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# An Example of the Influence of Modifying Genes in Neurospora

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# AN EXAMPLE OF THE INFLUENCE OF MODIFYING GENES IN NEUROSPORA*

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The influence of genetic modifiers, those genes which affect the expression of other genes, has been demonstrated in a wide variety of organisms. In *Neurospora crassa* the influence of modifiers is suggested by quantitative differences in growth substance utilization among different reisolates of a mutant strain (Good, Heilbronner, and Mitchell, 1950), and genetic suppression (Houlahan and Mitchell, 1947) may be regarded as an extreme type of modification. In addition, Houlahan and Mitchell (1948) and Mitchell and Mitchell (1952) have demonstrated extensive interactions among genes causing pyrimidine, lysine, and arginine requirements, and Srb (private communication) has found a case in which a single gene modifier appears to control the ability of a proline- or ornithine-utilizing strain of Neurospora to utilize arginine or citrulline. The data to be presented here deal with an instance in which the modification is such that different reisolates of a tryptophane- or nicotinic acid-utilizing mutant appear to be blocked at different steps in the pathway by which Neurospora synthesizes tryptophane and nicotinic acid. For some of the details of this pathway the reader is referred to the recent reviews of Mitchell (1950) and Horowitz and Mitchell (1951). Miss Dorothy Newmeyer, of Stanford University, who has used some of the strains which were used in this work, has also encountered some of the phenomena reported here.

## EXPERIMENTAL

### *Mutant strains*

Three types of mutants, differing in their abilities to utilize certain growth substances, were used in the crosses to be described here. For convenience these types have been designated 1, 2, and 3. The isolation number of the type 1 strains which were used in the crosses is C86 and that of the type 3 strains is 39401. Mutants C86 and 39401 have been described in the literature (Gordon, Haskins, and Mitchell, 1950). The origin of the type 2 strains which were used in the crosses will be described later in this report.

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All three of the mutant types utilize indole, tryptophane, kynurenine, 3-hydroxykynurenine, 3-hydroxyanthranilic acid, nicotinic acid, and nicotinamide. In addition, strains of type 2 utilize anthranilic acid, and strains of type 1 utilize anthranilic acid, phenylalanine, tyrosine, and quinic acid. Table 1 lists quantitative responses of the three types to minimal medium, phenylalanine, anthranilic acid, tryptophane, and nicotinamide.

*Crosses of the mutant types to wild type*

Asci were dissected from crosses of the three mutant types to wild type, and strains derived from each of the four spore pairs of each ascus were cultured on nicotinamide slants and then tested in 3 inch tubes of minimal

TABLE 1

FOUR-DAY DRY WEIGHTS PRODUCED BY MUTANT TYPES 1, 2, AND 3. GROWTH TESTS WERE CARRIED OUT AT 25°C. USING 125-ml. ERLENMEYER FLASKS CONTAINING 20 ml. OF LIQUID MEDIUM.*

| Mutant type | No. of ascospore cultures tested | Four-day dry weights (mg.) produced on | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | Minimal | DL-Phenylalanine 500 γ | | Anthranilic acid 200 γ | | L-Tryptophane 500 γ | | Nicotinamide 20 γ | |
| | | | Mean | S.E. | Mean | S.E. | Mean | S.E. | Mean | S.E. |
| 1 | 177 | trace | 42.6 | 0.6 | 64.9 | 0.6 | 76.3 | 0.8 | 76.7 | 0.5 |
| 2 | 107 | " | 9.5 | 0.9 | 55.1 | 0.9 | 71.8 | 1.2 | 69.4 | 0.8 |
| 3 | 243 | " | trace | | 1.1 | 0.2 | 49.0 | 0.6 | 70.9 | 0.5 |

*The minimal medium described by Beadle and Tatum (1945) was used in all the experiments described here.

medium and minimal plus nicotinamide. Those strains which appeared to be mutant were then further tested in 125-ml. Erlenmeyer flasks, as described in Table 1, and were classified as type 1, 2, or 3 (Table 2). Classification on this basis was relatively easy and straightforward except in the case of the cross of strain 3A × wild type Em 8815-3a. The progeny of this cross included a number of ascospore cultures which were borderline cases, and were therefore somewhat difficult to classify.

At the beginning of the investigation of these crosses and also of the intercrosses among mutant types (next section) both members of each mutant spore pair were tested. A total of 123 mutant pairs were tested in this way, and it was found that the agreement in growth response between the two members of any pair was excellent. Thereafter, ascospores were dissected out and tested in pairs rather than individually.

