HETEROCARYOSIS IN NEUROSPORA CRASSA[1]

G. W. BEADLE AND VERNA L. COONRADT
School of Biological Sciences, Stanford University, California

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IT IS possible by means of X-ray or ultraviolet treatment to produce mutant strains of *Neurospora crassa* that are deficient in their abilities to synthesize specific vitamins, amino acids, or other essential substances (BEADLE and TATUM 1941; TATUM and BEADLE 1942b). It occurred to us that it might be possible to determine whether the mutant genes concerned in such haploid strains are recessive or dominant to their normal alleles by producing heterocaryons, the cells of which would contain haploid nuclei from two mutant strains, one differing from wild type by one gene and the other differing by a second gene.

In our first attempt to produce a heterocaryon the mutant strains used were aminobenzoicless (unable to synthesize the vitamin *para*-aminobenzoic acid) and nicotinicless (unable to synthesize nicotinic acid). Growth rates were measured in special growth tubes (RYAN, BEADLE, and TATUM 1943) containing "minimal medium" (salts, sucrose, biotin, and agar, and containing neither *p*-aminobenzoic acid nor nicotinic acid). The two mutant strains were grown separately as controls. One experimental tube was inoculated with conidia of the two mutant strains of opposite mating types (sexes)—that is, the conidia were mixed at one end of the tube. It was supposed that the conidia would germinate and hyphae of opposite sex fuse. If both mutant genes were recessive, the two types of nuclei present in the fusion hyphae would complement each other, each type carrying the normal allele of the mutant gene in the other, and growth would occur. To determine the rate of any growth that might occur as a result of intercellular diffusion of complementary growth factors synthesized and excreted into the medium by separate hyphae of the two types, nicotinic acid by aminobenzoicless hyphae and *p*-aminobenzoic acid by nicotinicless hyphae, a tube was set up which was inoculated with conidia of the two mutants of the same mating type. Here no fusion was expected. However, the observed results immediately indicated that we were wrong in this expectation. The tubes in which like mating types were introduced gave growth rates similar to a wild type control, while growth was much slower in those in which different mating types were mixed. The control tubes in which single mutants were put showed little or no growth. Although we did not properly appreciate the fact at the time these experiments were carried

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out, hyphae which do not differ in sex reaction are known to fuse in many fungi (DE BARY 1887; WARD 1888; and many others), including species of Neurospora (KÖHLER 1929, 1930; SCHÖNEFELDT 1935; DODGE 1942). In many imperfect fungi such fusions can be shown, under experimental conditions, to lead to mixing of nuclei to give heterocaryotic strains (HANSEN and SMITH 1932; HANSEN 1938).

It was soon shown that unisexual heterocaryons can be produced at will in *Neurospora crassa* if the experimental conditions are so arranged that only heterocaryotic mycelia can grow. In this way the phenomenon of heterocaryosis provides a general method for determining dominance relations between mutant genes and their normal alleles. Furthermore, a method is made available by which tests for allelism in a haploid organism become as simple as in a diploid. It is the purpose of this paper to describe how heterocaryons are made, to offer proof that they really contain a mixture of two kinds of nuclei, to show how they can be used in studying dominance relations in a manner not possible in diploid organisms, and to point out some of the implications that have to do with the mechanisms of evolution in the fungi. The question as to whether mutant strains that are complementary in synthetic abilities could grow symbiotically if they did not fuse remains unanswered. Presumably they could, but since hyphae are actually found to be heterocaryotic, this condition is evidently more efficient than extracellular symbiosis.

PREVIOUS STUDIES

A remarkably clear demonstration of the occurrence of heterocaryosis in a fungus was made some years ago by HANSEN and SMITH (1932). These workers found that *Botrytis cinerea*, a species of the *Fungi Imperfecti*, contains an inconstant type called *x*. On culturing single multinucleate conidia of this type, it was found that some give rise to a constant type *a*, others give inconstant cultures, while still others give rise to a second constant type *b*. HANSEN and SMITH postulated that the *x* type is heterocaryotic, containing a mixture of two genetic types of nuclei. By chance assortment, some conidia contain only one type of nucleus, some only the other type, and some a mixture of both as expected on this assumption, type *a* strains give only type *a*, type *b* only type *b*, while type *x* gives *a*, *b*, and *x* types. This interpretation was shown to be correct by growing type *a* and *b* strains together. In this way type *x* was reconstituted through hyphal fusions and nuclear migrations. It should be emphasized that these phenomena, fusion of hyphae and random assortment of nuclei in the formation of conidia, are strictly asexual.

HANSEN (1938) has extended these observations to other species of the *Fungi Imperfecti*. In cultural studies of species from thirty genera, it was found that the available strains of more than half of these were heterocaryons. Single vegetative spore isolations gave rise to constant non-conidial forms, constant conidial forms, and inconstant mixed forms. The widespread occurrence of the phenomenon of heterocaryosis in certain groups of fungi is therefore

established beyond any reasonable doubt, although unfortunately the work of HANSEN and SMITH has received relatively little attention by investigators interested in this subject.

LINDEGREN (1934) reports that bisexual heterocaryons of *Neurospora crassa* may occur in nature and discusses the significance of these from the standpoint of the evolutionary economy of the species. He later (1942) discusses the possibility of unisexual heterocaryosis occurring in fungi either as a result of hyphal fusions or by mutation. It remained, however, for DODGE (1942) to produce unisexual heterocaryons experimentally in material that can be worked with genetically. By combining vegetatively the strains Dwarf 16 and C 8, both mating type *a* of *Neurospora tetrasperma* and both relatively slow growing types, a heterocaryotic strain was obtained that grew much faster and sporulated more abundantly than either component strain. Cultures derived from small conidia from such vigorous cultures gave rise to three types, C 8, Dwarf 16, and a vigorous type (the heterocaryon), thus indicating the presence within a single conidium of both C 8 and Dwarf 16 nuclei.

