STABILITY OF NUCLEAR PROPORTIONS DURING GROWTH OF NEUROSPORA HETEROKARYONS¹

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AN EXPLANATION for the stability of the normal phenotype which generally results from heterokaryosis between biochemical mutants of *Neurospora crassa* was suggested by BEADLE and COONRADT in 1944. They observed that the growth rate of such heterokaryons on minimal medium equalled that of wild type strains, whereas the proportions of the nuclear types varied widely, even among different heterokaryons composed of the same two mutants. Since the mutants cannot grow separately on minimal medium, it was obvious that at some nuclear ratio outside the observed range, the dosage of the normal allele which is carried only in the nuclei of the minority type would be insufficient to support normal growth of the entire culture. It was surmised that chance fluctuations in the local nuclear ratios might cause different growth rates among individual hyphae. Then, "If selection of rapidly growing hyphae is allowed to go on for some time, one would expect nuclear ratios to adjust themselves so as to give a maximum rate of growth". Thus the failure to observe submaximal growth rates might be explained on the basis of natural selection of nuclear ratios in the optimal range. This idea has become so widely accepted that it was stated (PROUT, *et al.* 1953) without qualification that hyphae with certain nuclear ratios are able to grow faster than others and "will, as a consequence, have a greater chance of forming the frontier of the mycelium and will contribute that favorable ratio of nuclear types to the new growth. Thus there will be a constant selection for a ratio of the two types of nuclei that will permit the maximum rate of growth". On the other hand, stable submaximal growth rates were noted by BARRATT and GARNJOBST (1949) in heterokaryons of unknown nuclear proportions between wild type and colonial-1 strains, in apparent contradiction to the notion of hyphal selection.

The opportunity to test this notion is now afforded by the development of methods for experimentally controlling the initial nuclear ratio. In mixed conidial inocula, heterokaryosis is not simply the result of a few pairwise fusions of conidia, but is a widespread anastomotic process by which large numbers of nuclei are intermingled (ATWOOD and PITTENGER 1955). Therefore, it is not surprising that a systematic relation exists between the nuclear ratio in the homokaryotic inoculum and the resultant heterokaryon over a wide range of ratios (PITTENGER *et al.* 1955). Preliminary experiments proved the feasibility of constructing heterokaryons with prolonged submaximal growth rates by the use of inocula with extreme input ratios, and, in several cases, no selection could be demonstrated after various intervals of growth (PITTENGER and ATWOOD 1954). These and other experiments, reported here in more detail, confirm the absence of selection for nuclear ratios capable of giving

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the normal growth rates. A quantitative estimate of the dominance of *pan*⁺ is given, based on the relation between nuclear proportions and growth rate.

MATERIALS AND METHODS

The homokaryotic stocks used were *pan, al-1*, A(5531, 4637); *nic-2, al-2*, A(4540, 15300); *lys-3*, A(4545), and *arg-6*, A(29997). The mutant loci are listed in the descriptive index of BARRATT *et al.* 1954. Their genetic backgrounds are such that all combinations with uncontrolled inocula give the immediate wild type response, that is, type I in the classification of HOLLOWAY (1955). Only one stock of each was used, so that the genetic background associated with a given marker is the same in all experiments. Stocks were grown at 29°C in 125 ml Ehrlenmeyer flasks, on synthetic medium supplemented with the appropriate growth factors. The preparation of heterokaryons having different nuclear ratios was as follows: (1) Conidial suspensions from homokaryotic cultures six to ten days old were mixed in various proportions; (2) mixtures were soaked overnight at 3°C in supplemented liquid medium, then incubated 2 to 4 hours at 29°C in the same medium; (3) mixtures were centrifuged; (4) the pellets were incubated 3 days at 29°C on supplemented sorbose agar; and (5) the coalesced pellets were transferred to growth tubes. It is important to note that all steps in the preparation of these heterokaryons were carried out on supplemented medium and that supplements are carried over in the final growth tube inoculum so that heterokaryosis, although established, does not become obligatory until the mycelium has grown a short distance from its starting point.

Growth rates were measured in horizontal growth tubes as described by RYAN *et al.* (1943). In some instances the growth rate did not become constant, and an average rate was determined in the region of the tube distal to that in which strong systematic trends were apparent. The growth tubes ordinarily used were 16 mm in diameter and 300 to 400 mm in length. In some experiments special growth tubes were used 1000 mm in length and 20 mm in diameter with sampling chimneys at 10 cm intervals. When growth was completed all the conidia were removed from the ends of the growth tubes or from sampling chimneys by aspiration into small glass tubes containing a tuft of glass wool. The aspirator tubes were flushed with sterile water to suspend the conidia for plating, or in some cases, the conidia were aspirated directly into a trap of sterile water. The synthetic medium of WESTERGAARD and MITCHELL (1947) was used in some experiments and Fries' #3 minimal with 3 percent washed agar (RYAN *et al.* 1943) in others. The maximum growth rate on Westergaard's medium with unwashed agar is considerably lower and somewhat more variable than on Fries' minimal with washed agar. Growth rates were measured at 29°C.