From table 2 it may be seen that each ascus from the crosses of the mutant types to wild type contains two wild-type spore pairs. Testing on the four growth substances in addition to minimal medium permits subdivision of the mutant offspring into types 1, 2, and 3; and it is found that all three types are recovered from the 3A × wild type crosses, while 1A × wild type and 2A × wild type both yield mutant types 1 and 2. This re-

TABLE 2

CLASSIFICATION OF ASCI FROM CROSSES OF MUTANT TYPES 1, 2, AND 3 TO WILD TYPE. (STRAINS 8a, 25a, AND Em 8815-3a ARE WILD TYPES.)

| Cross | No. of asci tested | Constitution of asci: Number of spore pairs of the indicated types. | | | |
|---|---|---|---|---|---|
| | | Wild | 1 | 2 | 3 |
| 1A × 8a | 18 | 2 | 2 | 0 | 0 |
| | 3 | 2 | 0 | 2 | 0 |
| | 1 | 2 | 1 | 1 | 0 |
| | 22 | | | | |
| 2A × 8a | 4 | 2 | 1 | 1 | 0 |
| | 1 | 2 | 2 | 0 | 0 |
| | 5 | 2 | 0 | 2 | 0 |
| | 10 | | | | |
| 3A × 8a | 4 | 2 | 0 | 0 | 2 |
| | 8 | 2 | 0 | 1 | 1 |
| | 2 | 2 | 1 | 0 | 1 |
| | 14 | | | | |
| 3A × 25a | 10 | 2 | 0 | 0 | 2 |
| | 1 | 2 | 0 | 1 | 1 |
| | 2 | 2 | 1 | 0 | 1 |
| | 13 | | | | |
| 3A × Em 8815-3a | 6 | 2 | 0 | 0 | 2 |
| | 1 | 2 | 0 | 2 | 0 |
| | 1 | 2 | 1 | 1 | 0 |
| | 1 | 2 | 2 | 0 | 0 |
| | 5 | 2 | 1 | 0 | 1 |
| | 14 | | | | |

covery of mutant offspring which are nutritionally different from the mutant parent suggests that genetic modifiers are influencing the phenotype of the mutant offspring. This suggestion is supported by the observation that in a total of 24 instances, two of the mutant types are found in a single ascus.

*Intercrosses among mutant types*

In further studies of the three mutant types, intercrosses were analyzed with the results shown in table 3. The 2A strain which was used in these crosses was derived from one of the type 3 × 1a crosses referred to below, and strain 2a came from the cross of 3R (anthran.) to wild type 8a. The origin of 3R (anthran.) is described in the next section. It will be seen from table 3 that crosses 2A × 2a and 3A × 2a gave rise to a new mutant phenotype, type 4. Type 4 strains are able to utilize kynurenine, 3-hydroxykynurenine, 3-hydroxyanthranilic acid, nicotinic acid, and nicotinamide, but, unlike the other types, they grow very poorly on indole and tryptophane. The average 4-day dry weights produced by 39 different type

TABLE 3

CLASSIFICATION OF ASCI FROM INTERCROSSES AMONG MUTANT TYPES 1, 2, AND 3

| Cross | No. of asci tested | Constitution of asci: Number of spore pairs of the indicated mutant types | | | |
|---|---|---|---|---|---|
| | | 1 | 2 | 3 | 4 |
| 1a × 1A | 11 | 4 | 0 | 0 | 0 |
| | 3 | 2 | 2 | 0 | 0 |
| | 14 | | | | |
| 1a × 2A | 5 | 2 | 2 | 0 | 0 |
| | 4 | 3 | 1 | 0 | 0 |
| | 1 | 1 | 3 | 0 | 0 |
| | 1 | 4 | 0 | 0 | 0 |
| | 11 | | | | |
| 1a × 3A | 7 | 1 | 1 | 2 | 0 |
| | 3 | 2 | 0 | 2 | 0 |
| | 1 | 0 | 2 | 2 | 0 |
| | 11 | | | | |
| 2a × 2A | 1 | 1 | 0 | 2 | 1 |
| | 2 | 2 | 0 | 0 | 2 |
| | 1 | 0 | 1 | 2 | 1 |
| | 2 | 1 | 1 | 1 | 1 |
| | 3 | 1 | 2 | 1 | 0 |
| | 2 | 1 | 2 | 0 | 1 |
| | 1 | 0 | 2 | 0 | 2 |
| | 3 | 0 | 1 | 3 | 0 |
| | 1 | 2 | 0 | 1 | 1 |
| | 2 | 0 | 2 | 1 | 1 |
| | 18 | | | | |
| 2a × 3A | 3 | 0 | 1 | 2 | 1 |
| | 10 | 0 | 0 | 2 | 2 |
| | 1 | 0 | 2 | 2 | 0 |
| | 1 | 0 | 0 | 3 | 1 |
| | 15 | | | | |
| 3a × 3A | 19 | 0 | 0 | 4 | 0 |

4 strains on 500$\gamma$ of L-tryptophane and on 20$\gamma$ of nicotinamide were 6.3 ± 1.2 mg. and 72.9 ± 1.3 mg., respectively.