The work reported in this paper indicates that the method of DODGE may be extended to the heterothallic species *Neurospora crassa*.

MATERIAL AND METHODS

The significant feature of putting the phenomenon of heterocaryosis on a predictable experimental basis is the establishment of conditions which give the heterocaryon a strong selective advantage over the component strains. With mutant strains deficient in particular syntheses and hence dependent on an outside source of vitamins, amino acids, or other substances, such conditions are readily arranged by growing cultures on a medium deficient in the growth factors required by the two strains. If the mutant genes are both recessive to normal, the two kinds of nuclei complement each other in the heterocaryon so that only heterocaryotic hyphae can grow, and the heterocaryotic condition is automatically maintained. The same principle may be applied to strains that have low growth rates for other reasons—for example, colonial types that advance more slowly over an agar culture medium than do wild type strains. If one or both components are types with normal growth rates, but otherwise distinguishable from wild type, it is possible to make tests for dominance and allelism by first genetically combining such types with mutants unable to synthesize growth factors but morphologically normal in the presence of the growth factors required. Heterocaryotic combinations can then be detected by their ability to grow on a minimal medium. This can be illustrated with the hypothetical mutants x and y, each of which grows at a normal rate. By making the double mutants $x\ a$ and $y\ b$, where a and b are biochemical mutants known to be recessive and non-allelic, the heterocaryon $(a+x)$ plus $(+b\ y)$ can be made. If this is morphologically normal, mutants x and y are recessive and non-allelic

In the experiments to be referred to, several mutants, all derived from wild

type strains of *Neurospora crassa* following treatment with X-rays or ultraviolet radiation, were used. Each of these behaves in crosses as though differentiated from the original wild type strain by one significant gene. Reference to mutants is made by numbers assigned at the time the original mutant spore was isolated. "Biochemical" mutants are named according to the substance they cannot synthesize, although it should be pointed out that in many instances it is not known that this is the immediate product of the blocked reaction. Thus a particular argininеless mutant might not be able to convert ornithine to citrulline (SRB and HOROWITZ unpublished) and therefore might more properly be called citrullineless.

The biochemical mutants made use of in studies reported here are as follows: Albino 1 (*al-1*)-4637, absence of pigmentation in mycelium and conidia.—albino 2 (*al-2*)-15300, phenotypically like albino-1 (MRS. M. V. G. HUNGATE unpublished).—aminobenzoicless (*pab*)-1633, unable to synthesize the vitamin *p*-aminobenzoic acid (TATUM and BEADLE 1942a).—argininеless (*arg*)-29997, unable to synthesize ornithine and hence citrulline and arginine (SRB and HOROWITZ unpublished).—lysineless (*lys*)-4545, unable to synthesize the amino acid lysine (DOERMANN unpublished).—nicotinicless-1 (*nic-1*)-3416, unable to synthesize the vitamin nicotinic acid (BEADLE, TATUM, HOROWITZ, and BONNER unpublished).—nicotinicless 2 (*nic-2*)-4540, unable to synthesize nicotinic acid. Genetically and biochemically different from nicotinicless-1 (BEADLE, TATUM, HOROWITZ, and BONNER unpublished).—pantothenicless (*pnt*)-5531, unable to synthesize pantothenic acid (BEADLE, TATUM, HOROWITZ, and BONNER unpublished).—tryptophaneless-1 (*tpt-1*)-10575, unable to synthesize the indole nucleus of the amino acid tryptophane (TATUM, BONNER, and BEADLE in press).—tryptophaneless-2 (*tpt-2*)-40008, unable to synthesize indole and hence tryptophane. Genetically and biochemically different from tryptophaneless-1 (TATUM, BONNER, and BEADLE in press).

In addition to these, four morphological mutants were used. These are referred to by number only. Brief descriptions follow: 221, a semi-colonial form that progresses along a growth tube at approximately one percent of the rate of a normal strain. Discolors medium somewhat.—2608, a colonial form that produces cauliflower-like buttons of growth. Rate of progression less than one percent of that of wild type.—3100, a colonial form like 2608. Turns dark and blackens the medium on which it grows.—5801, a semicolonial form, the mycelium of which gives the appearance of flowing over an agar surface. Rate of progression about one percent of that of wild type.

Mating types are designated by the letter *a* in upper or lower case. Thus 4637A is albino-1 of mating type or sex *A* which corresponds to the (+) type of LINDEGREN. Mating type *a* corresponds to the (−) sex of LINDEGREN.

Measurements of growth rates were made in special glass growth tubes as described in detail by RYAN, BEADLE, and TATUM 1943. In these the mycelium is allowed to progress along the surface of an agar medium. Measurements are made at 12-hour or other desired intervals. In general a minimal medium made up with Fries solution, sucrose (1.5 percent), biotin (0.005 μg/ml), and agar (3 percent) was used.

