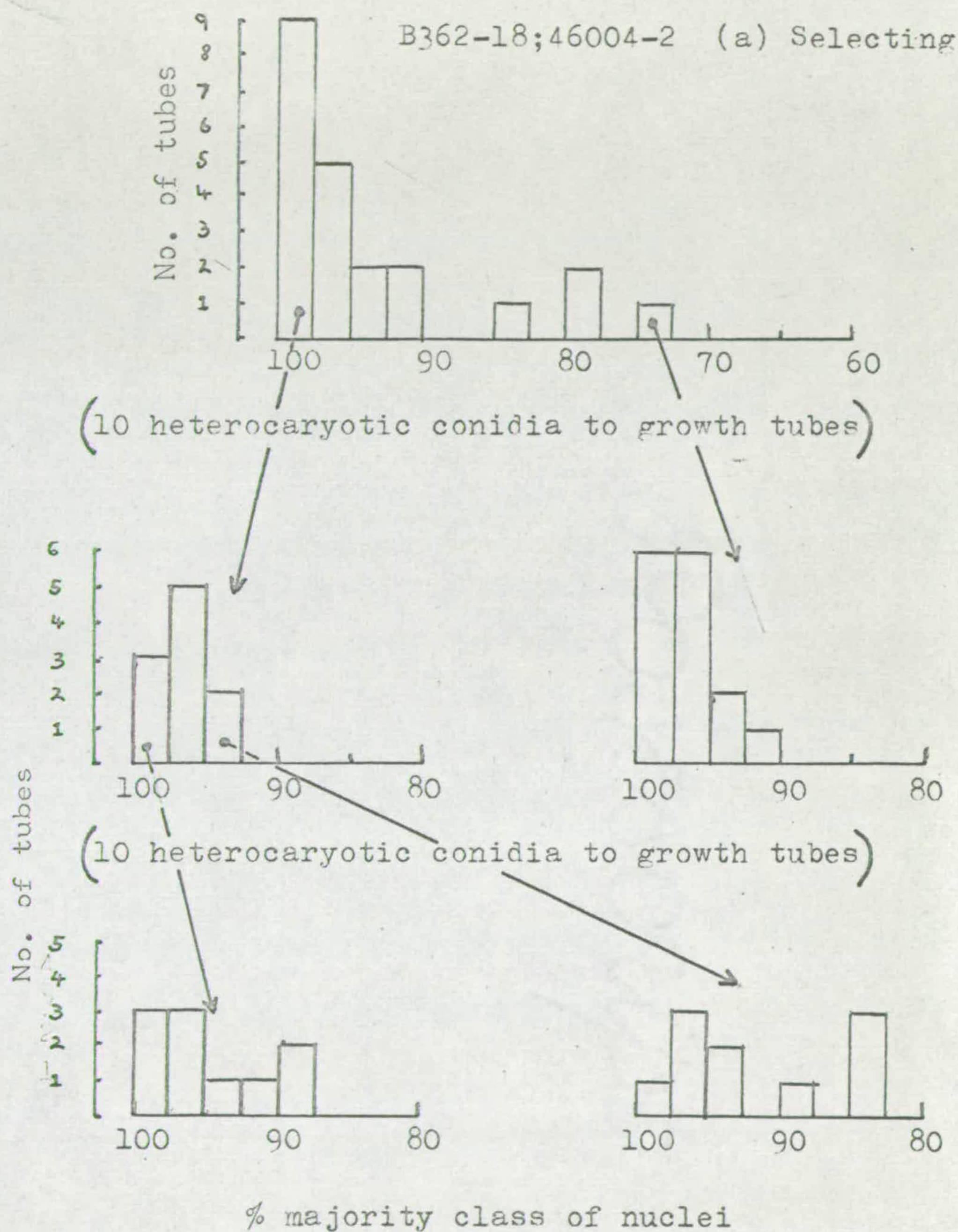


Fig.-18 Distribution of nuclear ratios of successive 'generations' of heterocaryons derived from a selecting mycelium (Fig.-17a)

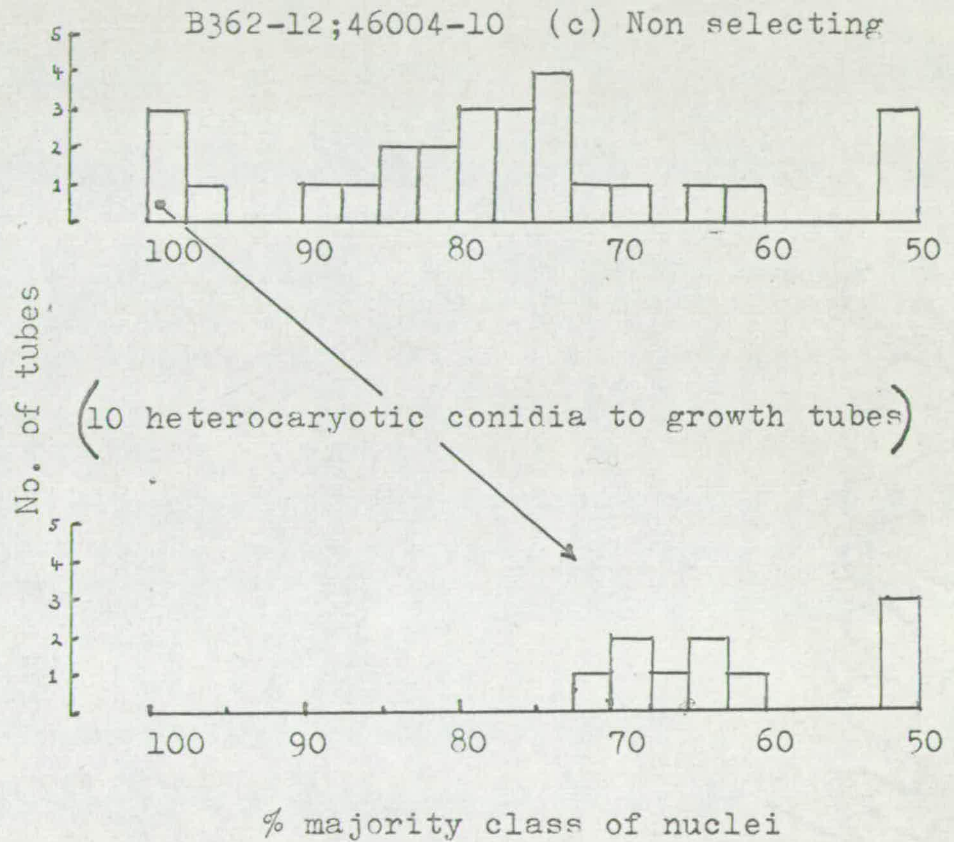


Fig.-19 Distribution of nuclear ratios of successive 'generations' of heterocaryons derived from a non selecting mycelium (Fig.-17c)

90% or more of one nuclear type, compared with an expectation of about 1%. When conidia from one of the latter mycelia were grown up and nuclear ratios measured, however, (Fig. 19), only intermediate ratios were found.

Conidial ratios from the 'parent' mycelium would appear not to have been strictly maintained and some degree of nuclear ratio change had occurred. Extreme ratio mycelia, however, did not generate further extreme ratio mycelia but generated a broad array of mainly intermediate ratios as expected from Fig. 7c. These extreme ratio mycelia were therefore seen to be different from other stocks, 'selectors', which had been found to generate further extreme ratio mycelia. The behaviour of these latter is described below.

"Selectors"

Nuclear ratio change, selection, during growth of a mycelium has been shown to account for some extreme ratios of 90% majority nuclear class or over. Conidia from an extreme ratio mycelium would be expected to generate an array as shown in Fig. 7b. If, however, a process of selection takes place, the resulting mycelia should be of extreme ratio. The nuclear ratios should fall between 90% majority nuclear type and whatever ratio marks the limit of selection, say 99%.

Twenty conidia from each of two stocks giving extreme ratio mycelia were grown up in growth tubes and nuclear ratios measured, the distribution of ratios being given in Fig. 17a

and 17 b. Not all conidia produced mycelia with more than 90% majority class of nuclei. Approximately 24% and 21% produced mycelia of intermediate ratio.

Conidia from two growth tubes of one distribution, one of extreme ratio and the other of intermediate ratio, were grown up and distribution of nuclear ratios measured. Only extreme ratio mycelia were produced. Conidia from two of these latter mycelia were also grown up and ratios were mainly extreme.

Fig. 18 shows the distributions of these nuclear ratios and the relationship between the distributions and the mycelium which provided the heterocaryotic mycelia whose ratios make up the distributions.

Although in some mycelia, derived from conidia from an extreme ratio 'parent' mycelium,selection to extreme ratios appeared not to be operating or had not come to the limit, these types were unstable in that they generate further mycelia which do select to extreme ratios.

The conclusions concerning these stocks then,is that the conidia, presumably having a starting ratio according to the distribution calculated in Fig. 7b, produce a mycelium in which a process of selection takes place, so that by the end of a growth tube extreme ratios are found. These few tubes which were found to be in the intermediate range were not intrinsically 'non selectors' in that they produced conidia which again produced a preponderance of extreme

ratio mycelia.

An alternative explanation for the proportion of intermediate ratio mycelia may be thought to lie in purely random fluctuations of a growing mycelium. If a single conidium is allowed to germinate and grow without restriction, it develops into a radially growing colony. A mycelium in a growth tube might be considered to be a strip or segment of such a colony. The differences in nuclear ratios, extreme or intermediate, between heterocaryons in different growth tubes may therefore reflect differences between segments developed in a single colony. This can be tested by taking samples of mycelium from around the perimeter of a growing colony. If differences arise by sectoring in the mycelium from a single conidium, then, of samples from the same colony, some will be of extreme ratio, others of intermediate ratio. That is to say, the differences found between growth tubes will be found between samples of the same colony. If, on the other hand, these differences are due to differences between conidia, then nuclear ratios taken from one colony should be more similar to each other than samples between colonies. On the basis of the growth tube results, it would be expected that all samples from one colony will have ratios of 90% or more of one nuclear class, while the samples from another colony will all be of intermediate ratio.

The next section, therefore, describes experiments designed to answer this question of mycelial heterogeneity,

within and between heterocaryotic colonies derived from the same parent mycelium.

Mycelial heterogeneity

In order to eliminate measurement variation in nuclear ratios from the following experiments, conidia from a heterocaryon were sampled, made up into a suspension and 11 streaks made on minimal plates. A X^2 test of the null hypothesis that the numbers of growing (heterocaryotic) and non growing (homocaryotic) conidia in each streak were from samples of the same population was carried out. The calculated X^2 was not significant and therefore there was no reason to reject the hypothesis. The test was carried out on conidia from both an extreme ratio and an intermediate ratio mycelium. (Table 4).

Ten 15 hour old colonies from a selecting mycelium were inoculated at the centre of a large petri plate 150 mm. in diameter. After 48 hours when the front had almost reached the edge of each plate, ten punch tube samples were removed from around the perimeter, at the front. Once the samples in the tubes had produced conidia, conidial suspensions were streaked and a total of 400 (growing and non growing) conidia in each streak were counted, Table 5a.

A X^2 test was made of the null hypothesis that numbers of growers and non growers from samples within colonies were samples of the same population. Despite the fact that all X^2

TABLE 4

Estimations of growing (heterocaryotic) and non-growing (homocaryotic) conidia by repeated streaks of the same conidial suspension.

SUSPENSION 1			SUSPENSION 2		
Streak No.	Non-Growers	Growers	Streak No.	Non-Growers	Growers
1	156	106	1	432	49
2	169	143	2	405	32
3	139	131	3	376	40
4	141	105	4	412	31
5	133	109	5	451	41
6	153	111	6	421	51
7	155	111	7	386	27
8	140	105	8	395	38
9	120	106	9	404	29
10	137	88	10	397	25
11	129	98	11	434	29
Combined X^2 - 7.8			Combined X^2 - 3.6		

X^2 for 10 degrees of freedom - 18.3

values but one were significant, i.e. there was heterogeneity of nuclear ratio within colonies, the differences were not large enough to account for the observed differences in the growth tube experiments. The ten samples from each of nine colonies had extreme ratios, greater than 90% of one nuclear type, and the tenth colony had an average ratio of 88% majority nuclear type. Since only 75 mm. growth had taken place, selection in this latter colony may still have been proceeding.

A second experiment using single conidia colonies from an intermediate ratio mycelium, similar to the one described in Fig. 17c, was carried out, Table 5b. Results were as before, significant mycelial heterogeneity but differences arose within colonies corresponding to the array in Fig. 17c.