The proportions of heterokaryotic and of the 2 types of homokaryotic conidia were determined by plating conidia in a thin top layer of agar on minimal and supplemented sorbose agar. At least 3 plates of each medium were used, at dilutions yielding about 200 to 300 colonies per plate. The average number of nuclei per conidium was determined from stained preparations according to HUEBSCHMAN (1952), about 500 conidia being scored for each determination. The nuclear ratios were estimated from these data by the approximation proposed by ATWOOD and

MUKAI (1955). In some cases estimates of average nuclear number per conidium for one heterokaryon were used for others having the same components and grown under similar conditions. The variation in average nuclear number was small regardless of the strains involved, and the estimated nuclear ratio is not a strong function of nuclear number within the range encountered.

NUCLEAR PROPORTIONS WITH MAXIMAL GROWTH RATES

Nuclear proportions at the beginning and end of growth were first studied in heterokaryons having normal growth rates, but differing in initial nuclear proportions, in order to test the stability of these proportions during normal growth of a variety of heterokaryotic combinations. In one such experiment four strains, *pan, al-1*; *lys-3*; *nic-2, al-2*; and *arg-6*, were mixed in combinations of 2 in various conidial ratios not exceeding 50:1. The growth rates of the resultant heterokaryons averaged 3.1 mm/hr on Westergaard's medium and none were definitely submaximal. The data in table 1a show that regardless of the initial nuclear ratios or the component strains of the heterokaryons, the differences between nuclear proportions at the beginning and end of growth were small; none were significant when tested by the method of paired comparisons and the *t* test. The results of a similar experiment with Fries' minimal medium are given in table 1b. The heterokaryons involved the same four mutant strains as in the previous experiment, but *pan, al-1* furnished the majority of nuclei in all but one case. The mean difference between the proportion of *pan, al-1* at the beginning and end of growth was only +.016, although eight of the ten heterokaryons showed a slight increase in *pan, al-1* nuclei during growth. If this increase is real, as is suggested by the *t* test, it is clearly nonadaptive since the nuclear proportions are approaching the range in which growth rate is limited. Both experiments show that the nuclear ratio changes very little during normal heterokaryotic growth, although the growth rate is maximal over a wide range of nuclear ratios.

NUCLEAR PROPORTIONS WITH SUBMAXIMAL GROWTH RATES

Inocula with conidial ratios ranging from 50:1 to 500:1 will frequently, though not always, produce heterokaryons with reduced growth rates. The nuclear proportions and the growth rates of 21 such heterokaryons are given in table 2. Although the shapes of most of the growth curves of heterokaryons having submaximal growth rates were similar, some variation was found. The curves presented in figure 1 are representative samples of those encountered in the experiments. The shape of growth curve 12c is typical, 14 of the 21 cultures showing this particular pattern while 6 others were similar to either 13c or 14c. Inspection of the growth curves reveals that growth was initiated with a somewhat reduced lag and a relatively high rate. Subsequently, the rates of individual cultures varied for periods up to 100 hours, the early variation following a fairly constant pattern which can reasonably be related to the experimental conditions. Preincubation of the inoculum accounts for the diminution of lag, and growth factor carried with the inoculum accounts for the high initial rate. Exhaustion or outdistancing of the growth factor supply then results in a temporary cessation or retardation of growth. Finally, growth is resumed at a sub-

TABLE 1

Growth rates at 29°C and proportions of nuclear types in 20 heterokaryons sampled at the beginning and the end of 300 mm growth tubes