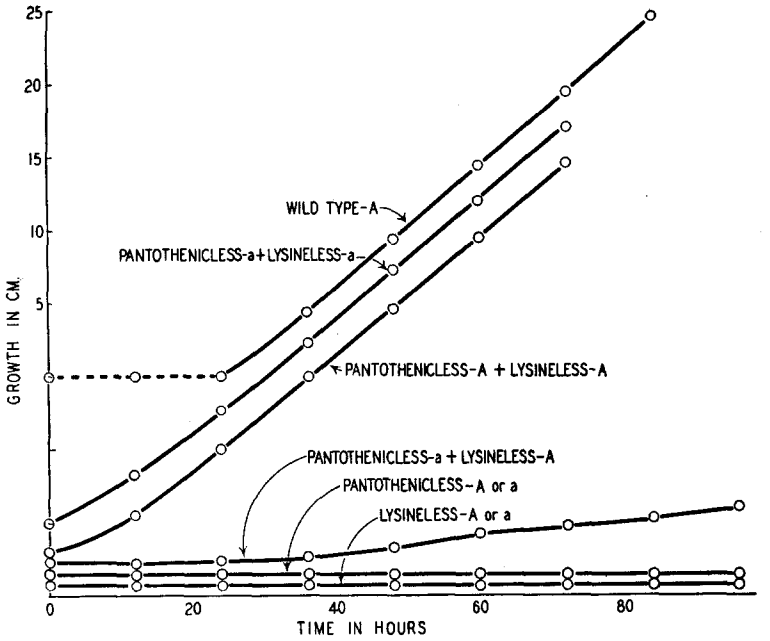

FIG. 1. Growth of lysineless, pantothenicless, wild type, and lysineless plus pantothenicless heterocaryotic strains of *Neurospora crassa*. For some reason the wild type culture did not start growing for 24 hours (dotted portion of growth curve), but this has no significance so far as the equilibrium rate is concerned.

GROWTH OF HETEROCARYONS

The behavior typical of heterocaryons made by combining two strains deficient in different growth factors is summarized in figure 1. The two components, pantothenicless and lysineless, show no measureable growth on minimal medium. When like-sexed strains of the two mutants are grown together on the same medium, however, the growth rate is as high as that of wild type—that is, about four millimeters per hour. When mutants of opposite sex are grown together, irregular growth occurs, averaging about 0.9 mm/hr.

Essentially similar results have been observed for other unisexual combinations, fourteen of which are listed in table 1. It is a general rule that bisexual heterocaryons show irregular growth as measured in tubes and have a lower average rate than do those that are unisexual. It is supposed that this lower growth rate of bisexual heterocaryons in *N. crassa* results from the observed tendency of hyphae containing nuclei of the two sexes to form fruiting bodies (perithecia) rather than to grow in the manner of strictly vegetative hyphae. The difference between the two types may well be mainly in orientation of growth rather than in its amount. In all combinations of group 1 and group 2 of table 1, like-sexed heterocaryons gave growth rates essentially the same as that of wild type controls—that is about 4 mm/hr. The combinations of two morphological mutants tested gave growth rates lower than normal. The two listed in table 1, group 3, gave rates of 2.5 and 2.0 mm/hr. These combinations will be discussed further in considering dominance relations.

TABLE 1

Combinations of mutants of the same sex that have been observed to give heterocaryons as judged by growth rates and phenotypic appearance.

STRAIN NUMBERS	NAMES OF MUTANTS	GROWTH RATES (mm/hr)
Group 1		
1633+ 3416	aminobenzoicless+nicotinicless-1	4.1
1633+ 4540	aminobenzoicless+nicotinicless-2	4.1–4.4
1633+ 5531	aminobenzoicless+pantothenicless	4.2–4.4
4540+ 5531	nicotinicless-2+pantothenicless	4.2–4.4
4545+ 5531	lysineless+pantothenicless	3.9–4.6
4545+10575	lysineless+tryptophaneless-1	4.1–4.3
4545+29997	lysineless+argininеless	4.4–4.5
5531+10575	pantothenicless+tryptophaneless-1	4.5
10575+29997	tryptophaneless-1+argininеless	4.2–4.4
Group 2		
221+ 5531	morphological+pantothenicless	4.2–4.4
2608+ 4545	morphological+lysineless	*ca.* normal
3100+ 5531	morphological+pantothenicless	4.2–4.4
5531+ 5801	pantothenicless+morphological	4.3–4.4
Group 3		
221+ 3100	morphological+morphological	2.2–2.6
221+ 5801	morphological+morphological	0.7–2.5

PROOF OF THE HETEROCARYOTIC CONDITION

In the case of five different heterocaryons, each made by combining two different mutant strains, bits of mycelia were removed from the frontier of the growing culture after these mycelia had migrated through growth tubes (about 30 cm). Such inocula were transferred to petri plates of minimal medium and allowed to develop for several hours at 25°C. During this time hyphae had grown out radially from the site of inoculation for a distance of several millimeters. Individual hyphal tips were cut off with a platinum-iridium microspatula and transferred to regular test tube slants of Difco cornmeal agar medium. Into these same tubes conidia of the wild type strain of unlike mating type were transferred. After fusion and production of ripe perithecia had taken place, samples of ascospores were removed and cultured individually on media fortified with the growth factors required by the components of the heterocaryon. If biochemical mutants were involved, such single ascospore cultures were transferred asexually (by means of conidia) to a minimal medium and to two sets of minimal media each fortified with a growth factor required by one of the components. This procedure as applied to the heterocaryon made by combining aminobenzoicless and nicotinicless strains is summarized as follows: (1) Aminobenzoicless and nicotinicless strains grown together.—(2) Combination culture grown through growth tube on minimal medium.—

TABLE 2

Recovery of mutant strains from crosses of wild type with single hyphal tip cultures derived from heterocaryons.