The main conclusion drawn from these results is that there is no reason to suspect that differences within colonies, developed within the growing mycelium, are large enough to explain the major differences (selection, non selection) found between the same mycelium derived from conidia of growth tubes. If colonies in growth tubes are regarded as segments of the colony which would develop were the conidium given room for unrestricted growth, there is no reason to believe that the segmentation which occurs is the cause of differences between colonies in growth tubes. Differences between colonies from both extreme ratio and intermediate ratio mycelia were

T A B L E 5

Numbers of growing conidia (i.e. heterocaryotic) out of a total of 400 conidia on streaks of conidia produced by punch tube samples from around the perimeter of selecting and non-selecting colonies.

a. Selecting colonies from single conidia

	Colony No.									
	1	2	3	4	5	6	7	8	9	10
	65	120	29	34	91	34	86	18	10	28
	52	142	57	18	105	43	77	3	12	12
	40	101	77	30	86	40	66	10	18	28
	36	120	73	35	108	58	83	6	16	8
	42	113	35	25	63	60	71	5	8	6
	55	144	34	48	80	49	50	6	14	6
	47	95	28	34	86	50	48	38	12	1
	50	128	14	40	66	43	50	21	22	19
	36	92	34	40	80	41	56	4	16	1
	44	92	40	29	74	38	56	2	6	30
Mean	46.7	114.4	41.1	33.3	83.9	45.6	64.3	11.3	12.4	12.9
X^2*	18.6	42.1	99.1	14.5	29.7	18.7	34.2	105.5	20.5	104.7
% majority class of nuclei (based on mean)	96	88	96.5	97.5	92	96	94.5	99	99	99

b. Non selecting colonies from single conidia

	1	2	3	4	5	6
	153	129	241	187	123	149
	215	145	194	172	189	154
	176	187	191	195	157	120
	138	184	173	199	167	130
	251	170	171	137	140	133
	188	176	167	225	142	173
	153	160	197	134	110	160
	176	177	171	132	140	148
	163	143	165	159	146	132
	204	185	195	164	114	186
Mean	181.7	165.6	186.5	170.4	143.3	149.6
X^2	105.0	38.5	47.5	89.5	57.0	39.6
% majority class of nuclei (based on mean)	77	80	76	79.5	84	83

continued overleaf

TABLE 5

(Continued)

c. Selecting colonies from pellet

	Colony No.						
	1	2	3	4	5	6	7
	154	220	187	184	208	188	209
	152	209	193	174	190	227	192
	146	191	200	191	216	199	224
	146	170	186	170	228	200	175
	143	148	193	192	216	206	217
	158	160	206	199	196	187	216
	158	182	201	170	192	180	187
	150	130	186	201	208	201	204
	130	197	206	172	198	221	195
	179	166	200	208	193	212	200
Mean	151.6	177.4	198.5	186.1	204.5	202.1	201.9
X^2*	15.3	70.9	5.5	17.8	14.5	20.3	20.6
% majority class of nuclei (based on mean)							
	82.5	78	74	76	73	73	73

* X^2 values are sum of X^2 for growers and non growers

X^2 for 9 degrees of freedom - 16.9

large but only one colony from the extreme ratio mycelium could be classified as a non selector, with reservations, and all colonies from the intermediate ratio mycelium were non selectors. Nevertheless, even in the absence of more convincing differences between colonies,it seems likely that differences in behaviour between mycelia produced by conidia from one mycelium are due to differences in the conidia themselves.

Within colony Variation

Minor differences in nuclear ratio of samples from the same colony could arise in a number of ways. (1) They could be due to sampling variation of nuclei into branches, assuming that a random sample of nuclei of the 'main stem' contribute to the new nuclei in the branch, (2) they could be due to different rates of selection within the colony, (3) a combination of the above could result in nuclear ratio differences.

Process (1) could be of importance during the early stages of germination and growth when numbers of nuclei are small,and very diverse nuclear ratios could occur by chance in new hyphae. The difficulty here, of course, is to decide whether the effective size of the population sampled at branch points changes during growth even though total number of nuclei does. Pittenger and Atwood (1956) have shown in a stable extreme ratio heterocaryon, that hyphal tips,

picked at random, differ greatly in nuclear ratio. Such differences could conceivably be due to hyphal sampling variation. Such sampling effect could also be reflected by variation in nuclear ratios of mycelial transfers of a selected heterocaryon, Fig. 15.

To test whether rate of selection varied within a colony (2) selecting colonies were allowed to grow to the edge of large petri plates and punch tube samples were then removed along two radii on each plate. The advantage of this method over successive sampling of the growing front was that it caused no distortion of the front and approximately the same hyphae could be sampled and followed back to the inoculum.

The results for samples in two plates are given in Table 6. It can be seen that within one colony, ratios are all extreme although there is heterogeneity. This is in contrast to the previously found regular progress towards extreme ratios. Results such as these might have been the results of secondary or 'filling up' growth behind the front allowing selection to continue and extreme ratios to be reached. This would correspond to the previously described perimeter punching experiments. Results obtained by Gillie (unpublished) show that mycelium in a growth tube increases rapidly in dry weight behind the front until a maximum is reached 5 - 7 cms. behind the front, where the dry weight is of the order of 5 times greater than

TABLE 6

Numbers of growing conidia i.e. heterocaryotic out of a total of 400 in streaks of punch tube samples taken along radii of single colonies.

	Plate 1		Plate 2	
	Radius 1	Radius 2	Radius 1	Radius 2
	29	24	49	22
	6	30	40	20
	22	25	57	26
	69	12	50	57
	61	41	36	37
	30	25	15	57
	49	41	36	45
Mean	38	28.3	40.4	37.7
X^2	476.8	23.8	28.0	39.8
% majority class of nuclei (based on mean)	97.0	98.0	96.5	97.0

X^2 for six degrees of freedom - 12.6

at the front. Visual inspection of such tubes indicates that increase in weight is due to a proliferation of hyphae. These results support the hypothesis that growth behind the front is sufficient to allow further selection. The problem of variation in nuclear ratio between samples of mycelium from the same colony remains largely unresolved, in view of the failure to measure the progress of selection in different segments of the same colony. Sampling differences at branch points cannot be rejected as a source of ratio difference.

Differences between colonies when all are either selectors or non selectors could be due to the same influence as mentioned above, and in addition due to differences in initial ratios i.e. different conidial ratios.

It would be expected that in selecting heterocaryons there is a limit to the extent of selection, at some point before homocaryosis is reached. At this point differences due to initial ratio differences would disappear. At some intermediate stage, however, these differences should be detectable as in the plate colonies where only 75 mm. growth occurs before sampling. In non selecting colonies initial ratio differences, despite random fluctuation in ratio, should persist. But as pointed out before, the final ratios of non selecting colonies do not conform to the expected distribution of conidial ratios, Fig. 7. Although only six were studied in this case, ratios were all between 76% and 84% majority class (Table 5b) of nuclei, when the expected

ratios would be mainly in the 50% to 70% range (Fig. 7c). (These results will be discussed later).

Pellet Experiments

Since it was not possible to control the starting ratio of a mycelium from a conidium, it was decided to construct pellets of known and controllable composition. This eliminates initial ratio differences between colonies. Large plates were inoculated with pellets containing equal numbers of conidia of the two homocaryons. Due to inviability of arg.-10$^-$ conidia, determined by plating the suspensions of conidia used in making up the pellets, the effective ratio in the pellets was 70% arg.-1$^-$, 30% arg.-10$^-$. The pellets were made up of the same stocks as were used in the single conidia experiments of Table 4a.

The average nuclear ratio of punch tube samples from the front of colonies at the edge of the plates was 76% majority class of nuclei. It was presumed that arg.-10$^-$ nuclei were in the majority, since other pellets of this input ratio are known to select towards a majority of arg.-10$^-$ nuclei, Section V. It is therefore assumed that these colonies have selected from a 70:30 starting ratio towards the 97:3 ratio typical of this stock, and had reached 76:24 at the edge of the plate.

Ratio differences as shown by X^2 within colonies were lower than in single conidia selecting colonies.

Numbers 1, 3 and 5 were homogeneous at the 5% level of expectancy, and differences between colonies appear to be reduced also, Table 5c.

It was concluded from these results that similarity of initial ratios accounted for reduction of differences between colonies. Any initial small number effect of sampling differences of ratio in branches would also be eliminated in these pellet colonies, since growth on to plates starts from a well formed mycelium with large numbers of nuclei. The fact that differences within colonies were reduced lends support to this hypothesis.

Summary

Conidia from a selecting mycelium results in heterocaryons of which approximately 20% have intermediate ratio. Conidia from these 'non selectors' produce only selecting heterocaryons. These latter heterocaryons in their turn again generate selecting heterocaryons. The non selecting heterocaryons from a selecting heterocaryon are unstable in that further vegetative generations of heterocaryons are selectors. Similarly, conidia from a non selecting heterocaryon produced approximately 17% extreme ratio mycelia which generated only non selectors.

This variation in selectability appears to be due to variation between conidia, and is not the result of variation or sectoring in the mycelia of colonies from single conidia.

Minor variation in nuclear ratio between and within colonies seems to be due to differences in initial starting ratios, and sampling variation at branch points within the mycelium.

Section III

While working on heterocaryons between mutant isolates from the backcross to 3 A, inconsistencies in the results became apparent. Double inocula had been set up between the various isolates and a single heterocaryotic conidium from each tested for selection, by measuring the ratio at the end of a growth tube. At intervals of four or five weeks the heterocaryons were constructed with freshly set up double inocula of the isolates which had been transferred at least twice during this time. Some combinations which started off as selecting heterocaryons, i.e. had extreme ratios, produced non selecting heterocaryons, i.e. had intermediate ratios, at the third construction.

When conidial heterogeneity with respect to selectability was investigated it was realised that the above results could have been the result of heterogeneity, since single growth tubes could yield such results. Transfers from the single conidial cultures of the first construction were available, so conidia from these were grown up and tested. All mycelia from the conidia of this original construction were of extreme ratio. Similarly, single conidia cultures of the same combination, from the third construction of double inoculum, were tested and found to produce mycelia of intermediate ratio. It was concluded that selectability had indeed been lost. It was decided to

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test the hypothesis that the ability of the homocaryons to form selecting heterocaryons changed with time or on transfer.

Serial transfer of homocaryons

A heterocaryon, B362-32;46004-2, a transfer from a single conidium colony of the first series of tests on backcross mutants, was broken down and the homocaryons recovered. This involved plating conidia from the heterocaryon on arginine sorbose plates, picking colonies, which were then divided into two, one half being put on arginine supplemented medium and the other on minimal medium to separate homocaryons from heterocaryons. The two homocaryons were distinguished by complementation tests.

Two arg.-10$^-$ and one arg.-1$^-$ breakdown isolates were inoculated separately on to supplemented slants and grown up at 30^{0}C. to increase growth rate. Within 24 hours mycelium had reached the end of the agar and a small portion of the front, about 1 mm.^{2}, was cut out and used to inoculate a fresh slant. Twenty-four successive transfers were carried out. At intervals, double inocula of conidia from the homocaryons were set up and from these, single heterocaryotic colonies were grown up in growth tubes and nuclear ratios measured. Fig. 20 shows the distributions of nuclear ratios obtained from combinations of untransferred mutants and 5th, 8th, 13th and 24th transfer mutants. The results for the two heterocaryons from the three mutants (arg.-10$^-$a;arg.1$^-$ and

ABSTRACT OF THESIS

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Address Institute of Animal Genetics, King's Buildings, West Mains Road, Edinburgh.

Degree Ph.D. *Date* 12th August, 1964.

Title of Thesis STUDIES ON HETEROCARYONS IN NEUROSPORA.

The subject under investigation was nuclear ratio change in mycelium of interallelic heterocaryons between arg.-1$^-$ and arg.-10$^-$ homocaryons. These homocaryons are mutants of the arginine cycle of Neurospora crassa.

(1) In some heterocaryons, originating from single heterocaryotic conidia, nuclear ratios changed during growth of the mycelium from initial intermediate conidial ratios, 1:1, 2:1 etc., to extreme ratios where the majority nuclear type in the mycelium constituted 90% or more of the total. In all heterocaryons tested, the majority nuclear type was arg.-10$^-$. This change in nuclear ratio (selection) was followed in growing mycelium was found to be a regular, continuous process which stopped at some point short of complete homocaryosis.