Heterokaryon no.	Nuclear types	Proportions of nuclear types in opposite ends of growth tubes			Growth rate mm/hr
		Beginning	End	Difference	
a. Grown on Westergaard's medium, unwashed agar*					
30	<i>pan, al-1</i>	0.974	0.968	+0.006	3.1
	<i>arg-6</i>	0.026	0.032		
18	<i>pan, al-1</i>	0.920	0.946	-0.026	3.3
	<i>lys-3</i>	0.080	0.054		
26	<i>arg-6</i>	0.739	0.769	-0.030	2.8
	<i>pan, al-1</i>	0.261	0.231		
21	<i>arg-6</i>	0.696	0.747	-.051	3.0
	<i>nic-2, al-2</i>	0.304	0.253		
6	<i>arg-6</i>	0.749	0.729	+.020	3.3
	<i>lys-3</i>	0.251	0.271		
2	<i>lys-3</i>	0.796	0.813	-.017	3.3
	<i>arg-6</i>	0.204	0.187		
9	<i>lys-3</i>	0.785	0.834	-.049	3.2
	<i>nic-2, ol-2</i>	0.215	0.166		
14	<i>lys-3</i>	0.802	0.780	+.022	2.9
	<i>pan, al-1</i>	0.198	0.220		
24	<i>nic-2, al-2</i>	0.948	0.937	+.011	3.2
	<i>arg-6</i>	0.052	0.063		
12	<i>nic-2, al-2</i>	0.906	0.882	+.024	3.2
	<i>lys-3</i>	0.094	0.118		
b. Grown on Fries' minimal medium, washed agar†					
P/N # 1	<i>pan, al-1</i>	0.680	0.708	-.028	4.9
	<i>nic-2, al-2</i>	0.320	0.292		
7c	<i>pan, al-1</i>	0.899	0.944	-.045	5.0
	<i>nic-2, al-2</i>	0.101	0.056		
8c	<i>pan, al-1</i>	0.847	0.872	-.025	5.0
	<i>nic-2, al-2</i>	0.153	0.128		
5c	<i>pan, al-1</i>	0.961	0.976	-.015	4.9
	<i>nic-2, al-2</i>	0.039	0.024		
15c	<i>pan, al-1</i>	0.951	0.954	-.003	4.9
	<i>arg-6</i>	0.049	0.046		
P/L # 2	<i>pan, al-1</i>	0.341	0.412	-.071	4.8
	<i>lys-3</i>	0.659	0.588		
17c	<i>pan, al-1</i>	0.859	0.874	-.015	4.9
	<i>lys-3</i>	0.141	0.126		
21c	<i>pan, al-1</i>	0.950	0.963	-.013	4.8
	<i>lys-3</i>	0.050	0.037		
23c	<i>pan, al-1</i>	0.852	0.824	+.028	4.8
	<i>lys-3</i>	0.148	0.176		
24	<i>pan, al-1</i>	0.769	0.745	+.024	5.0
	<i>lys-3</i>	0.231	0.255		

* $\bar{X}_d = -.009$.

† $\bar{X}_d = -.016$.

TABLE 2

Growth rates at 29°C and the proportions of nuclear types in 21 heterokaryons having submaximal growth rates sampled at the beginning and the end of 300 mm growth tubes

Heterokaryon no.	Nuclear types	Proportion of nuclear types in opposite ends of growth tubes			Growth rate mm/hr*
		Beginning	End	Difference	
a. Grown on Westergaard's medium, unwashed agar†					
44A	<i>pan, al-1</i>	.994	.984		
	<i>nic-2, al-2</i>	.006	.016	+.010	1.0
44B	<i>pan, al-1</i>	.992	.984		
	<i>nic-2, al-2</i>	.008	.016	+.008	0.9
55A	<i>pan, al-1</i>	.977	.940		
	<i>lys-3</i>	.023	.060	+.037	2.0
55B	<i>pan, al-1</i>	.974	.945		
	<i>lys-3</i>	.026	.055	+.029	1.9
66A	<i>pan, al-1</i>	.992	.967		
	<i>arg-6</i>	.008	.033	+.025	2.6
66B	<i>pan, al-1</i>	.989	.953		
	<i>arg-6</i>	.011	.047	+.036	2.1
77A	<i>lys-3</i>	.941	.912		
	<i>pan, al-1</i>	.059	.088	+.029	2.4
77B	<i>lys-3</i>	.960	.905		
	<i>pan, al-1</i>	.040	.095	+.055	2.6
88A	<i>lys-3</i>	.922	.945		
	<i>nic-2, al-2</i>	.078	.055	-.023	2.2
88B	<i>lys-3</i>	.964	.934		
	<i>nic-2, al-2</i>	.036	.066	+.030	2.1
99A	<i>lys-3</i>	.919	.905		
	<i>arg-6</i>	.081	.095	+.014	2.3
b. Grown on Fries' minimal medium, washed agar‡					
2c	<i>pan, al-1</i>	.981	.972		
	<i>nic-2, al-2</i>	.019	.028	+.009	0.97
3c	<i>pan, al-1</i>	.985	.982		
	<i>nic-2, al-2</i>	.015	.018	+.003	0.98
4c	<i>pan, al-1</i>	.982	.973		
	<i>nic-2, al-2</i>	.018	.027	+.009	0.97
11c	<i>pan, al-1</i>	.988	.970		
	<i>arg-6</i>	.012	.030	+.018	1.23
12c	<i>pan, al-1</i>	.983	.975		
	<i>arg-6</i>	.017	.025	+.008	1.26
13c	<i>pan, al-1</i>	.979	.964		
	<i>arg-6</i>	.021	.036	+.015	1.16
14c	<i>pan, al-1</i>	.963	.974		
	<i>arg-6</i>	.037	.026	-.011	3.36
18c	<i>pan, al-1</i>	.967	.939		
	<i>lys-3</i>	.033	.061	+.028	1.87
19c	<i>pan, al-1</i>	.961	.914		
	<i>lys-3</i>	.039	.086	+.047	4.42
20c	<i>pan, al-1</i>	.983	.950		
	<i>lys-3</i>	.017	.050	+.033	1.44

* Growth rates of the controls of each heterokaryotic combination were 3.0 to 3.1 mm/hr on Westergaard's and 4.9 mm/hr on Fries' medium.