COMPONENT 1 OF HETEROCARYON	COMPONENT 2 OF HETEROCARYON	NUMBER OF HYPHAL TIPS ISOLATED	NUMBER GIVING BOTH MUTANT TYPES
1633a, aminobenzoicless	3416a, nicotinicless-1	10	7
1633A, aminobenzoicless	4540A, nicotinicless-2	5	5
1633A, aminobenzoicless	5531A, pantothenicless	5	2
4545A, lysineless	2608A, morphological	5	4
221a, morphological	5801a, morphological	6	6

(3) Bit of mycelium transferred to minimal medium in petri plate.—(4) Single hyphal tip cultures isolated and crossed with wild type of opposite mating type.—(5) Ascospores isolated and single spore cultures established on a medium containing both *p*-aminobenzoic acid and nicotinic acid.—(6) Asexual transfers made to a minimal medium containing neither *p*-aminobenzoic nor nicotinic acid. Cultures are thus classified as normal or mutant.—(7) Mutant cultures transferred asexually to two media, one containing minimal requirements plus *p*-aminobenzoic acid, the other the same except with nicotinic acid instead of *p*-aminobenzoic acid. Those that grow on *p*-aminobenzoic acid are aminobenzoicless; those that grow on nicotinic acid are nicotinicless.

If the mycelium is a true heterocaryon, at least some of the hyphal tips removed from it should contain both types of nuclei, and both mutants should be recoverable from the cross with wild type. Conversely, recovery of both component mutants constitutes proof of the heterocaryotic condition.

Results of this procedure applied to five heterocaryons are summarized in table 2. In all combinations both component mutants were recovered from at least some single hyphae. In the case of hyphae from which only wild type or only one type of mutant was recovered, the numbers of mutant cultures recovered were so small that sampling errors could reasonably account for the failure to recover both components.

These observations show that in the combinations studied our interpretation that growth is due to a true heterocaryotic condition is correct. There can be no reasonable doubt that it is a general truth for those pairs of slow or non-growing mutants that in combination show a normal or greatly increased growth rate.

DOMINANCE RELATIONS

If a heterocaryon made up of two mutant strains, each of which fails to grow at a normal rate, shows a normal growth rate, it seems justifiable to conclude that each of the two mutant types concerned is recessive to the alternative wild type condition. This is the situation with all the eleven

mutants listed in table 1. Each of them is a component of at least one heterocaryotic combination that shows normal growth.

It is found, however, that the two combinations involving two morphological mutants do not give normal rates. Yet each of the three mutants concerned is completely recessive in a combination with pantothenicless (group 2, table 1). To account for this seeming inconsistency, it is necessary to appreciate the fact that a Neurospora mycelium is made up of hyphal cells which are multi-

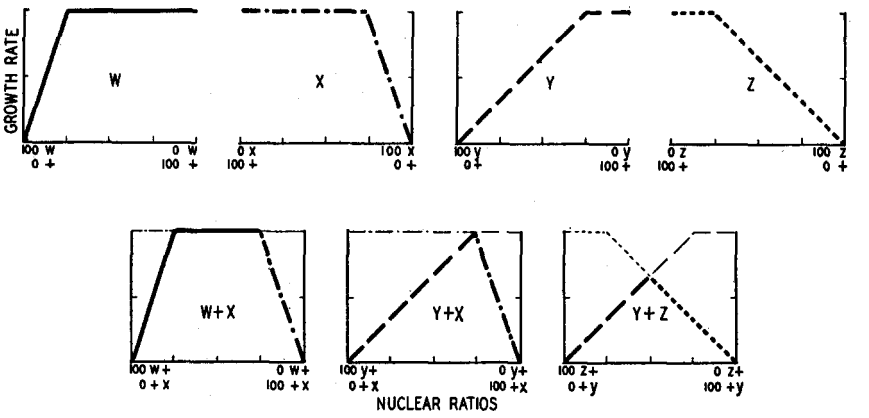

FIG. 2. Possible relations between nuclear ratios and growth rates of hypothetical heterocaryons.

nucleate and which are separated by crosswalls, each of which has a central perforation large enough to allow passage of nuclei from cell to cell. This means that in a heterocaryotic strain, in the absence of selection, there is no fixed ratio between the two (or more) types of nuclei present such as exists between chromosome sets in ordinary diploid or polyploid organisms. Instead, the ratio of the two kinds of nuclei in any "cell" of a two-component heterocaryon is free to shift continuously from nuclei all of one type to all of the other. If there is no selective advantage of hyphae with one nuclear ratio over those with another, the ratio in a given hypha will be governed by chance. However, in a heterocaryon made up of two components, each of which by itself has a sub-maximal rate of growth, there will be selection against hyphae with too great a proportion of either type. *A priori* there is no way of knowing within what range of ratios growth will be normal, but it should be possible to determine this experimentally. Some of the theoretically possible conditions for two-component heterocaryons are indicated in figure 2. Here four mutants *w*, *x*, *y*, and *z* are assumed to show the dominance relations represented in graphs W, X, Y, and Z. In these it is assumed that growth rates are linearly related to the proportion of wild type nuclei, but it should be emphasized that a linear relation is not necessary for the general interpretation. (See WRIGHT 1934, 1941, for discussion of physiological theories of dominance.) Strain *w* by itself—that is, with 100 percent *w* nuclei—does not grow. In heterocaryons with increasing proportions of wild type nuclei the growth rate increases until

at some ratio of the two kinds of nuclei a maximum growth rate is reached. This ratio of nuclei is assumed to be three mutant to one wild type in curve W. Any greater proportion of wild type nuclei would be as favorable. Here, then, a ratio of wild type to mutant nuclei of 1:3 or greater would be selected. Mutant *x* is assumed to show the same relation with wild type. Mutants *y* and *z*, on the other hand, are less recessive than *w* or *x* and therefore have less margin of safety. In heterocaryons with wild type they require that at least

TABLE 3

Summary of data on nuclear ratios in heterocaryons made by combining strains deficient in ability to synthesize specific growth factors.