Supplementation of such heterocaryons with arginine had no effect upon selection, indicating that it was not due to any metabolic consequences of the arginine mutations themselves. Experiments using a wide range of supplements failed to show that selection was due to any simple supplementable cause.

(2) Heterocaryotic conidia from a selecting mycelium were found to be heterogeneous with respect to selection in the mycelium which they produced. Approximately 20% of conidia produced mycelium of intermediate final ratios, i.e. non selecting. These, however, were unstable in that they generated further selecting mycelia. This heterogeneity was not correlated with the nuclear ratio of the conidium from which the mycelium originated but with some other conidial difference.

Ability to form selecting heterocaryons was progressively lost over a number o serial transfers of homocaryotic mycelium from the growing front. This loss was confined to the arg.-1$^-$ homocaryon. The loss appeared to be stable and further heterocaryons from conidia of such heterocaryons also produced non selecting heterocaryons.

(3) There appeared to be a threshold value at around 10% arg.-10$^-$; 90% arg.-1$^-$ where there was no selection or very slow selection towards extreme ratios in favour of arg.-10$^-$ nuclei.

(4) To explain these and other experimental results the hypothesis was advanced that selection was due to interaction between arg.-10$^-$ nuclei and cytoplasmic element resulting in inhibition of division of arg.-1$^-$ nuclei. Below a certain threshold level of elements no selection occurs and no replication of elements takes place.

(5) In non selecting, intermediate ratio heterocaryons it was assumed that initia conidial ratios were maintained during growth. On comparison of the array of nuclear ratios of heterocaryons from conidia of a non selecting heterocaryon with the expecte distribution, certain discrepencies were found. Further investigation suggested that there was in fact selection towards 70:30 ratios. This selection process could be prevented by supplementing heterocaryons with arginine. At around 70% arg.-10$^-$ nuclei it appeared that the optimum levels of each of the two arginine cycle enzymes was reached.

Use other side if necessary.

Fig.-20 (a) Serial transfers of homocaryons

Untransferred isolates

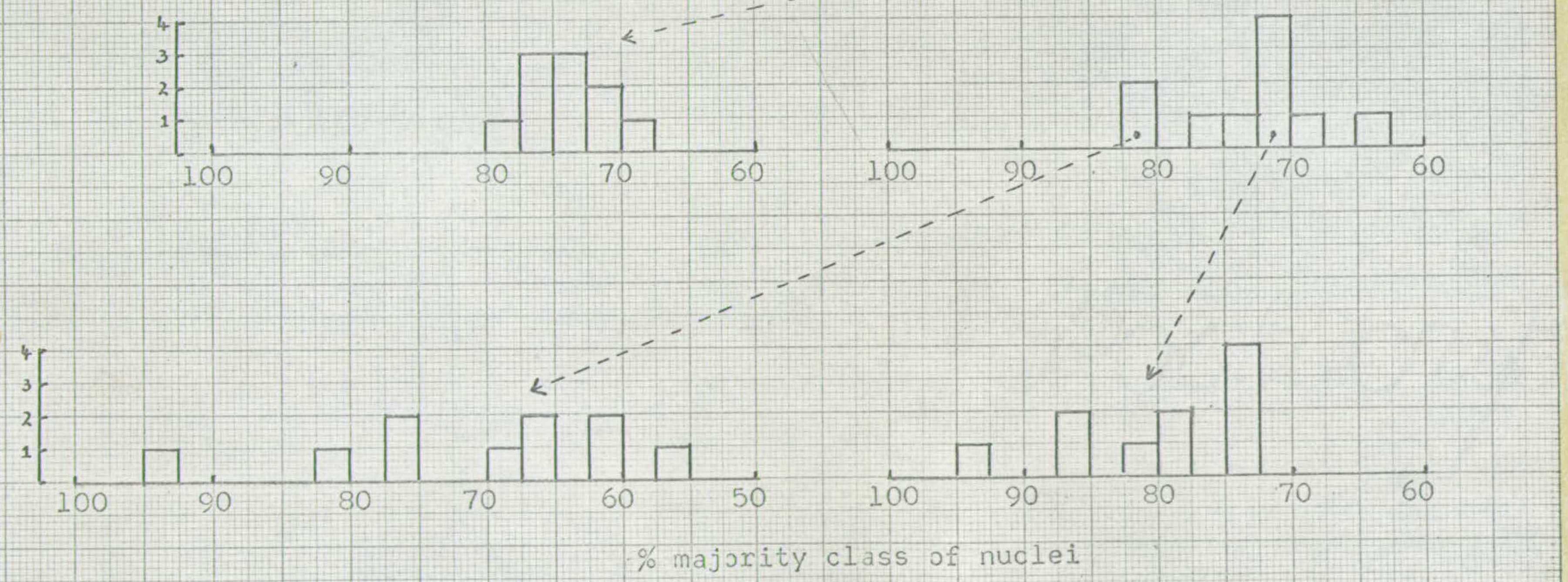
arg-1⁻ — 5th Transfer — 8th Transfer — 13th Transfer

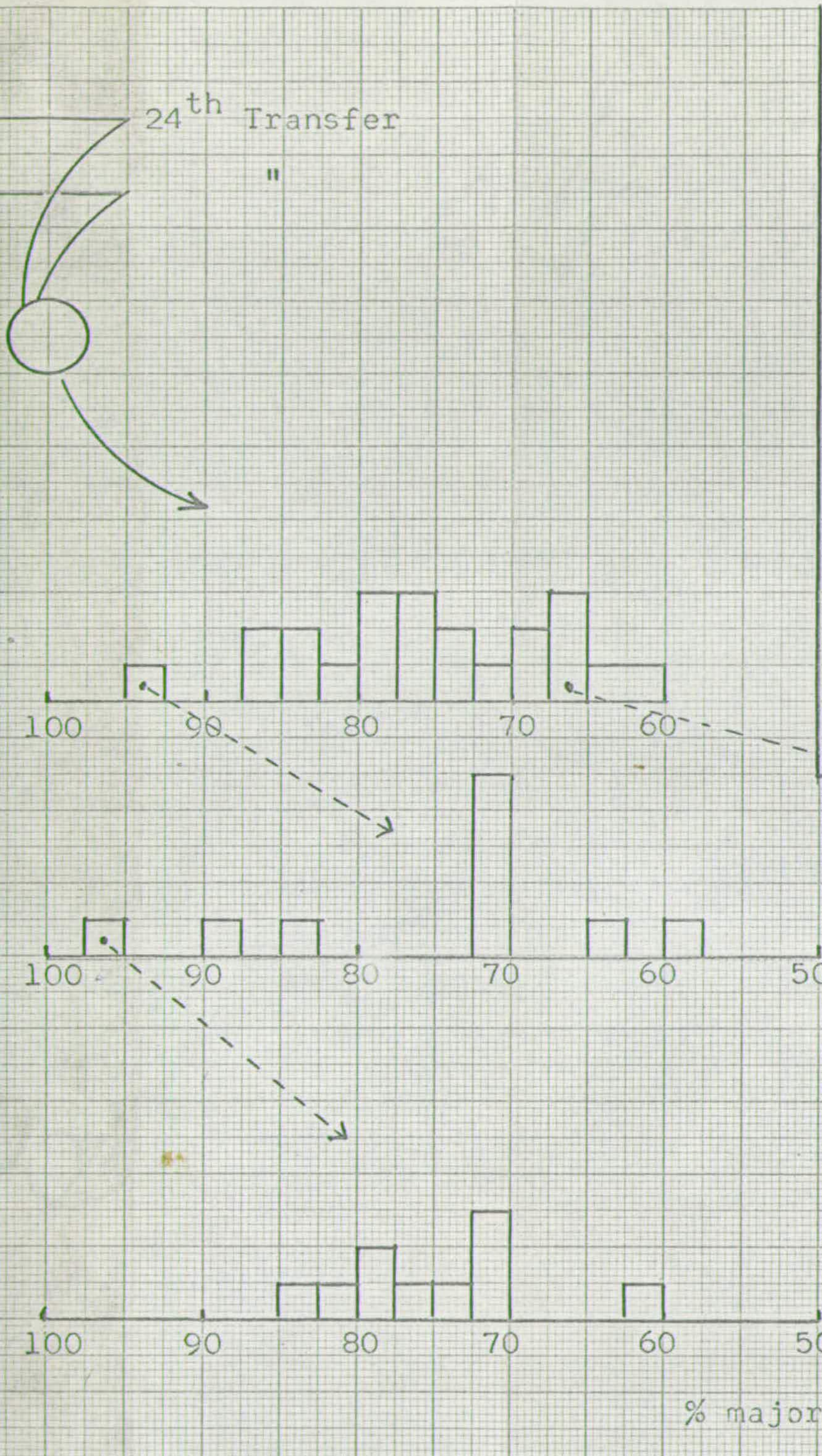
arg-10⁻ — " — " — "

Double Inoculum

Conidia to Growth tubes

No. of tubes





(b) Back test of transfers

24th Transfer arg-10⁻

Untransferred arg-1⁻

7 6 5 4 3 2 1

100 90 80 70 60 50

24th Transfer arg-1⁻

Untransferred arg-10⁻

4 3 2 1

100 90 80 70 60 50

% majority class of nuclei

100 90 80 70 60 50

100 90 80 70 60 50

% majority class of nuclei

arg.-10^{-}b;arg.-1^{-}) were so similar that the results were pooled. The results showed that by the 13th transfer, half the conidia from the double inocula produced intermediate ratio mycelia. By the 24th transfer only one conidium out of 22 had produced an extreme ratio mycelium compared to the untransferred mutants, whose double inoculum produced conidia the majority of which resulted in extreme ratios showing that selection was operating (Fig. 20a).

Converging lines indicate mutants combined into double inocula cultures (circle) and solid lines indicate the array of nuclear ratios of mycelia derived from conidia from the double inoculum. Dashed lines connect particular mycelia to the array of nuclear ratios generated by its conidia.

Stability of Conidia

Conidia from two intermediate ratio mycelia from the 13th transfer double inoculum were grown up in growth tubes and nuclear ratios measured (Fig. 20c.). Only intermediate ratios were obtained. A third generation was produced from conidia, as shown, and again mainly intermediate ratio heterocaryons resulted. Unlike intermediate ratio mycelia produced by some conidia from a selecting mycelium (Fig. 18) these intermediate ratio mycelia are stable in that they generate mainly intermediate ratio mycelia.

At the 24th transfer, intermediate ratio mycelia generated mainly intermediate ratio mycelia. The one extreme

ratio mycelium from this distribution was used to generate further mycelia and again only one of these was of extreme ratio. Conidia from this latter mycelium produced only intermediate ratios when grown up. As at the 13th transfer, intermediate ratio mycelia were stable. It is thus seen that these extreme ratio mycelia acted in the same way as did extreme ratio mycelia from non selecting mycelium (Fig. 19).

The conclusion drawn from these results was that selectability of the mutants was progressively lost during frequent transfer and rapid growth.

Back tests

To determine whether both mutants or only one was involved in the loss of selectability in heterocaryons, double inocula were set up between untransferred arg.-10⁻ and transferred arg.-1⁻ and between untransferred arg.-1⁻ and transferred arg.-10⁻. Conidia from the former combination, produced only intermediate ratio or non selecting mycelia, and from the latter combination 9 out of 10 conidia produced extreme ratio or selecting heterocaryons. The ability to form selecting heterocaryons therefore had been lost only in the arg.-1⁻ mutant.

An obvious explanation for such results is contamination of the arg.-1⁻ mutants with another stock capable of growth on minimal medium and therefore indistinguishable from heterocaryotic colonies. A heavy concentration of spores from the 24th

transfer was streaked on a minimal plate. After 15-20 hours a small proportion, a fraction of one percent, had germinated and formed colonies. Ungerminated conidia were picked to arginine slants, grown up and a double inoculum made with this purified mutant and untransferred arg.-10⁻. As before, no selecting heterocaryons were produced by the conidia of this culture. No contamination was found in transfers earlier than the 21st.

A further check on the effects of contamination was provided by the following experiment. A non selecting mycelium from the double inoculum of 13th transfer mutants was broken down and homocaryons isolated as explained before. Three arg.-1⁻ and three arg.-10⁻ homocaryons were set up in double inocula with untransferred arg.-10⁻ and arg.-1⁻ respectively. The results in Table 7 correspond with previous results, in that transferred arg.-1⁻ is no longer able to form a selecting combination with an arg.-10⁻ mutant, with which, previous to transfer, it formed a selecting heterocaryon, but the reciprocal heterocaryon did select.