† $\bar{X}_d = +.023$.

‡ $\bar{X}_d = +.016$.

maximal rate with some irregularity. Several considerations which will be mentioned in this and succeeding sections suggest that the final resumption of growth is not caused by a change in the nuclear proportions, but by some other adaptive process.

The differences between the nuclear proportions at the beginning and end of these cultures are of no greater magnitude than those in cultures with maximal growth rates. However, in all but two cases the direction of change is toward an increase of the minority type; the differences are significant by *t* test. These differences, in general, show no obvious correlation with the corresponding average growth rates, although the lowest growth rates tend to be associated with the smallest changes in nuclear proportions. This creates some difficulty for the hypothesis that the increase of the minority type is brought about continuously by natural selection, since one would expect strong selection pressure at the slowest growth rates.

FIGURE 1.—Representative growth curves of heterokaryotic cultures illustrating the variation in shape and growth rate encountered among individual heterokaryons having submaximal growth rates. All cultures, including the control (15c) were (*pan, al-1* + *arg-6*) except 19c which was (*pan, al-1* + *lys-3*). See tables 1b and 2b for the nuclear proportions of the respective cultures.

SUCCESSIVE SAMPLING OF INDIVIDUAL CULTURES

Additional experiments were carried out in growth tubes so designed that nuclear ratios could be determined at frequent intervals during a long growth period. The purpose of these experiments was to determine whether irregularities in growth rates are correlated with changes in nuclear ratio which would not be detected by sampling the ends of ordinary growth tubes, and also whether nuclear selection would become apparent after a longer growth period. The special tubes were 1000 mm in length, with sampling chimneys every 10 cm along the top of the tube.

Six heterokaryons, *pan, al-1* + *arg-6*, *pan, al-1* + *nic-2, al-2*, *lys-3* + *pan, al-1*, *nic-2, al-2* + *lys-3*, *pan, al-1* + *lys-3*, and *lys-3* + *nic-2, al-2*, were prepared by mixing conidia in a ratio of approximately 500:1. The first of these was grown on Fries' minimal medium. The other 5 were grown on Westergaard's medium, and just prior to inoculation of each of these five, a few drops of a solution containing the growth factors of the mutants of the respective heterokaryons, were placed on the agar in the beginning of the growth tubes. This prolonged the initial period of unrestricted growth and made possible the determination of the growth rates and nuclear ratios of the heterokaryons on medium in which the growth factors of the respective mutant strain were not limiting. However, since the growth rates of the heterokaryons were faster than the diffusion rate of the growth factors in the medium, the growing fron-

tier had advanced beyond the influence of the growth factor within about 10 cm of the point of inoculation. When growth was complete, conidia were removed from most of the sampling chimneys, where they had formed in abundance, and the proportion of nuclear types determined. Four of the six cultures achieved normal rates within about 20 cm, and averaged 3.2 mm/hr on Westergaard's medium. Two of the heterokaryons, *pan, al-1 + arg-6* grown on Fries' medium, and *pan, al-1 + nic-2, al-2* grown on Westergaard's, had submaximal growth rates which averaged 1.9 and 1.4 mm/hr, respectively. The growth curves are shown in figure 2 with an additional growth curve showing a maximal rate on Fries' medium as a control for the *pan, al-1 + arg-6* culture. The nuclear proportions along the lengths of the growth tubes are given in table 3.

Although the growth rates varied considerably during the first few days of growth in the cultures having submaximal rates, the nuclear ratios were remarkably constant throughout the length of the tubes and gave no indication of changing in the direction expected to increase growth rate. Cultures having normal growth rates showed somewhat more variation in nuclear ratio, but the changes were definitely nonselective and appeared as random fluctuations except near the beginning of the tubes. In all cases where growth was initiated on medium supplemented at the proximal end with a solution of the growth factors, the major nuclear type decreased,

FIGURE 2.—Growth curves of 6 heterokaryons grown in 1000 mm growth tubes at 29°C, No.'s 1-5 on Westergaard's medium, No.'s 6 and 15c on Fries' medium. Growth rates and nuclear ratios for successive samples of cultures 1-6 are given in table 3. Culture 15c, grown in 300 mm tube, is included to show control rate of culture 6. The beginning of growth tubes 1-5 were supplemented with the growth requirements of the mutants just prior to inoculation. KEY: 1 = *lys-3 + pan, al-1*; 2 = *lys-3 + nic-2, al-2*; 3 = *nic-2, al-2 + lys-3*; 4 = *pan, al-1 + lys-3*; 5 = *pan, al-1 + nic-2, al-2*; 6 and 15c = *pan, al-1 + arg-6*.