MUTANT 1 MUTANT 2	*pab A* *pnt A*	*pab a* *pnt a*	*pab a* *pnt a*	*lys A* *pnt A*	*lys A* *pnt A*	*lys a* *pnt a*	*pnt A* *nic-2 A*	*pnt A* *nic-2 A*	*pab a* *nic-2 a*	*pab A* *nic-2 A*
Growth rate (mm/hr)	4.2	4.2	4.4	4.2	4.6	4.5	4.2	4.3	4.1	4.2
No. isolated	395	487	498	444	466	501	413	400	400	400
non-germinated	12	65	58	25	43	49	34	35	23	24
germinated	383	422	440	419	423	452	379	365	377	376
Wild type	201	217	218	213	231	242	251	186	227	239
Total mutants	182	205	222	206	192	210	128	179	150	137
Mutant 1	11	11	86	23	78	38	40	149	39	19
Mutant 2	171	194	136	183	114	172	88	30	111	118
Ratio mutant 1 / mutant 2	1 / 15.5	1 / 17.6	1 / 1.6	1 / 8.0	1 / 1.5	1 / 4.5	1 / 2.2	5.0 / 1	1 / 2.9	1 / 6.2

three-quarters of the nuclei be wild type to give a normal growth rate. In all these cases, heterocaryons, in which wild type is one component, might by chance variation revert to wild type homocaryons. If, however, heterocaryons are made up of any two mutants showing the characteristics of *w*, *x*, *y*, or *z*, natural selection of rapidly growing hyphae will, under the appropriate conditions, automatically maintain the heterocaryotic condition. If mutants *w* and *x* are combined, the simplest prediction is indicated in curve W+X where it is seen the ratio of *w*+ to +*x* nuclei must remain between 3:1 and 1:3 for maximum growth. In other words, selection would be expected to maintain the ratio within these limits. In the same way it can be seen that if mutants *x* and *y* are combined (curve Y+X), the ratio would tend to be maintained at one *y*+ to three +*x* nuclei, the only ratio that gives maximal growth. Mutants *y* and *z* would give results as shown in curve Y+Z. Here no ratio gives a growth rate equal to wild type, but a maximum rate is given at a 1:1 ratio of the two types of nuclei.

On the basis of these considerations, the experimentally obtained growth rates for heterocaryons are understandable. Mutants 221 and 5801 each give normal rates with 5531 but with each other give a rate that is only about half that of a normal strain. Presumably pantothenicless (5531) is relatively more

recessive, like *w* and *x* of figure 2, while the morphological mutants 221 and 5801 are relatively less recessive to their normal alleles, like *y* and *z* of figure 2.

An indication that the nuclear ratios in a heterocaryon might deviate rather widely from a 1:1 was obtained in collecting the data of table 2. In testing for the recovery of the two components of heterocaryon 1633+5531 (aminobenzoicless+pantothenicless), of 33 mutant strains recovered, only two were aminobenzoicless. This indicates a difference in relative dominance of the normal alleles of the two mutant genes.

More extensive studies on the nuclear ratios characteristic of various heterocaryons are summarized in table 3. The method used in obtaining these results was as follows: Heterocaryons were allowed to grow through growth tubes and conidia permitted to form at the end opposite the site of inoculation. These conidia were then dusted onto wild type protoperithecia of the opposite mating type grown on cornmeal agar. Perithecia were allowed to ripen and discharge their ascospores. From tubes in which at least several hundred perithecia had developed, a random sample of 400 to 500 discharged ascospores was removed and the spores cultured individually. Half of these were expected to give rise to wild type cultures and the other half to mutants of one or the other of the two types combined to give the heterocaryon. These were then differentiated by growing asexual transfer cultures on media containing known supplements as described on page 296.

There are several possible sources of error. It is assumed that the nuclear ratio does not change during the process of formation of conidia. While this appears to be an entirely reasonable assumption, there is no proof that it is correct. Differential germination of ascospores of the two mutant types will give rise to spurious ratios. A check on this is had in the observed ratio of wild type of mutant cultures. If this is one-to-one as expected, it is a reasonable deduction that spore germination was satisfactory. This test was fairly satisfactorily met by all combinations in table 3 except those involving nicotinicless-2. This mutant is known from independent evidence to show reduced ascospore viability. In this respect, this particular mutant was an unwise choice.

It is seen that the ratios vary rather widely for different combinations and also in different determinations within one combination. This would suggest that the situation in these particular combinations is similar to curve W+X of figure 2—that is, in each there is a rather wide range of ratios that give wild type growth rates.

In the eight determinations in which pantothenicless was one component of the combination, the estimated percentage of pantothenicless nuclei varied from 31.2 to 94.6 percent. This means that the mutant gene concerned is relatively recessive. In the instance in which the percentage of pantothenicless nuclei was 94.6, one normal allele per seventeen was apparently sufficient to give a normal growth rate.