To eliminate the effect of aging, in loss of selectability, the original untransferred homocaryons were retested some eight weeks after the start of the transfer experiment. No change in the type of heterocaryon, produced by conidia of the double inoculum, could be detected i.e. all were selecters, Fig. 21.

T A B L E 7

Nuclear ratios of single conidia mycelium from double inocula of untransferred arg.-1$^-$ and arg.-10$^-$ with arg.-10$^-$ and arg.-1$^-$ recovered from non-selecting mycelia of the 13th transfer.

Double inoculum of "breakdown" arg.-1$^-$ and untransferred arg.-10$^-$	Single conidium colony number	% majority class
Isolate No.		
1	1	68.5
	2	66.5
	3	74.0
	4	72.0
2	1	50.0
	2	67.0
	3	65.0
	4	65.5
3	1	50.0
	2	72.0
	3	72.0
	4	65.0
"Breakdown" arg.-10$^-$ and untransferred arg.-1$^-$		
1	1	90.0
	2	89.0
	3	90.5
	4	95.0
2	1	94.0
	2	96.0
	3	90.0
	4	96.0
3	1	98.0
	2	84.5
	3	96.0
	4	90.0

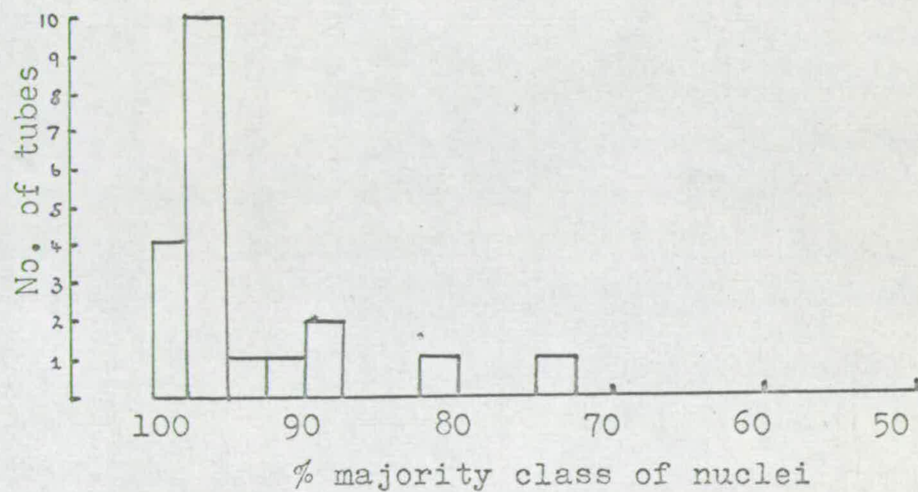


Fig.-21 Distribution of nuclear ratios of mycelia from conidia of a double inoculum culture of untransferred homocaryons eight weeks after start of serial transfer experiment. (Cf. Fig.-20a)

Summary

Rapid serial transfer of homocaryons, which form a selecting heterocaryon, results in a permanent loss of selectability after a maximum of 24 transfers of mycelium. After 13 transfers, one half of the conidia, produced by a double inoculum culture, resulted in intermediate ratio mycelia. These mycelia generated further intermediate ratio cultures. Twenty-fourth transfer arg.-1$^-$, untransferred arg.-10$^-$ double inocula, produced only non selecting heterocaryons while 24th transfer arg.-10$^-$, untransferred arg.-1$^-$ double inocula, produced selecting heterocaryons. Only arg.-1$^-$ had lost the ability to form selecting heterocaryons.

Age and contamination effects to account for loss of selectability on transfer were largely ruled out.

Section IV.

Breakdown and resynthesis of heterocaryotic mycelia.

The following experiments resulted from a misfortune when the only pair of backcross isolates so far found to form a fast growing, selecting heterocaryotic mycelium were lost, along with the heterocaryon. Both had been crossed to 3 A but were required for progeny tests. Each of the mutants had been set up in combinations separately with other mutants, but none of the mycelia from conidia of double inocula selected. A number of them were broken down and the homocaryons in question isolated. But no matter what the origin of the homocaryons no selecting combinations were found.

Since the original mutants now no longer existed it was impossible to confirm that they did originally form selecting mycelia. Progeny from the cross to 3 A had been isolated so it was possible to test whether constituents of a selecting mycelium lost the ability to form such a mycelium after they had been in a non selecting mycelium.

Four isolates from the above cross were known to form the following arg.-10$^-$; arg.-1$^-$ heterocaryotic mycelia; B362-32;46004-2 and B362-14;46004-5 selecting, and B362-32;46004-5 non selecting. When these combinations were originally tested, only one conidium from each double inoculum was grown up on a race tube to measure nuclear ratio. Transfers of the single

conidial cultures had been made and nuclear ratios of mycelia from conidia of these transfers confirmed the original classification. B363-32;46004-5 was broken down and the homocaryons recovered. Three B362-32 and three 46004-5 were set up in double inocula with the original untransferred mutant isolates 46004-2 and B362-14. A single conidium from each was grown up and ratio measured. The results and a schematic explanation of these operations are given in Table 8 and Fig. 22. The mycelia containing the recovered B362 mutants were of extreme ratios but those with the recovered 46004-5 were of intermediate ratio.

The problem of conidial heterogeneity had not been realised at this stage, so further experiments were carried out to confirm the indication that an arg.-1$^-$ mutant had apparently lost its ability to form a selecting mycelium after passage through a non selecting mycelium.

Breakdown isolates of B362-32; 46004-2 when put together resynthesise a selecting mycelium. Breakdown per se is therefore not the source of loss of selectibility. When a 46004-2 mutant, recovered from a non selecting mycelium is used, no selecting mycelia are produced, Table 9, Fig. 23. Only one breakdown 46004-2 was tested and it is not known whether all recovered mutants would behave in this way.

To test whether a gain in selectability could be achieved, a non selecting mycelium, B362-31;46004-2, was broken down and

homocaryons recovered. The homocaryons recombined to form a non selecting mycelium. When an arg.-10⁻ mutant, recovered from a selecting mycelium, was combined with the arg.-1⁻ then again no selection resulted. On the other hand, arg.-1⁻ mutants from a selecting mycelium formed a selecting mycelium no matter from which kind of mycelium the arg.-10⁻ mutant (B362-31) originated. The arg.-10⁻ mutants, recovered from a non selecting heterocaryon, when tested with transferred and untransferred arg.-1⁻ mutants (Section III) formed non selecting and selecting heterocaryons (Table 10).

Summary and Conclusions

When a heterocaryotic mycelium is formed from breakdown isolates of other mycelia, the origin of the arg.-10⁻ mutants has no influence on selectability of the mycelium made between the breakdown isolates. Arg.-1⁻ mutants, on the other hand, suffer an apparent loss or gain of the ability depending on whether they were recovered from non selecting or selecting mycelia. Rapid transfer of an arg.-1⁻ homocaryon appears to have the same effect, loss of selectability, as passage through a non selecting mycelium.

B362-32;46004-2 and B362-31;46004-2 were originally set up at the same time using conidia from one slant of 46004-2 derived directly from an ascospore. Apparent loss or gain of selectability could have been due to heterogeneity in the original ascospore isolate and/or stable variations in selectability of the conidia from the original double inocula.

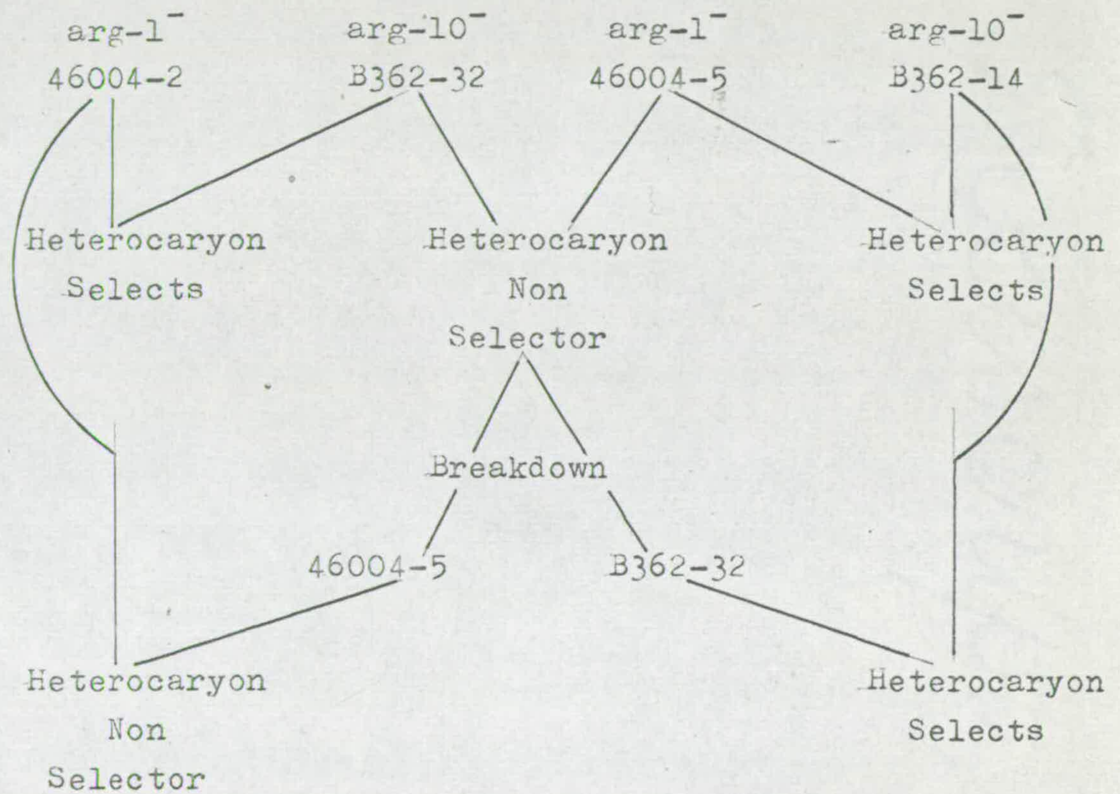


Fig.-22 Origin of mutant ascospore isolates forming heterocaryons whose nuclear ratios are given in Table 8

TABLE 8

Nuclear selection in heterocaryotic combinations of "Breakdown" homocaryons and original ascospore isolates

Double inoculum	Nuclear ratio of conidial colony from double inoculum
(a) Breakdown B362-32 homocaryons from 18362-32;46004-5 with original mutant isolate 46004-2	
1	99.5
2	97.5
3	95.5
(b) Breakdown 46004-5 homocaryons from B362-32;46004-5 with original mutant isolate B362-14	
1	65.0
2	73.0
3	80.0

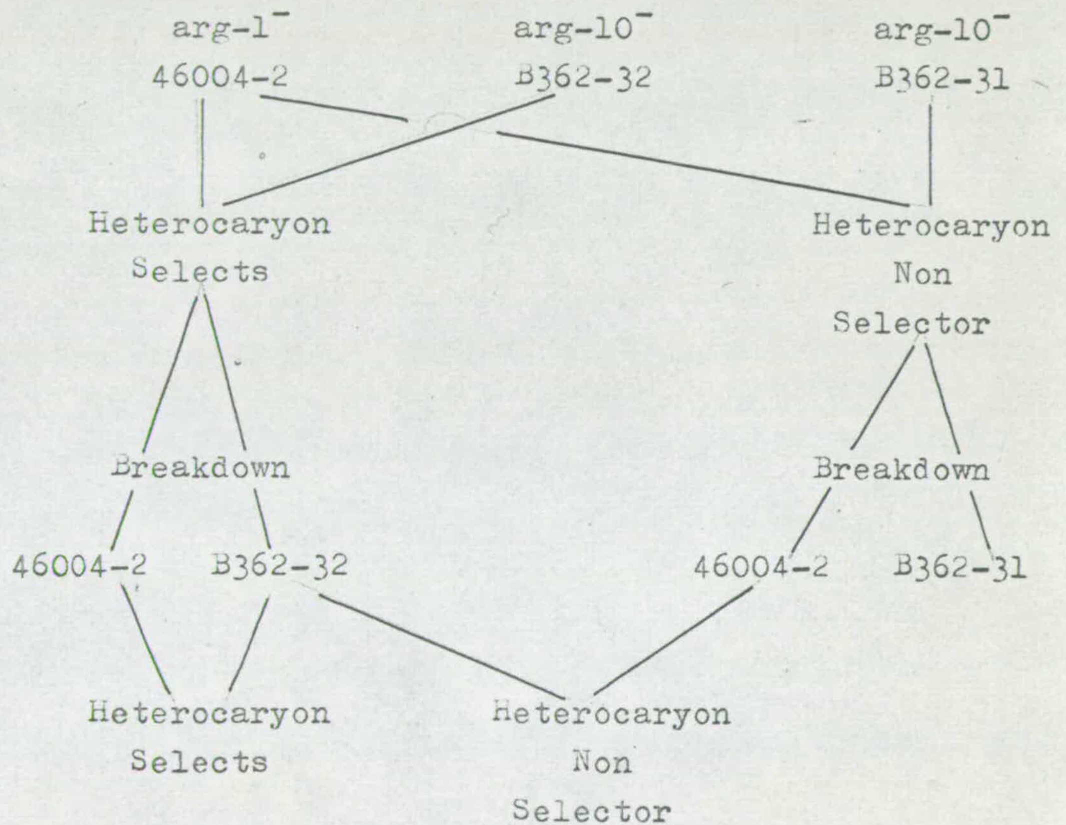


Fig.-23 Origin of mutant ascospore isolates forming heterocaryons whose nuclear ratios are given in Table 9a