TABLE 3

*Growth rates at 29°C and proportions of nuclear types in six different heterokaryons sampled at 100 mm intervals along 1000 mm growth tubes**

Heterokaryon no.	Nuclear types		Proportion of major nuclear type in the indicated sampling chimney											Average growth rate mm/hr
	Major	Minor	1	2	3	4	5	6	7	8	9	10	11	
1	<i>lys-3</i>	<i>pan, al-1</i>	.968	.936	.919		.858			.861	.924	.937	.903	3.2
2	<i>lys-3</i>	<i>nic-2, al-2</i>	.953	.926			.958	.934		.928	.962	.961	.890	3.2
3	<i>nic-2, al-2</i>	<i>lys-3</i>	.988	.939			.963			.913	.914	.914	.813	3.4
4	<i>pan, al-1</i>	<i>lys-3</i>	.970	.969	.945	.941	.945		.936		.936		.944	3.0
5	<i>pan, al-1</i>	<i>nic-2, al-2</i>	.997	.989	.990	.994	.994	.990	.986	.971	.994	.992	.985	1.4
6	<i>pan, al-1</i>	<i>arg-6</i>	.965	.976	.980	.975	.969	.966	.975	.961				1.9

* All heterokaryons were grown on Westergaard's medium except No. 6 which was grown on Fries' medium. The beginning of growth tubes Nos. 1-5 were supplemented with the growth requirements of the mutants just prior to inoculation. See figure 2 for growth curves of these heterokaryons.

although not always significantly, between the first and third sampling chimneys, or roughly coincident with the change from supplemented to minimal medium. From these results it may be inferred that consistent differences between the ends of the ordinary growth tubes in table 2 also originate near the beginning of growth, the nuclear proportions remaining fairly constant thereafter. Finally, the nuclear proportions at various sampling points in the same tube show no correlation with the growth rates locally in effect beneath these points, even in the regions where nuclear ratios and growth rates are changing most rapidly.

INFLUENCE OF EXOGENOUS GROWTH FACTORS

The presence in the medium of growth factors required by the mutant components of a heterokaryon can render the culture independent of the complementary actions of these components. Therefore it seemed possible that such growth factors would have important effects on growth rate and nuclear selection in heterokaryons. In order to observe these effects, various heterokaryotic combinations were grown on minimal and supplemented media and the growth rates and nuclear ratios were determined. In separate experiments four different heterokaryons, prepared in ratios expected to give submaximal growth rates, were grown in duplicate or triplicate on Westergaard's minimal medium and on medium singly supplemented with the growth requirements of the respective mutants. Heterokaryons within the same experiment had approximately the same nuclear ratio. The results, given in table 4, show that the cultures grown on medium supplemented with the growth factor required by the major nuclear component had normal growth rates, whereas those grown on either minimal medium or medium supplemented with the growth factor of the minor nuclear component had submaximal growth rates. The differences in the growth rates of the heterokaryons in which the *nic-2, al-2* component was predominant were not significant, but are included to show that pantothenate in the medium did not increase the growth rate, except where the nuclear proportions were already limit-

TABLE 4

The average growth rate and the proportion of nuclear types in the beginning and the end of 300 mm growth tubes of heterokaryons grown at 29°C on minimal and supplemented media in four separate experiments

Heterokaryon no.	Growth factor added to medium	Nuclear types in heterokaryon		Proportion of major nuclear types in opposite ends of growth tubes		Average growth rate mm/hr
		Major	Minor	Beginning	End	
VI-1	Minimal			.944	.941	2.3*
VI-2	Minimal			.970	.977	2.3*
VI-3	<i>pan</i>	<i>pan, al-1</i>	<i>arg-6</i>	.976	.977	3.3
VI-4	<i>pan</i>			.971	.990	3.2
VI-5	<i>arg</i>			.969	.979	2.1*
VI-6	<i>arg</i>			.966	.979	2.3*
4-1	Minimal			.948	.983	2.3*
5-2	<i>nic</i>	<i>pan, al-1</i>	<i>nic-2, al-2</i>	.931	.964	1.9*
6-1	<i>pan</i>			.985	.970	3.1
PIII	Minimal			.948	.940	2.6*
PII	<i>lys</i>	<i>pan, al-1</i>	<i>lys-3</i>	.870	.866	2.3*
PI	<i>pan</i>			.939	.916	3.4
10-2	Minimal				.624	3.5
11-2	<i>pan</i>	<i>nic-2, al-2</i>	<i>pan, al-1</i>	.653	.684	3.4
12-1	<i>nic</i>			.653	.619	3.6