Since heterocaryons combining 3100 with 221 give low growth rates, it is probable that a rather high proportion of normal alleles is necessary to "cover"

each of these two mutants. An attempt was made to determine the ratio of 3100 to 5531 nuclei in a heterocaryon made up of these two mutants. The results were unsatisfactory because of poor viability of 3100, but they did suggest a marked preponderance of pantothenicless over 3100 nuclei, possibly as much as 50:1. This is consistent with the assumption that the allele of

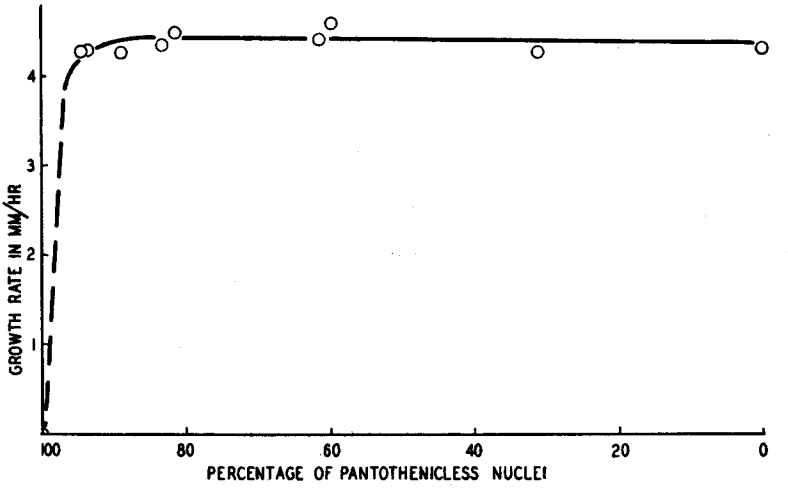

FIG. 3. Observed growth rates of heterocaryons in which pantothenicless was one component and in which the indicated percentages of pantothenicless nuclei were found.

pantothenicless is strongly dominant over its mutant allele, but that the normal allele of 3100 is only weakly dominant over the 3100 mutant allele.

Heterocaryons that do not grow at normal rates should have a more or less constant nuclear ratio if the theoretical considerations presented here are sound. Unfortunately, data that bear directly on this are not available.

If the general interpretation expressed in this paper is correct, it should be possible in heterocaryons to study dominance relations quantitatively and in detail. Given growth rate data and nuclear ratios on a sufficient number of combinations one should be able to draw complete growth rate-dosage curves for the mutants concerned. On the basis of the data presented it is already possible to define to a certain extent the relation between growth rate and the ratio of mutant to normal alleles of the pantothenicless gene. This is done in figure 3, where growth rates at experimentally determined ratios are recorded.

USE OF HETEROCARYONS IN TESTS OF ALLELISM

Three examples will serve to illustrate the use of heterocaryons in tests for allelism. Nicotinicless-1 and nicotinicless-2 are both sex-linked but appear to be biochemically different in that under certain conditions nicotinicless-2 produces a yellow pigment which is excreted into the medium, whereas nicotinicless-1 does not. It was found that when a mixed inoculum of these two mutants is put on a minimal medium, growth occurs which, when measured in

growth tubes, is approximately normal in rate. This strongly suggests that the component nuclei are *nic*-1+ and +*nic*-2. Direct confirmation of this was had by crossing the two mutants. Of 89 cultures produced from the resulting ascospores, 34 were wild type. With independent assortment of the two gene pairs, 25 percent wild type would be expected, with linkage, less than this. The observed excess is consistent with independent evidence that nicotinicless ascospores of both types show a lower germination percentage than do wild type ascospores.

The two tryptophaneless mutants, 10575 and 40008, show a similar relation. When like sexes are put together on minimal medium, growth is markedly accelerated over that of the single mutants, each of which grows slowly under these conditions. That they are genetically different is shown by the fact that from crosses between them, wild type strains are recovered. They are biochemically different as shown by the fact that 40008 will grow on anthranilic acid (*ortho*-aminobenzoic acid) while 10575 fails to do so (TATUM, BONNER, and BEADLE, in press). The evidence is clear that different genes have mutated to give rise to these two tryptophaneless strains.

A third example comes from the work of MRS. M. V. G. HUNGATE who kindly permits it to be cited prior to publication. It is observed that when albino-1 and albino-2 strains are grown together on a regular yeast extract-malt extract-agar medium, yellow pigment is produced in the conidia. These mutants are both sex-linked. From crosses between them, wild type strains are recovered with a low frequency indicating close linkage. However, there is genetic evidence suggesting that albino-1 is associated with a chromosome aberration of some sort so that the two genes may not be as close together as the data might indicate. Here again two phenotypically similar mutants appear to be differentiated from wild type by different genes. In this case mutual heterocaryotic dominance is expressed in pigment production rather than by an increased growth rate.

Other examples could be cited in which mutant genes of independent origins are indicated by heterocaryon tests to be allelic. A negative result, however, must be interpreted with caution. The mutant types may each require such a high ratio of normal alleles to give good growth that the required ratios are impossible to attain in the heterocaryon between them (an extreme case of the kind of relation illustrated in figure 2, curve Y+Z). This is equivalent to saying that one or both of the two genes is dominant in the ordinary sense in which this term is used in diploid organisms.

Although there is no fusion of nuclei, dominance relations presumably have the same physiological basis in heterocaryons as in ordinary diploid or polyploid organisms. For many characters, at least, it does not matter that homologous chromosomes carrying the two alleles of a given gene are separated by nuclear membranes. This relation has been shown to hold for many fungi as indicated by numerous reports in the literature, for a few special cases in higher plants, and for at least one instance in animals (see BULLER 1941 and POWERS 1943 for discussion and references). In the latter case it is shown that

individuals of *Euplotes patella* with two sets of diploid nuclei, each homozygous for mating types genes, show the same mating type as do animals with a single set of diploid nuclei heterozygous for the same mating type alleles (POWERS 1943).