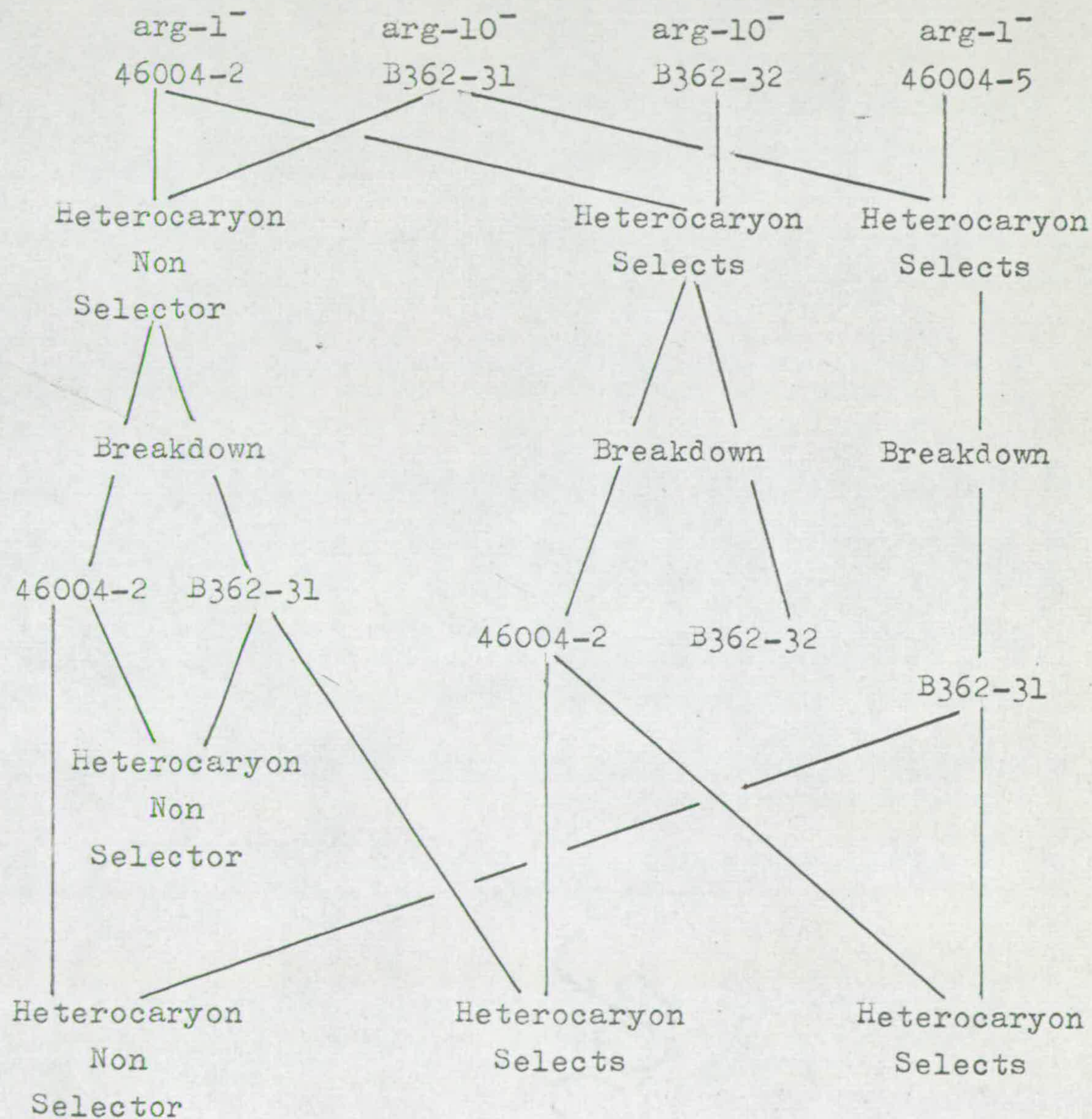


Fig.-24 Origin of mutant ascospore isolates forming heterocaryons whose nuclear ratios are given in Table 9b

TABLE 9

Nuclear selection in various combinations of breakdown homocaryons

Double inoculum	Origin of homocaryons (from selecting (S) or non selecting (NS)		Nuclear ratio of single conidia cultures from double inoculum
	B362 Arg.-10⁻	46004 Arg.-1⁻	(% Majority class)
a. B362-32:4600-2	S	NS	71.5 68.5 56.0
	S	S	99.0 98.0 97.5
b. B362-31:46004-2	NS	NS	71.0 67.5 68.5 64.0 50.0
	S	NS	70.5 84.0 65.0 70.0 63.0
	S	S	98.0 97.5 96.5 91.0 99.5
	NS	S	97.0 97.5 98.0 99.0 98.5

TABLE 10

Nuclear selection in heterocaryotic combinations of "breakdown" homocaryons and homocaryons which had undergone rapid serial transfer.

a. Three arg.-10$^-$ homocaryons from non selecting heterocaryons B362-31;46004-2 set up in double inocula with untransferred 46004-2 homocaryon

Double inoculum No.	Nuclear ratio of single conidia cultures from double inoculum (% majority class)
1	97.0
	94.5
	99.0
2	95.0
	99.0
	99.5
3	97.5
	94.0
	99.0

b. The above three homocaryons set up in double inocula with 46004-2 after twenty-four serial transfers.

Double inoculum No.	Nuclear ratio of single conidia cultures from double inoculum (% majority class)
1	91.0
	72.0
	58.0
	72.0
2	74.0
	86.0
	72.0
	73.0
3	56.0
	58.0
	76.5
	72.0

Section V.

Input/Output nuclear ratios of a selecting heterocaryon

Pittenger and Brawner reported that in mycelium from conidial pellets containing 70% I nuclei and 30% i nuclei no selection took place and ratios remained stable. Table 11 gives the result of an attempt to find a similar state of affairs in the system under consideration. Pellets were made up by the method of Atwood and Pittenger in ratios from 99% B362-32, 1% 46004-2 to 1% B362-32, 99% 46004-2. After three days incubation on arginine sorbose plates the pellets were transferred to growth tubes and nuclear ratios measured at beginning and end of the tubes. The nuclear ratio of the pellets themselves could not be measured because of very poor conidiation. The suspensions of conidia used in making the pellets were plated to check that numbers of viable conidia corresponded to the input ratios.

All pellets except those with input ratios of 10% and 1% arg.-10^{-} conidia, remained at, or selected to, extreme ratios where the majority class of nuclei was arg.-10^{-}. The exceptions remained at, or were only very slowly selecting away from, their input ratios. Ratios at beginning and end of the tubes were very similar even in tubes where the mycelium had selected from intermediate to extreme ratios. This was presumably due to the fact that a considerable amount of growth

TABLE 11

Input and output nuclear ratios of selecting mycelia from pellets containing varying proportions of homocaryotic conidia.

Heterocaryon B362-32;46004-2

% B362-32 (arg.-10$^-$) conidia in pellet	Nuclear ratios in growth tubes (% arg.-10$^-$)	
	Beginning	End
99	98.0	99.0
90	98.0	99.5
80	98.5	98.5
70	95.0	98.0
60	96.0	96.0
50	97.5	99.0
40	95.0	93.0
30	94.0	98.5
20	91.0	82.0
10	5.0	5.5
1	8.5	6.0

had taken place around the point of inoculation before conidia were formed.

Input/Output ratios of non selecting heterocaryons

Before it was realised that selectability had been lost from the mutant isolates of the backcross to 3 A, attempts were made with these stocks to determine whether Pittenger and Brawner's selection threshold was operating. No matter what the ratio of homocaryotic conidia in the pellets, however, the nuclear ratios at the ends of the growth tubes were intermediate ones.

Table 12 gives the output nuclear ratios at the end of growth tubes of pellets made up from various proportions of conidia from (a) B362-32 and 46004-5, conidia from the double inoculum of these homocaryons producing only intermediate ratio heterocaryons and (b) B362-32 and 46004-2 which at first set up of the homocaryons in double inocula was classified as a selector but 'lost' the ability to select thereafter.

Samples of mycelium from the ends of the B362-32; 46004-2 pellet tubes were transferred to fresh tubes and ratios remeasured at the end. The mycelial transfers were washed free of conidia in sterile water. In the mycelium from the pellet with a 99% arg.-10^{-} input, the nuclear ratio in the second tube dropped to 72%:28% from 94%:6% in the first tube. Of the other pellets some ratios became more extreme others less extreme after the second period of growth.

A second experiment using B362-32 and 46004-2 conidial pellets was carried out and this time pellets with 1% and 10% arg.-10$^-$ conidia produced mycelium which remained at extreme ratios with a majority of arg.-1$^-$ nuclei, Table 13. The other cultures remained at approximately the same ratios or changed to intermediate ratios, but all with arg.-10$^-$ nuclei predominating.

It was decided to retest the ratios of mycelium from extreme ratio pellets. Three different combinations of mutants were used, two having been consistently classified as non selectors by measurement of ratios of single heterocaryotic conidia and the third being B362-32:46004-2 which had 'lost' the ability to select. The results in this case, Table 14, were that those mycelia from pellets with a 99% input of arg.-10$^-$ conidia remained at extreme ratios and those with a 99% input of arg.-1$^-$ conidia became less extreme. Washed mycelium from two extreme ratio heterocaryons was transferred to growth tubes containing minimal and arginine medium but no further change in ratio was observed.

Finally, extreme ratio pellets of B362-32 and 46004-2 conidia and of B362-12 and 46004-2 conidia were made up and after the usual incubation period each pellet was divided into six portions, three being used to inoculate growth tubes with arginine medium and three to inoculate minimal growth tubes. In all cases, Table 15, the pellets placed on arginine medium produced mycelium of extreme ratio whose majority class of

nuclei was the same as the majority class of conidia in the pellet. On minimal medium, only those pellets with a high input of arg.-10^{-} conidia remained at, or very close to extreme ratios, while high input arg.-1^{-} cultures changed to intermediate ratios.

In all the above experiments the concentration of viable conidia in the pellets was checked by plating the conidial suspensions.

To check that heterocaryotic mycelium does not break-down into its component homocaryons on arginine medium a 50:50 pellet of conidia from B362-12 and 46004-10 was made up. After incubation on sorbose medium, the pellet was divided into two arginine and two minimal tubes. This is a non selecting heterocaryon, so any reduction in numbers of heterocaryotic conidia produced on arginine compared with minimal must be due to breakdown and not ratio change. The final ratios on the arginine tubes were 50% and 50.5% majority class of nuclei, and on minimal, 50% and 52%. Therefore no breakdown had occurred on arginine medium and presumably, extreme ratios of mycelia grown on arginine in previous experiments were not due to breakdown.

Extreme ratio pellets of B362-12 and 46004-10 were allowed to grow on minimal plates and punch tube samples removed from the fronts at intervals. The nuclear ratios of each series of samples are given in Figs. 25-27, and show the changes in ratio of the mycelia as it grows. The

funnel tube method was also employed to show this change.