* Growth rate not constant during course of experiment.

ing. Changes in the nuclear ratios between the beginning and end of growth were not significant, regardless of the medium on which the cultures were grown. Thus, in heterokaryons with the stocks used, the presence of an exogenous growth factor does not confer a selective advantage on nuclei carrying the mutation for requirement of the growth factor. The absence of selection for the minor nuclear component in these experiments demonstrates again that adaptive nuclear ratio changes do not ordinarily occur whether or not growth factors are limiting.

GROWTH CURVES OF *pan, al-1* HOMOKARYONS

It seems reasonable to assume as a working hypothesis that the growth limiting effect of nuclear disproportion in heterokaryons is equivalent to a deficiency of the growth factor required by the major component. It is therefore of interest to compare the growth curves of a homokaryotic mutant under conditions of limiting growth factor supply with those of heterokaryons having the same mutant as their major component and limited by nuclear disproportion. Figure 3 shows representative growth curves of *pan, al-1* on Fries' medium supplemented with amounts of calcium pantothenate varying from 0.2 to 0.5 μg per ml. The inocula were prepared in exactly the same way as in the heterokaryon experiments except that no conidia other than *pan, al-1* were added. It is apparent that the same sequence of changes in

FIGURE 3.—Growth curves of *pan, al-1* homokaryons grown at 29°C on Fries' minimal supplemented with various amounts of calcium pantothenate. KEY: A = 0.50 µg/ml; B = 0.35 µg/ml; C = 0.30 µg/ml; D = 0.25 µg/ml; and E = 0.20 µg/ml. For the average growth rate of the respective cultures in triplicate, see figure 4.

growth rate occurs with the homokaryon as with various heterokaryons having *pan, al-1* as the major component. The growth rates of the homokaryon on limiting pantothenate are, if anything, less constant than those of the heterokaryons with limiting nuclear proportions. Since none of the rate fluctuations of the homokaryon can be caused by changes in nuclear proportion, it is not necessary to assume that the similar fluctuations in heterokaryons are caused by such changes.

DOMINANCE OF *pan*⁺

Under conditions of prolonged stability of the nuclear proportions, a subnormal growth rate may be looked upon as a measure of the degree of dominance of the wild type allele present only in the nuclei of the minor component. For the purpose of correlating average growth rate with nuclear ratio and thus arriving at an estimate of dominance of *pan*⁺, the data in tables 1b and 2b are plotted in figure 4. Cultures grown on Fries' medium afford a more reliable correlation than those on Westergaard's, because of their greater submaximal range and their less variable maximum rate. Because of the small range of nuclear proportions over which the growth rate is depressed, the frequency of the minor component is most conveniently plotted on a logarithmic scale. The frequencies of the minor component in the proximal and distal ends of the tubes were averaged, a procedure which seems justified because the differences are small in most cases, and the true frequency may often lie between these values. Actually, the results are very similar if only the distal ends are used. The average growth rates of triplicate samples of *pan, al-1* homokaryons grown on various amounts of calcium pantothenate are also plotted in figure 4 with the concentration of calcium pantothenate on the same axis as the frequency of the minor component.

The maximum growth rate of *pan, al-1* is reached with 0.5 µg of calcium pantothenate per ml and is the same as the growth rate on minimal Fries' medium of heterokaryons in which the nuclear proportions are in the optimal range. Below the optimal range, the growth rate falls precipitously with decreasing proportion of *pan*⁺ nuclei, or with decreasing concentration of supplement. It is noteworthy that the curves are, at least roughly, of the same form; that is to say, that changing the con-

FIGURE 4.—Growth rate on Fries' minimal vs. proportion of *pan*⁺ nuclei in heterokaryons and growth rate vs. concentration of calcium pantothenate in medium for the *pan, al-1* homokaryon. KEY: ○ = *pan, al-1* homokaryon; ▲ = *pan, al-1 + arg-6*; △ = *pan, al-1 + lys-3*; and ● = *pan, al-1 + nic-2, al-2*.

centration of pantothenate by a certain factor has the same effect on the growth rate as changing the proportion of *pan*⁺ nuclei by the same factor. Thus the proportion of *pan*⁺ nuclei can be equated to the concentration of pantothenate in the medium. From the data in figure 4 it appears that 0.1 μg of calcium pantothenate per ml is approximately equivalent in growth promoting effect to 1 percent of *pan*⁺ nuclei.