USE OF HETEROCARYONS IN CROSSING MUTANT STRAINS

It is often observed that mutant strains of Neurospora form perithecia poorly and hence are difficult to cross. For example, the tryptophaneless strains 10575 and 40008 do not cross readily with each other. On the other hand, the unisexual heterocaryon in which both 10575 and 40008 nuclei are present is essentially wild type in behavior. It forms protoperithecia abundantly and can be crossed with the opposite sex of either tryptophaneless component. From such crosses, two types of asci are, of course, expected: those which segregate for both tryptophaneless gene pairs and hence give spores of wild type constitution, and those from ascospore mother nuclei homozygous for the tryptophaneless strain to which the heterocaryon was crossed.

This method, which can be used generally, is analogous to using heterozygotes in crosses of diploid organisms.

POSSIBILITY OF HETEROCARYONS WITH MORE THAN TWO COMPONENTS

It should be possible experimentally to produce heterocaryons with three kinds of nuclei all of which must be present to maintain normal growth. If three non-allelic recessive mutants *a*, *b*, and *c*, each of which fails to grow or grows slowly under the conditions selected, are combined in pairs to give the three strains $ab+$, $a+c$, and $+bc$, there should be formed, on growing the three double mutants together, a heterocaryon dependent for its growth on one normal allele in each of the three component nuclei. Natural selection would maintain the system as long as growth remained an advantage.

In a similar way with four mutant strains, four-component heterocaryons should be possible. Heterocaryons with more than two components might well be useful in studies on dominance relations.

SIGNIFICANCE OF HETEROCARYOSIS IN RELATION TO HETEROSIS

It is generally accepted that heterosis in diploid organisms is the result, at least in part, of dominant or semi-dominant favorable growth genes in one of the constituent chromosome sets complementing those of the other set (JONES 1942, see also EAST 1936). ROBBINS (1941) has suggested the possibility that increased vigor in excised hybrid tomato roots as compared with those of the parent strains may be the result of increased ability of the hybrid through complementary gene action to produce a full quota of growth factors and has suggested to DODGE (1942) that a similar interpretation might be applied to the cases of rapidly growing heterocaryons reported in *Neurospora tetrasperma*.

This interpretation applies to the observations presented here—for example, in the case of a heterocaryon made up of pantothenicless and lysineless (fig. 1) it is clear that ability to grow is the result of the complementary action of the

normal alleles of the two mutant genes concerned. Pantothenicless nuclei carry normal alleles of the lysineless gene, whereas the nuclei containing lysineless carry normal alleles of pantothenicless. The heterocaryon is thereby enabled to synthesize both pantothenic acid and lysine. Here, then, is what amounts to an extreme case of heterosis, clearly resulting from the presence of genetically complementary nuclei in a common cytoplasm. It is an instance in which it can be stated with confidence that the complementary growth factors are to be referred to the complementary genes concerned with the production of the vitamin pantothenic acid and the amino acid lysine.

The physiological basis of this cooperative gene action is with little doubt similar to that for hybrid vigor, or heterosis, in diploid organisms (DODGE 1942).

EVOLUTIONARY SIGNIFICANCE OF HETEROCARYOSIS

Accepting the text-book accounts of its life history, it is difficult to imagine how a haploid heterothallic fungus like *Neurospora crassa* could possibly compete in an evolutionary sense with a homothallic relative like *N. tetrasperma* in which two sets of chromosomes in separate nuclei are maintained in the mycelium. In *tetrasperma*, mutant genes which are deleterious in the combination in which they happen to first arise but potentially useful in other combinations can be saved through protection by their normal alleles. In *crassa* the opportunities for "trying out" new combinations would be greatly restricted if all mycelia were homocaryotic.

LINDEGREN (1942) has clearly pointed out that the phenomenon of heterocaryosis provides a mechanism by which this difficulty can be avoided. As a matter of fact, heterocaryons, with the possibility of several components and with no restrictions on the proportions of different chromosome sets present are even more flexible than diploids. Their formation in nature is inevitable. Hyphae of a single mycelium can be seen to fuse and anastomose freely. The same is true of hyphae from different mycelia. Dominance relations would be expected to give rise to "heterocaryotic vigor" following such interstrain fusions, and this vigor is all that is needed to maintain the heterocaryotic condition through natural selection.

That strains with normal growth rates, and with no apparent differences from wild type strains except those of pigmentation, do actually fuse and produce heterocaryons when grown together on a medium on which both components are able to grow is demonstrated by the behavior of albino-1 and albino-2 mutants as observed by MRS. M. V. G. HUNGATE. Here the selective advantage of the heterocaryon is not apparent, but, judging by pigmentation, the heterocaryotic condition invariably arises when the two strains are grown together on a yeast extract-malt extract-agar medium.

POSSIBLE RELATION OF HETEROCARYOSIS TO EVOLUTION OF SEXUAL REPRODUCTION

By greatly increasing the rate of formation of new combinations of genes, sexual reproduction has an obvious selective advantage over asexual means