Summary and Conclusions

It was concluded from these results that ratios of intermediate ratio heterocaryons were not due in every case to maintenance of initial ratios. This conclusion having been pointed to when ratios of heterocaryons, produced by conidia from an intermediate ratio mycelium, were compared to the expected conidial ratios from such a mycelium. It appears then that in mycelium from pellets with disproportionate numbers of the two types of conidia that nuclear ratios tend to become less extreme. Using extreme ratio pellets it was found that when arg.-10$^-$ nuclei were in the majority, the extreme ratio was maintained in the mycelium. When arg.-1$^-$ nuclei were in the majority ratios in the mycelium became less extreme. Mycelial transfers from the extreme ratio arg.-10$^-$ colonies showed no further change after growing the length of a second growth tube containing minimal medium or arginine medium. These changes were also demonstrated by nuclear ratios of punch tube samples from the fronts of mycelia originating from both types of extreme ratio pellets.

Extreme ratio pellets grown on arginine supplemented medium remained at extreme ratios while samples of the same pellets inoculated on to minimal medium showed a change in ratio towards intermediate ratios when the pellet contained a high proportion of arg.-1$^-$ conidia.

Non selecting heterocaryons in fact select towards intermediate ratios when the initial ratio is an extreme one in favour of arg.-1$^-$ nuclei. The fact that this selection seems to be prevented on arginine medium points to the conclusion that this selection has some connection with the metabolic consequences of the arginine mutations.

TABLE 12

Input/output nuclear ratios of a non-selecting mycelium and of a mycelium in which selectability has been lost.

% arg.-10⁻ conidia in pellet	Output ratio at end of growth tube (% majority class)	Ratios of mycelial transfers at end of second growth tube
(a) Heterocaryon B362-32;46004-5		
99	55	
90	76	
80	67	
70	50	
60	69	
50	50	
40	74	
30	61	
20	50	
10	70	
1	70	
(b) Heterocaryon B362-32;46004-2		
99	94	72
60	50	65
50	70	77
40	68	72
1	63	81

TABLE 13

Input/output nuclear ratios of a mycelium in which selectability has been lost.

Heterocaryon B362-32;46004-2

%arg-10⁻ conidia in pellet	Output ratio at end of growth tube (% arg.-10⁻ nuclei)
99	97
90	82
80	79
50	70
20	50
10	16
1	3

TABLE 14

Extreme input nuclear ratios of three non-selecting mycelia.

	% B362 conidia in pellet	Output ratio at end of race tube (% majority class)
Heterocaryon B362-32;46004-5		
1	1	67
2	99	89
Heterocaryon B362-32;46004-2		
3	1	73
4	99	98
Heterocaryon B362-12;46004-10		
5	1	64
6	99	95

Mycelium from tubes 4 and 6 to fresh race tubes with minimal and arginine medium.

4	arginine	97
		92
		93
	minimal	95
		97
		98
6	arginine	97
		95
		97
	minimal	96
		98
		94

TABLE 15

Nuclear ratios of mycelia from extreme ratio pellets of non-selectors grown on arginine and minimal medium.

	% arg.-10⁻ conidia in pellet	Output ratio at end of race tube (% majority class) arginine	minimal
Heterocaryon B362-32;46004-2			
	1	88 arg.-1⁻	69
		99	73
		99	83
	99	96 arg.-10⁻	94 arg.10⁻
		96	95 arg.10⁻
		95	95
Heterocaryon B362-12;46004-2			
	1	86 arg.-1⁻	84
		96 "	69
		99 "	71
	99	98 arg.-10⁻	81
		98 "	97 arg.10⁻
		97 "	98 arg.10⁻

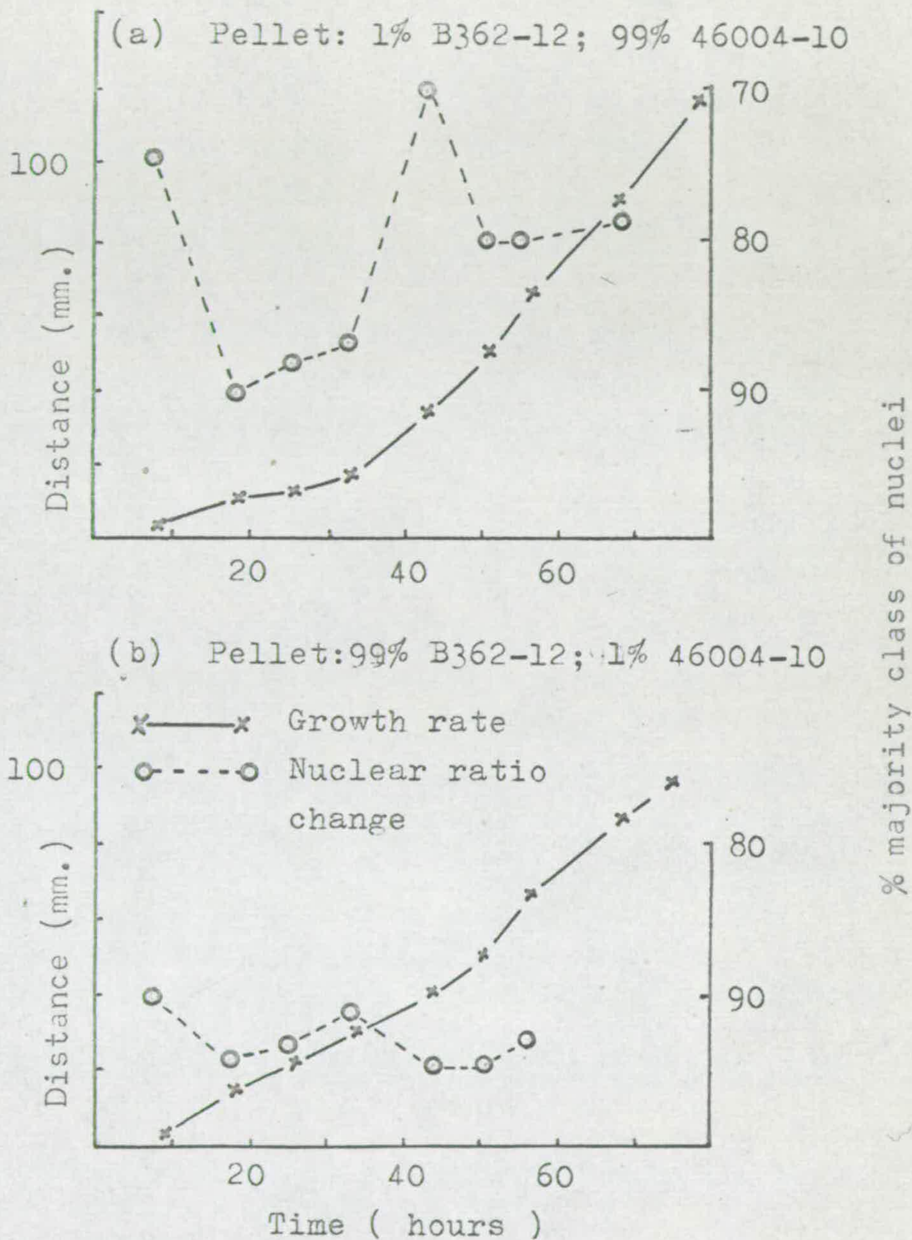


Fig.-25 Nuclear ratios of punch tube samples from fronts of non selecting colonies originating from pellets of following composition

(a) 1% arg-10$^-$; 99% arg-1$^-$

(b) 99% arg-10$^-$; 1% arg-1$^-$

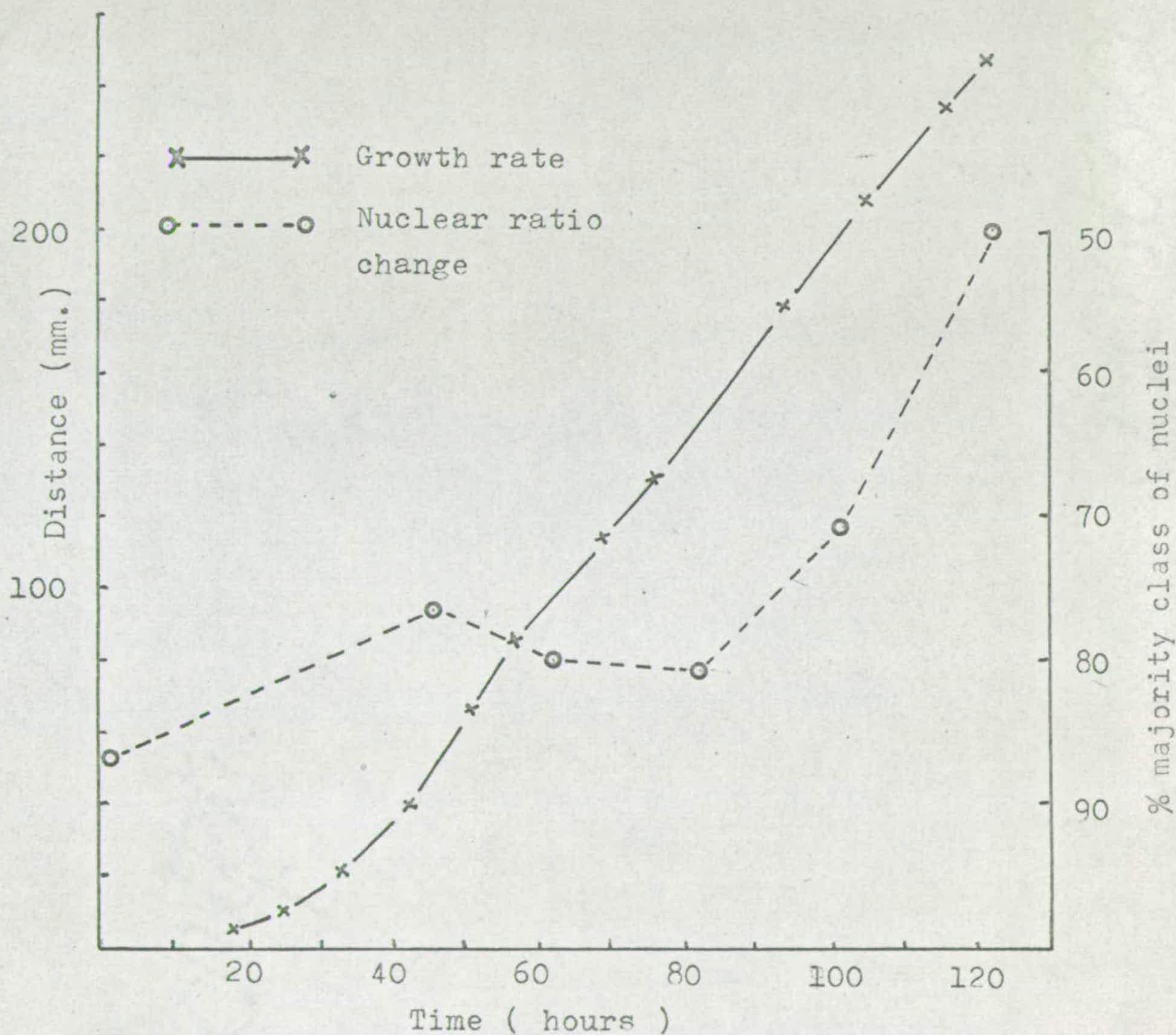


Fig.-26 Growth rate and nuclear ratios of non selecting mycelium originating from a conidial pellet of the following composition 1% B362-12 (arg-10⁻) 99% 46004-10 (arg-1⁻). Measured on a funnel tube.