The heterokaryons having *lys-3* as the minor component appear to require more *pan*⁺ nuclei for an equivalent growth response than those having either *nic-2, al-2* or *arg-6*, and this effect is also apparent among the heterokaryons grown on Westergaard's medium (nos. 55A and 55B in table 2a). Although it is uncertain whether this effect is real, it is possible that this *pan*⁺ is less dominant than the others, or that modifying factors in the genetic background of *lys-3* are responsible.

If dominance is defined as the reciprocal of the frequency of the minor component just sufficient to promote normal growth, then the dominance of *pan*⁺ is approximately 25 to 30; that is, the maximum activity of *pan*⁺ is some 25 to 30 times greater than that necessary for a normal growth rate. It would be of interest to learn whether the system always functions with this extra capacity, or whether the peak activity is evoked by the growth-limiting conditions. This cannot be determined with the type of information at hand, but since the growth responses to pantothenate and *pan*⁺ nuclei are similar, it can be inferred that the activity per nucleus does not change appreciably within the suboptimal range.

DISCUSSION

During sustained growth the nuclear proportions in heterokaryons having a wide range of nuclear ratios have proved to be stable under a variety of conditions. Since the mutants used in these studies were not chosen for any special properties other than their compatible genetic backgrounds, it is safe to assume that their stability

in heterokaryons is by no means unique. The stabilizing mechanism is nonadaptive; that is, it operates to maintain the status quo even when a change in nuclear proportions would be advantageous. Certain initial shifts toward an optimal nuclear ratio, and also a rather consistent sequence of growth rate changes in the early stages of growth cannot, for the following reasons, be caused by the mechanism of selection suggested by BEADLE and COONRADT: (1) The initial trend toward increase of the minor component is not continued in a subsequent period of growth at submaximal rate; (2) during the period of initial change, the growth rate at a given point is not correlated with the nuclear proportions at that point; (3) when the *pan, al-1* homokaryon is grown on partially deficient medium the same series of rate changes is observed as in the heterokaryons.

The initial rate fluctuations are most economically ascribed to growth factor carried with the inoculum or intentionally added to the beginning of the tube. When this supply is outdistanced, an adaptation of unknown nature occurs involving a temporary lag in growth. Since this lag also occurs in the *pan, al-1* homokaryon, it is probably not related to the biosynthesis of pantothenate, but rather to processes requiring the co-factor activity. The initial shifts in nuclear proportions occurring at the boundary zone of the inoculum are probably related to the causes of dissimilarity between input and final ratios which have been discussed in a previous publication (PITTENGER *et al.* 1955). The presence of growth factor in the medium is shown to have no effect on nuclear selection during sustained growth. However, the passage of the mycelium across a boundary between supplemented and minimal medium, particularly when near the inoculum where transport mechanisms are probably poorly developed, may selectively retard the nuclei in large homokaryotic segments. The successive sampling experiments (table 3) indicate that when systematic nuclear ratio changes occur they are associated with the passage of this boundary, and later discontinued. In the indefinite period of stability which follows, it is clear that selection of fast growing hyphae does not occur.

In order to account for the stability of the nuclear ratios it is important to consider the nuclear constitution of individual hyphae at the growing frontier and the opportunity for active transport of materials from regions proximal to the frontier. As pointed out by PROUT *et al.* (1953), some of the hyphal tips isolated from the growing frontier of a heterokaryon appear to be homokaryotic. Microscopic examination of stained preparations of mycelium shows that the number of nuclei per unit length is such that it would be hardly possible, by the usual techniques, to isolate viable hyphal tips containing less than about 100 nuclei. Even if only a fraction of these nuclei contribute their progeny to a culture derived from such an isolate, the finding that such cultures are sometimes homokaryotic implies that local nuclear proportions in different hyphal tips of the same frontier must be extremely variable. At the same time, it is evident that in the heterokaryotic culture as a whole, a variation of only a few percent in the suboptimal range (fig. 4) would mean the difference between maximal growth rate and complete cessation of growth. If the growth rates of individual hyphae were dependent on the local nuclear constitution, the optimal conditions would be almost immediately restored by natural selection. However, since suboptimal nuclear proportions remain stable, it is concluded that the growth rate of

an individual hypha is not determined by its local nuclear proportions, but by the proportions within a larger domain, so that random variations are negligible. This conclusion is supported by direct examination of the growing frontier, which gives no indication of autonomous behavior of the hyphae. The configuration of the frontier changes continually during growth; sometimes it is uniform, sometimes one or more hyphae are distinctly in advance of the average position, but these hyphae do not maintain their advantage and do not replace their competitors in forming the new frontier. The growth of heterokaryotic and wild type strains is indistinguishable in this respect.