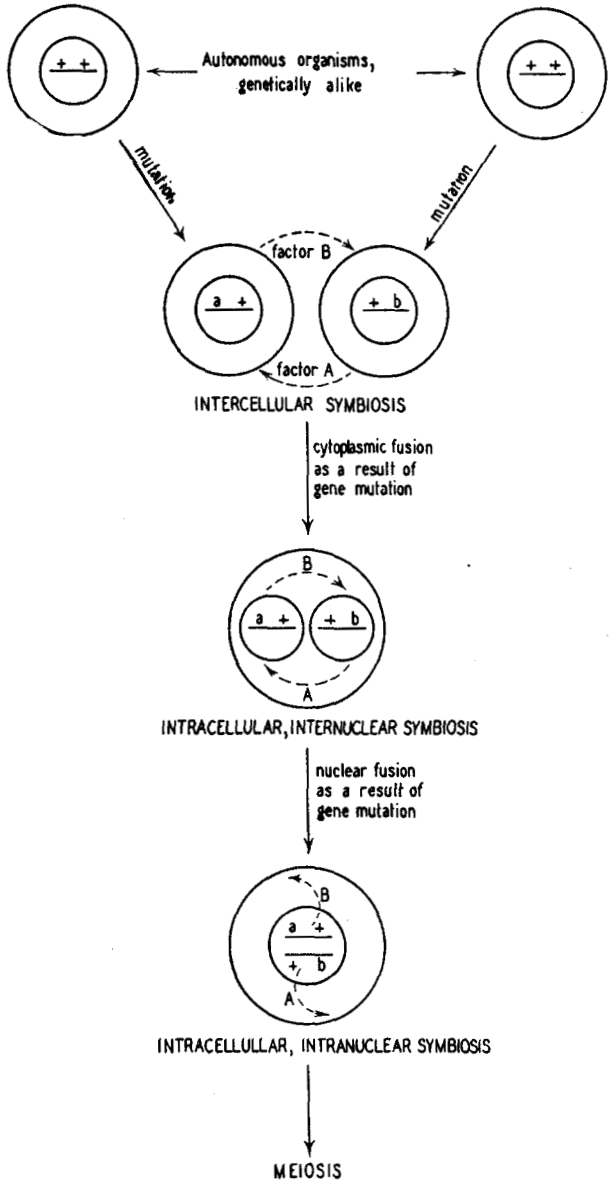

FIG. 4. Schematic representation of postulated steps in the evolution of sexual reproduction.

of multiplying individuals of a species. In considering possibilities as to how mechanisms of sexual reproduction evolved, DARLINGTON (1939) points out that nuclear fusion and meiosis must have arisen simultaneously since one without the other would be not only useless but indeed detrimental. He suggests that meiosis is a consequence of nuclear fusion and that the ability of a diploid nucleus to divide mitotically is a derived condition. In this way the

two events, fusion and meiosis, could arise as a result of a single evolutionary change. There still remains, however, the question of how cellular and nuclear fusion evolved. It seems possible that heterocaryosis, as it occurs in Neurospora, might have been one of the steps in this process. By this we do not mean to imply that sexual reproduction arose first in the fungi or that it arose only once. The hypothesis suggested could apply in principle to any of a number of primitive organisms. For example, myxomycetes, many algae, and numerous protozoa, particularly those with multinucleate cells, would appear to offer favorable opportunities for the phenomenon of heterocaryosis.

The scheme presented here proposes a series of successive steps, each of which, under appropriate circumstances, would have a selective advantage over the preceding condition. These conditions are represented in figure 4 and outlined as follows:

1. Organisms homogeneous and relatively autonomous in growth factor synthesis.

2. Differentiation of individuals through gene mutation into two groups deficient in abilities to synthesize growth factors. This could occur under environmental conditions such that these growth factors were not necessary for survival—that is, under conditions in which they were available from an external source. In fact, it is possible that it may be more economical than not for an organism to lose the ability to synthesize a substance that it can obtain in sufficient amounts from an external source.

3. Intercellular symbiosis arising from a change in environment which would reduce the availability of externally supplied growth factors, thus making survival of two deficient types dependent on the symbiotic relation between them. Such symbiotic pairs of organisms have been set up experimentally where the complementary forms are different species *cf.* SCHOPFER 1943 for discussion and references). There is no apparent reason why strains of a single species should not cooperate in a similar way.

4. Vegetative fusion of cells without nuclear fusion with an increase in the efficiency of the symbiotic relation. This condition represents a stable condition that could persist more or less indefinitely, but one that is reproductively inferior to the succeeding stage.

5. Nuclear fusion with a still further increase in the efficiency of the complementary gene action. This step would presumably lead to an unstable condition at the next nuclear division such that chromosome pairing would occur, meiosis rather than mitosis would result, and the cycle could then be repeated.

It is seen that each step in the process outlined reduces the size of the combining units through the series: cell, nucleus, chromosome, and gene. Each advance would therefore increase the evolutionary flexibility of the species.

SUMMARY

Mutant strains of *Neurospora crassa* of the same mating type undergo vegetative fusion to form heterocaryons—that is, mycelia containing a mix-

ture of two types of nuclei. If each mutant involved is recessive, the heterocaryon may be phenotypically normal. If the two mutant components are deficient in their abilities to synthesize specific growth factors, such as vitamins or amino acids, the heterocaryon will be maintained by natural selection if it is grown on a medium that does not supply the growth factors necessary for growth of the individual components.

By crossing a strain derived from a single hyphal tip of a heterocaryotic mycelium to wild type, both component mutant strains used to make the heterocaryon can be recovered. This proves that the single hypha actually contained nuclei of both mutant types.

The ratio of nuclei in a heterocaryon is not mechanically fixed. 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. Such ratios have been measured in a number of heterocaryotic combinations and certain deductions made as to the dominance relations of the genes involved.

Heterocaryons can be used as tests for allelism and are often useful in crossing mutant strains that are otherwise difficult to hybridize.

It is theoretically possible to make heterocaryons with three or more components that will be maintained by natural selection.

Heterocaryotic vigor is believed to be similar in its physiological basis to heterosis in diploid or polyploid organisms.

The phenomenon of heterocaryosis serves to give the haploid mycelia of the heterothallic species of Neurospora at least some of the advantages enjoyed by diploid organisms in evolutionary competition.

It is suggested that a heterocaryotic condition might have been one of the steps in the evolution of sexual reproduction.

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