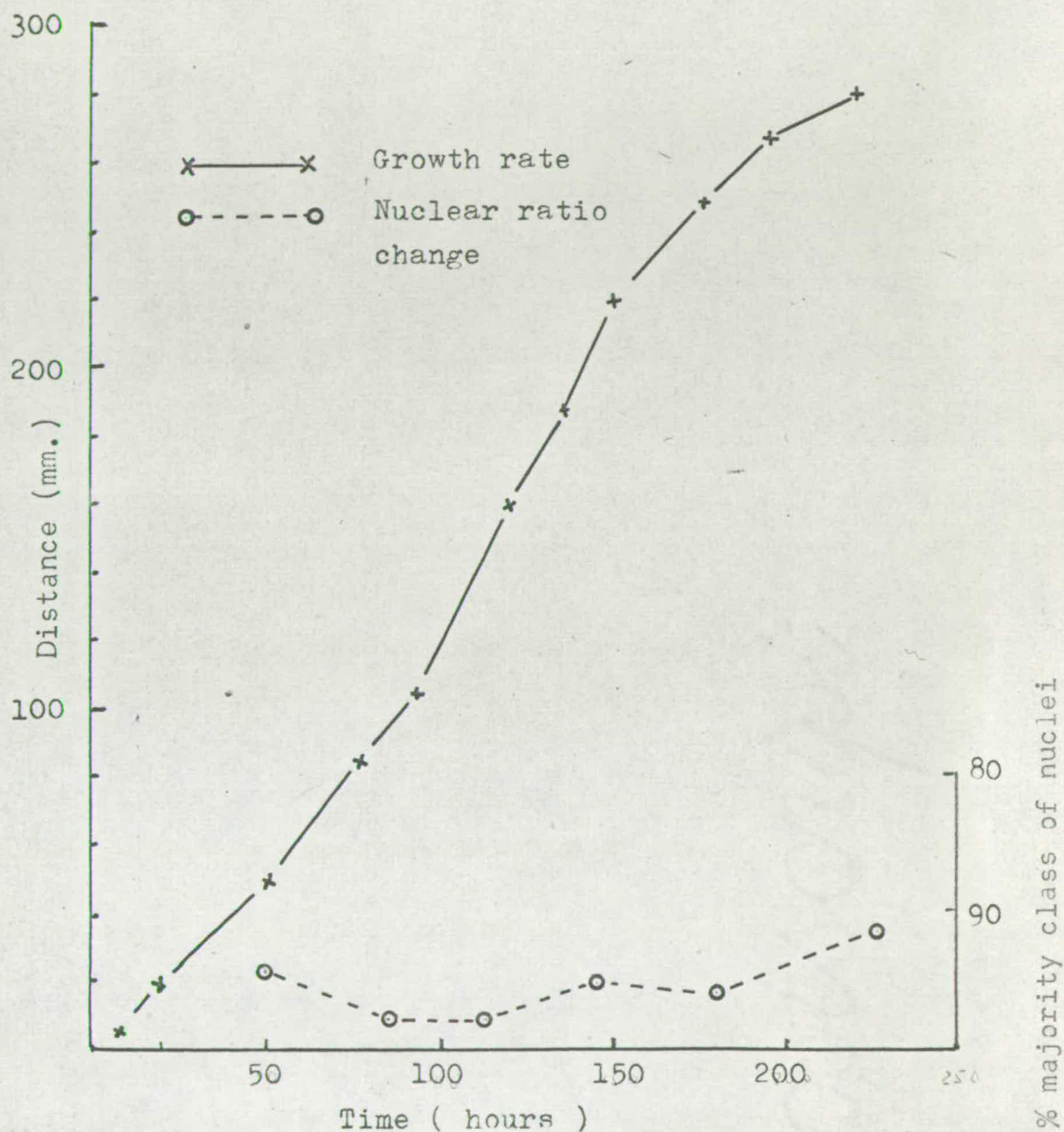


Fig.-27 Growth rate and nuclear ratio of non selecting mycelium originating from a conidial pellet of the following composition 99% B362-12 (arg-10⁻) 1% 46004-10 (arg-1⁻). Measured on a funnel tube.

D I S C U S S I O N

1. Selecting heterocaryons

The system under discussion is one in which a heterocaryon, between two non allelic mutants, arg.-1$^-$ and arg.-10$^-$ of the arginine cycle, undergoes during growth a change in nuclear ratio (selection). The selection process has been shown to be regular and continuous from intermediate mycelial nuclear ratios to extreme ratios. Homocaryosis is not the end point of selection but once values of 90% or more of arg.-10$^-$ nuclei have been reached only small fluctuations in ratio are observed over many transfers of mycelium.

Supplementation with arginine at levels which allows the homocaryons to grow at wild type growth rates has no effect upon selection. A variety of other supplements was also found to be ineffective in influencing selection. Selection was not, therefore, due to the metabolic consequences of the arginine mutations. It was not due either, apparently, to any other, simply supplementable, deficiency.

It therefore remains to decide whether selection to extreme ratios is due to nuclear interactions or to nucleo-cytoplasmic interactions. In the first case, selection could be due simply to differential division rates of the two nuclei or due to inhibition of one nucleus by a nuclear product of the other. In the second case something like inhibition of one nucleus by the other via an intermediate cytoplasmic

element is envisaged.

Two of the reported experiments could decide between these alternatives. The first of these two experiments is that which demonstrated progressive loss of ability in an arg.-1$^-$ homocaryon to form selecting mycelia with a given arg.-10$^-$, Fig. 20. After 13 successive serial transfers of mycelium from the front of the arg.-1$^-$ homocaryon, a double inoculum culture of conidia from this arg.-1$^-$ and an arg.-10$^-$ was set up. A number of conidia from the double inoculum were grown up and tested, one half produced extreme ratio mycelium and the other half intermediate ratio mycelium. These intermediate ratio mycelia generate, in turn, only intermediate ratio mycelium. After 24 transfers, however, the majority of conidia from the double inoculum culture produced stable intermediate ratio mycelia. A few extreme ratio mycelia were produced but these generated only intermediate ratio mycelium.

The second experiment, Figs. 17 and 18, demonstrated that conidia from extreme ratio, selecting mycelia were heterogeneous with respect to selectability. Conidia from selecting mycelia produced some intermediate ratio mycelia but these were unstable in that they generated from their conidia only extreme ratio mycelia.

Any theory put forward to explain selection must explain loss of selectability and also conidia heterogeneity with respect to selectability.

Firstly, assume that selection is due to differential nuclear division rates of the two types of nuclei, possibly due to the different backgrounds of the types. Should a mutation occur in the arg.-1$^-$ homocaryon which eliminates this differential, the heterocaryon of this homocaryon and an arg.-10$^-$ homocaryon will not show change to extreme ratios. Rapid transfer of arg.-1$^-$ is associated with loss of selectability, and according to this hypothesis then, transfer must be associated with an increase in mutant nuclei. Secondly, the mutation could be such as to effect some interaction in the heterocaryon, but unless it is also associated with increased division rate in the homocaryon, as with the first type of proposed mutation, it is difficult to imagine how serial transfer of mycelium would allow its accumulation.

Two other considerations count against the mutation theory. Firstly, the mutation rate would have to be very high to account for the fact that more than one homocaryon lost the ability to form selecting heterocaryons. Secondly, assume that heterogeneity of conidia from heterocaryons of untransferred homocaryons is also due to this mutation. Arg.-1$^-$ homocaryons presumably contain a mixed population of 'selecting' and 'non selecting' nuclei which are distributed in conidia of the heterocaryon at random; the result being that some conidia will produce selecting mycelia and others will not. To explain the fact that some non selecting mycelia from a selecting strain generate conidia

which produce selecting mycelia, some hypothesis of reversibility of the effect of the mutant at this stage has to be proposed. The only other alternative is to assume that conidial heterogeneity with respect to selectability is due to a cause other than that proposed to explain loss of selectability on serial transfer.

Any theory concerning changes in or of the nucleus must have to explain conidial heterogeneity and either an additional assumption of reversibility or additional hypotheses must be considered. While there is no evidence to reject such theories it is felt that a more acceptable theory to explain the results of the two experiments is one in which nuclear behaviour in selecting heterocaryons is directly dependent on alternative states of the cytoplasm. Such a theory fits the experimental results and there is supporting evidence from other work in Neurospora and in other filamentous fungi that such interactions exist.

Jacob et al (1960) have pointed out that the danger of episomes is, that they can be invoked to explain almost any complex nucleo-cytoplasmic interaction. The same could be said for cytoplasmic elements, but nevertheless they shall be invoked to explain the results reported in this thesis.

First of all the applicability of such a theory to the experimental results will be considered in operational terms, leaving certain questions open for further consideration.

Assume that a population of cytoplasmic elements interact with nuclei in growing, selecting mycelia in such a way that there is a decrease of arg.-1⁻ nuclei, without specifying the mechanism of this interaction. Below some threshold level of elements there is no detectable effect on nuclear ratio.

During conidiation, the elements would be distributed at random into conidia, some conidia having less than the effective number of elements to initiate change in nuclear ratio during germination and growth of the subsequent mycelium. Only until such times as the effective concentration is built up will any change in the ratio be seen. In some mycelia, starting with a low number of elements this point may not be reached until the culture has almost reached the end of a growth tube and conidiation is proceeding, too late to effect mycelial nuclear ratio. An intermediate ratio will be found in this mycelium but in subsequent mycelia from its heterocaryotic conidia, in which the cytoplasmic elements will now be distributed normally, extreme ratios will be expected.

This assumption could be tested by repeating the experiments on conidial heterogeneity but allowing more growth than is possible in a single growth tube before conidiation. If the above explanation holds then a decrease in conidial heterogeneity would be the expected result.

The cytoplasmic elements are presumed to be introduced into the heterocaryon by the arg.-1⁻ homocaryon. In the

homocaryon, under the conditions of the serial transfer experiment, numbers are presumed to increase more slowly than nuclei. Consequently,over a number of transfers, concentration of elements decreases, with corresponding increase in conidial heterogeneity, until eventually all elements are lost or reduced to a level where there is no effect upon nuclear ratios in the heterocaryons containing transferred arg.-1⁻ nuclei and cytoplasm.

An irregularity in the behaviour of homocaryons used in transfer experiments is that, when they were first isolated as single ascospore cultures, selectability was lost very rapidly over two or three mass transfers of conidia and hyphae. In an arg.-1⁻ homocaryon, reisolated from a selecting mycelium, it took 13 transfers of mycelia before any loss in selectability became apparent. Similarly, over four mass conidial transfers of this arg.-1⁻, no loss in selectability was noted. This difference in behaviour could be explained by the fact that some ascospores contain very few of the cytoplasmic elements, due to unequal distribution of cell contents at meiosis when the ascospores are formed. Ascospores were germinated and activated on small slants, conidia being produced at the point of inoculation of the ascospores. It is conceivable that, by the time this culture produced conidia there were still so few elements that there was great heterogeneity between conidia. The chance of loss

of elements or reduction to an ineffective level, even by mass transfer of conidia, would be large.

Such heterogeneity would explain why two heterocaryotic mycelia made up from ascospore cultures, containing nuclei of different arg.-10⁻ isolates of the same cross, but nuclei of the same arg.-1⁻ isolate, should differ in selectability. When the arg.-1⁻ was recovered from the selecting mycelium it formed selecting mycelia with both arg.-10⁻ homocaryons. Similarly an arg.-1⁻ homocaryon formed a selecting mycelium with one arg.-10⁻ isolate but not with another but when the arg.-1⁻ was recovered from the non selecting mycelium it would no longer form selecting mycelia with either arg.-10⁻. This loss or gain of selectability, confined solely to arg.-1⁻ homocaryons, is probably due to heterogeneity with respect to cytoplasmic elements in the conidia of the original ascospore isolates.

The following examples are given to show that in other systems, cytoplasmic determinants are distributed into conidia in such a way as to cause heterogeneity of conidia with regard to expression of these determinants, and in other cases such a situation has been invoked to explain experimental results. Continued vegetative propagation has also been shown to account for cytoplasmic differentiation.

In _Aspergillus nidulans_, Jinks (1954) has shown that the continued propagation by conidia, which are uninucleate,

or by hyphal tips, causes readjustment of the cytoplasm resulting in a loss of capacity for sexual reproduction. Briault (1956) has reported heterogeneity of conidia for cytoplasmic elements which determine the ability to adapt to new media.

Mahoney and Wilkie (1962) studying strains of A. nidulans found that conidia and ascospores give rise to the same proportions of a mutant called alba which fails to produce perithecia. Crosses indicate cytoplasmic determination of the mutant. They postulate that at sporogenesis there is random segregation of self reproducing particles into conidia. Some conidia with no particles produce stable mutants, others which have less than some threshold value of particles produce mutant types also, but these revert under various conditions. Reversion occurs at low temperatures, when it is assumed that production of particles outstrips production of nuclei, and the threshold value is reached. A gene, f, is also involved which may determine the threshold value in different strains. An alternative hypothesis being that f affects the relative stability of different metabolic states within the cytoplasm.

Fitzgerald (1963) suggests that phenotypic variation of a double mutation of Neurospora crassa, ranging in expression from reduced female fertility to complete sterility with production of a black pigment, could be due to different

equilibrium states between cytoplasmic elements. Intensificiation of phenotypic expression was brought about by rapid growth and serial transfer of mycelium. The extreme phenotype was found to be stable on subculturing.