Although individual hyphae of a frontier do not exhibit permanent differences in growth rate, their behavior when isolated from the proximal mycelium is quite different. This point is illustrated by the following experiment: A *pan, al-1 + nic-2, al-2* heterokaryon was prepared with a conidial input ratio of about 500:1 and duplicate samples of the pellet transferred to minimal agar in a growth tube and a petri plate. The growth tube culture grew at a stable rate of 1.3 mm/hr. Twenty-five hyphal tips approximately 1 mm in length were isolated from the plate culture and transferred to minimal agar growth tubes. After a prolonged lag period, eleven grew normally with a mean rate of 5 mm/hr. Eight had submaximal rates averaging 2.2 mm/hr with a range of 0.90 to 3.9 mm/hr. Six failed to grow and hence were either homokaryotic or inviable for some other reason. It may be inferred that the isolated tips possessed different nuclear ratios permitting a wide range of growth rates, or alternatively that isolation of the hyphae allowed the establishment of different nuclear proportions.

That the growth of the terminal hyphae should be influenced by the activity of more proximal regions is not surprising in view of the active transport process which has long been recognized. Microscopic examination of actively growing cultures reveals a continuous protoplasmic streaming toward the frontier of growth, the cytoplasm with its entrained particulates passing freely through the perforated septa of the mycelium. Transport of growth factors has been reported by RYAN *et al.* (1943), for distances up to 1 cm, and, under some conditions, much longer distances are involved, for instance, aerial hyphae may grow as much as 20 cm from their source of supply. A transport system extending 1 cm proximally would make available to the frontier the combined activity of enormous numbers of nuclei.

The existence of an active transport system explains the failure of hyphae to respond to local variations in nuclear ratio, but it is apparent that this is not the only prerequisite for stability. In addition, the nuclei of different components must divide at the same rate. Biosynthetic activities of the type usually affected in auxotrophic mutants have an indirect relation to nuclear division since mutant nuclei will divide normally in cytoplasm conditioned by the presence of other nuclei of complementary genotype. Limitation by nuclear disproportion may be regarded as a relative deficiency caused by the competition of a large number of nuclei for a specific biochemical activity which is facilitated by a much smaller number. This condition provides an unusual opportunity to determine whether nuclei carrying the wild type allele are as sensitive to the relative deficiency as those carrying the mutant allele. It is clear that where the suboptimal nuclear ratio is stable all the nuclei respond equally to the

biochemical deficiency whether or not they contain the allele which facilitates the synthesis of the limiting factor.

It seems certain that combinations of nuclei of some genotypes will not fulfill the condition of equal division rates, and instability of the nuclear proportions would then result. The type III heterokaryons of HOLLOWAY (1955) are examples of apparent instability; they grow normally for a limited time, then appear to have become homokaryotic at the point of cessation of growth. A nonadaptive overgrowth of *leu-1* has been reported by RYAN and LEDERBERG (1946) in heterokaryons between *leu-1* and wild type growing on limiting concentrations of leucine. Despite repeated trials, we have not succeeded in producing a heterokaryon in which growth is limited by a scarcity of *nic-2⁺* nuclei, or in which the proportion of *nic-2⁺* is sustained at less than about five percent. A possible explanation is that *nic-2⁺*, unlike *pan⁺* and *lys-3⁺*, has a selective advantage under conditions of relative deficiency. The products of the activity controlled by *nic-2⁺* might be more immediately available to nuclei which initiate this biosynthetic system than to other nuclei. Genetic control of the nuclear response to conditions in the cytoplasmic milieu, and of autonomously determined nuclear division rate, is to be expected; hence the mechanism of instability may be quite different in different cases, frequently nonadaptive, and frequently associated with the genetic background rather than with the markers under observation. The techniques of estimating and controlling the nuclear proportions give promise of elucidating such mechanisms.

SUMMARY

1. The nuclear proportions in heterokaryons with normal growth rates were stable between the ends of horizontal growth tubes.
2. Heterokaryons prepared from the same stocks had submaximal growth rates when extreme disproportion of the components was produced by controlling the conidial proportions in the inocula. In these heterokaryons the submaximal growth rates and extreme nuclear ratios were stable.
3. Supplementation with the growth factor, required by the major component, gave normal growth rates in heterokaryons which would otherwise have had submaximal rates, but did not alter the nuclear ratios. The growth factor of the minor component had no effect.
4. The growth responses to *pan⁺* nuclei and to supplementation with pantothenate were approximately of the same form. The dominance of *pan⁺* was estimated by correlating the nuclear proportions and growth rates of heterokaryons.
5. No evidence was found of selection of hyphae having more favorable nuclear ratios. It is suggested that this mechanism rarely, if ever, operates and that systematic changes in the nuclear ratio during sustained growth can usually be attributed to differences between the division rates of the components.

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