Given that the main assumption holds, namely the involvement of cytoplasmic elements in the process of selection, a number of questions can be asked and discussed within the limits of the results of experiments done to date: (1) do cytoplasmic elements replicate? (2) Is the majority nuclear class always arg.-10$^-$ nuclei? (3) what is the role of the majority nuclear class in selection, and what happens to the minority nuclei, are they left behind during growth or is division inhibited and slowed down?

(1) It would appear very unlikely that the cytoplasmic elements do not replicate. The arg.-1$^-$ homocaryons used in serial transfer experiments were isolated as single conidia from a selecting mycelium. After eight transfers of the homocaryons, conidia from double inocula of the homocaryons still produced selecting mycelia. The number of e lements must therefore have increased from the quantity originally present in the conidium from which the arg.-1$^-$ arose. In selecting mycelia,successive production of further selecting mycelia from single conidia has been reported Fig. 18. A selecting mycelium used in a previous investigation was transferred twelve times by massive conidial

inocula without loss of selectability. Elements can be assumed to replicate in the homocaryons as well as in heterocaryons.

Once the elements are lost or drop below a certain level no further multiplication is possible. This is assumed since,after serial transfer and loss of selectability, normal growth and culture of homocaryons and heterocaryons does not generate selecting strains. The occasional extreme ratio heterocaryon does not generate further extreme ratio heterocaryons, Fig. 20, and this is consistent with the occasional extreme ratio starting conidium.

To test whether loss of cytoplasmic elements is the only change taking place in transferred homocaryons a conidial pellet containing arg.-10$^-$ conidia, transferred (non selecting) arg.-1$^-$ conidia and a small proportion of untransferred (selecting) conidia should select to an extreme ratio given sufficient time for the cytoplasmic elements introduced by untransferred arg.-1$^-$ conidia, to build up to an effective level. The experiment was carried out using different input proportions of the three kinds of nuclei but all output ratios were intermediate. The control culture of selecting arg.-1$^-$ with arg.-10$^-$ was also of intermediate ratio suggesting that selectability had been lost in this arg.-1$^-$ also.

(2) In all heterocaryons which have been fully tested, in the experiments reported in this thesis and in other unreported experiments, the majority class of nuclei

in selecting heterocaryons has always been arg.-10$^-$. Assuming that in all these cases the cytoplasmic elements have been introduced by the arg.-1$^-$ homocaryon i.e. are always associated with the minority class of nuclei, this would suggest either a self replicating element associated with the arg.-1$^-$ nucleus, or an element whose replication is controlled by the arg.-1$^-$ nucleus. Since all mutants studied were of the same mating type there was no opportunity to make arg.-10$^-$ x arg.-1$^-$ crosses. It would be interesting to learn whether from such crosses, recombinant types could be isolated which would form selecting heterocaryons in which arg.-1$^-$ were the majority class of nuclei.

(3) When discussing data concerning crosses of arg.-1$^-$ and arg.-10$^-$ to 3 A, given in Table 2, it must be remembered that only a single heterocaryotic conidium was used to test selectability of each mutant isolate. In the later cases, where conidial heterogeneity was measured, 20% of conidia from selecting mycelia produced non selecting mycelia and approximately the same proportion of conidia from a non selecting mycelium produced selecting mycelia. After 13 transfers of an arg.-1$^-$ homocaryon with a resulting reduction in cytoplasmic elements, of the conidia produced by a double inoculum culture of transferred homocaryons, one half produced selecting mycelia the other half non selectors. As mentioned before there may be large

variations in numbers of elements in conidia from mycelia derived directly from an ascospore. Consequently there may be a large variation in selectability in conidial cultures derived from a double inoculum of such heterogeneous conidia. Therefore measurement of one conidial derived culture from a double inoculum is not a reliable measure of the ability of the homocaryons to form selecting mycelia.

The mutant isolates from a cross of arg.-10⁻ to 3 A apparently segregated in a 1:1 ratio with regard to selectability, when combined in a heterocaryon with one arg.-1⁻ mutant, by the single conidial test. Bearing in mind the qualifications upon the reliability of such results, the apparent result that a single gene was segregating, and that this gene is the one, which by interaction with the cytoplasmic elements causes the relative decrease of arg.-1⁻ nuclei, cannot be said to be experimentally established.

As to the fate of the minority nuclear class in selecting heterocaryons little can be said at this juncture. They are certainly not left behind during growth since the mycelium does not become homocaryotic. They must divide, and increase from the initial numbers present in the heterocaryotic conidia, doing so at a decreased rate or at a normal rate but with a proportion of nuclei being destroyed or inactivated. It would be very difficult to distinguish

experimentally between these two possibilities.

While the assumption of interaction between gene and cytoplasmic elements remains highly speculative it would be profitless to extrapolate further and consider the chemical nature of such interactions at this stage.

Finally in the light of these assumptions it is interesting to consider Pittenger and Brawner's selecting systems. Most of their results fit in with their theory that nuclear selection is due to two genes, I and i, not yet convincingly located in a linkage group. I nuclei are presumed to inhibit the replication of i nuclei but only when the initial proportion of I nuclei is 30% or greater. This is attributed to weak dominance of I nuclei, whatever dominance may mean at this level. In the present case no selection was found in mycelium from conidial pellets which contained 10% or less of nuclei forming the majority class in selecting heterocaryons. Since only one such experiment was carried out the difference between 10% and 30% can be ignored. The important fact is that a threshold exists in both cases. In explaining conidia heterogeneity, however, their theory is less convincing. Assuming that conidial ratios of 3i:1I do not produce selecting heterocaryons they can explain why a number of selecting heterocaryons produce between 10%and 26% heterocaryotic conidia which do not select to extreme ratios. They give no data upon which to

calculate an expected figure for such conidia and give no expected figure themselves. Assuming that the heterocaryons which produced the conidia have selected to extreme ratios i.e. majority I nuclei, it seems likely to expect that the proportion of conidia with a 3:1 majority of i nuclei would be less than 10%, certainly less than 26%. Approximate expected frequencies of conidia of these ratios, calculated for a heterocaryon whose frequency distribution of nuclei in conidia is given in Fig. 7, are 10% from 50:50 ratio mycelium and 3% from 90:10 ratio mycelium. There is also a report of abnormal results in testing the progeny of certain crosses for selectability but no details are given. This could be interpreted as due to loss or gain of some cytoplasmic elements. Since Pittenger and Brawner have concerned themselves mainly with the genetic aspect of selection, this thesis and their study probably represent different sides of the same coin.

2. <u>Non selecting heterocaryons</u>

The distribution of final nuclear ratios of mycelia, originating from heterocaryotic conidia from intermediate ratio mycelium, was seen to be different from the expected distribution of ratios, calculated on the assumption that in heterocaryons of intermediate ratio the initial conidial ratios were maintained in the growing mycelium. This

difference was apparent whether the heterocaryons were made up from homocaryons which had lost selectability or of homocaryons which had, as ascospore isolates, formed heterocaryons of intermediate ratio. The observed frequencies of nuclear ratios differed from the expected in that there was a deficiency of 50%:50% and 66%:33% ratios and an excess of ratios between 90% and 70% majority class, Fig. 19. It was concluded that some degree of ratio change was taking place.

When pellets containing conidia from homocaryons which formed intermediate ratio heterocaryons, were grown up, in most cases, no matter what the input ratio of the pellet the ratio of the mycelium was intermediate after a period of growth. Further experiments showed that extreme input ratio pellets (99% one class of conidia) remained at extreme ratios on arginine supplemented medium. On minimal medium the mycelium from pellets with 99% arg.-1$^-$ conidia became less extreme. To explain these results it is assumed that, at proportions of 70% or more of arg.-10$^-$ nuclei, the optimum proportions of synthetase and argininosuccinase are reached. Low concentrations of argininosuccinase, synthesised under the control of arg.-1$^-$ nuclei being less critical to the organism than synthetase. Growth rate differences, if any, of heterocaryons with extreme and intermediate ratios are not measurable. Very small linear growth rate differences might be enough to allow selection for intermediate ratio

hyphae. On the other hand, hyphae of intermediate ratio could perhaps produce more branches than other hyphae, i.e. growth rate measured as weight increase may differ between extreme and intermediate ratio mycelia. This would result in an increase of intermediate ratio hyphae by virtue of their faster 'rate of reproduction'.

A few extreme ratio mycelia generated by a small proportion of conidia from intermediate ratio mycelia could be due to the conidia themselves being of extreme ratio. Drift of ratio in the mycelium could be a contributary factor. At values greater than 70% arg.-10$^-$ nuclei, there will be no selective force operating to stop ratios becoming even more extreme by chance. When numbers of nuclei are small, during germination and the initial period of growth, there is a possibility of random drift of nuclei into branch points of the mycelium, so that some hyphae will have more extreme ratios than others. If those hyphae with extreme ratios establish the front in a growth tube then as growth proceeds, and the numbers of nuclei increase, it is likely that the extreme ratios are maintained.

In one pellet experiment, Table 13, mycelium from pellets containing 10% or less arg.-10$^-$ conidia remained at ratios in favour of arg.-1$^-$ nuclei after growth along a growth tube. Since this occurred in only one experiment it is not possible to say what this means, whether it is, for example,

a rate phenomenon or an indication of the existence of another stable equilibrium position with regard to the enzymes. In mycelia which select to extreme ratios this enzymatic selection must also be operating and may account for the difference in threshold value found in this study and in Pittenger and Brawner's investigation. If the value at which there are too few arg.-10⁻ nuclei to produce an effective interaction with the cytoplasmic elements is really 30%, below this level, enzymatic selection will serve to increase the proportion of arg.-10⁻ nuclei until the threshold value is reached and selection to extreme ratios takes over.

A possible consequence of there being two types of selection operating would be, that if by appropriate crosses recombinant types were produced, in which extreme ratio selection favoured arg.-1⁻ nuclei, the presence of a selective force operating in the opposite direction might prevent extreme ratios from being reached. Only on arginine medium would identification of such types be unequivocal.

S U M M A R Y

The subject under investigation was nuclear ratio change in mycelium of interallelic heterocaryons between arg.-1⁻ and arg.-10⁻ homocaryons. These homocaryons are mutants of the arginine cycle of _Neurospora crassa_.

(1) In some heterocaryons, originating from single heterocaryotic conidia, nuclear ratios changed during growth of the mycelium from initial intermediate conidial ratios, 1:1, 2:1 etc., to extreme ratios where the majority nuclear type in the mycelium constituted 90% or more of the total. In all heterocaryons tested, the majority nuclear type was arg.-10⁻. This change in nuclear ratio (selection) was followed in growing mycelium and found to be a regular, continuous process which stopped at some point short of complete homocaryosis.

Supplementation of such heterocaryons with arginine had no effect upon selection, indicating that it was not due to any metabolic consequences of the arginine mutations themselves. Experiments using a wide range of supplements failed to show that selection was due to any simple supplementable cause.

(2) Heterocaryotic conidia from a selecting mycelium were found to be heterogeneous with respect to selection in the mycelium which they produced. Approximately 20% of conidia produced mycelium of intermediate final ratios,

i.e. non selecting. These, however, were unstable in that they generated further selecting mycelia. This heterogeneity was not correlated with the nuclear ratio of the conidium from which the mycelium originated but with some other conidial difference.

Ability to form selecting heterocaryons was progressively lost over a number of serial transfers of homocaryotic mycelium from the growing front. This loss was confined to the arg.-1⁻ homocaryon. The loss appeared to be stable and further heterocaryons from conidia of such heterocaryons also produced non selecting heterocaryons.

(3) There appeared to be a threshold value at around 10% arg.-10⁻;90% arg.-1⁻ where there was no selection or very slow selection towards extreme ratios in favour of arg.-10⁻ nuclei.

(3) To explain these and other experimental results the hypothesis was advanced that selection was due to interaction between arg.-10⁻ nuclei and cytoplasmic elements, resulting in inhibition of division of arg.-1⁻ nuclei. Below a certain threshold level of elements no selection occurs and no replication of elements takes place.

(4) In non selecting, intermediate ratio heterocaryons it was assumed that initial conidial ratios were maintained during growth. On comparison of the array of nuclear ratios of heterocaryons from conidia of a non selecting heterocaryon

with the expected distribution, certain discrepencies were found. Further investigation suggested that there was in fact selection towards 70:30 ratios. This selection process could be prevented by supplementing heterocaryons with arginine. At around 70% arg.-10$^-$ nuclei it appeared that the optimum levèls of each of the two arginine cycle enzymes was reached.

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