It is noteworthy that all of the ascospores from the dissected asci gave rise to mutant strains. In addition, approximately 15,000 random ascospores from crosses of two different type 3 strains to strain 1a were plated on minimal agar using an adaptation of the method described by Lein, Mitchell, and Houlahan (1948). Approximately 97 per cent of the spores germinated and no wild types were found. Thus it appears that the mutations which prevent the growth of the various types on minimal medium are either allelic or else very closely linked. In agreement with this conclusion is

the fact that attempts to form phenotypically wild heterocaryons among the four mutant types have been unsuccessful.

*Adaptation of a type 3 strain to anthranilic acid*

As shown in table 1, strains of type 3 do not ordinarily utilize anthranilic acid. It was found, however, that a certain reisolate of this type, designated 3R, which had been maintained in the stocks for a period of several years occasionally would start growth after two or three days of incubation in liquid culture on anthranilic acid. This reisolate was cultured on slants of minimal agar supplemented with 5$\gamma$ of anthranilic acid/ml. and although it was somewhat slow to start on this medium, the strain eventually produced abundant growth. The response of this culture to phenylalanine, anthranilic acid, tryptophane, and nicotinamide in liquid medium appeared to be unchanged after a single transfer to anthranilic acid agar. Transfers to anthranilic acid slants were continued, however, and after the seventh consecutive transfer the strain was again tested, this time on quinic acid, tyrosine, phenylalanine, anthranilic acid, indole, tryptophane, and nicotinamide. All of these substances were utilized, and it was found that, within limits, increasing the concentration of any of them produced increased growth of the mold. Having acquired the ability to utilize all these compounds, 3R now resembled a type 1 strain. This adapted strain will be referred to as 3R(anthran.). Mutant progeny from a cross of 3R(anthran.) to wild type 8a were found to be predominantly of type 3, while those from the cross of strain 1A to wild type 8a (Table 2) included no type 3 strains. Thus it is apparent that 3R(anthran.) differs genetically from strain 1A although it is nutritionally similar to the type 1 strains.

*A genetic suppressor*

In the course of attempting a further genetic analysis of 3R(anthran.) this adapted strain was crossed with mutant strain 1a, and approximately 4000 ascospores were plated on minimal agar. Germination of the plated ascospores was approximately 98 per cent and on the basis of hyphal length, approximately 5 per cent appeared to be wild type. Five of these spores were transferred to agar slants, and testing of the resulting cultures showed them indeed to be phenotypically wild. In an effort to learn whether this behavior might be the result of genetic suppression, the five ascospore cultures were crossed to wild types 7A or 8a. Mutant spores were found among the progeny of each cross, indicating that genetic suppression had occurred. A similar case has been described by Houlahan and Mitchell (1947).

Asci were then dissected, a suppressor-carrying wild-type strain and two suppressed-mutant strains were selected, and crosses were made as shown in table 4. The action of the suppressor was tested on strains 10575 and E5029 as well as on strains of types 1, 2, and 3. Strain 10575 requires either indole or tryptophane for growth, and strain E5029 requires 3-hydroxykynurenine, 3-hydroxyanthranilic acid, nicotinic acid, or nico-

TABLE 4

CROSSES INVOLVING THE SUPPRESSOR (S); SUPPRESSED MUTANT (SM); MUTANT STRAINS 1a, 2a, 3a, 10575, AND E5029; AND WILD TYPES 8a AND 7A.

| Cross | No. of asci tested | No. of asci with the indicated ratio of wild-to mutant-spore pairs | | |
|---|---|---|---|---|
| | | 4:0 | 3:1 | 2:2 |
| S × 8a | 15 | 15 | 0 | 0 |
| S × SM-a | 14 | 14 | 0 | 0 |
| S × 1a | 17 | 2 | 7 | 8 |
| S × 2a | 13 | 0 | 3 | 10 |
| S × 3a | 15 | 0 | 5 | 10 |
| SM-a × 7A | 14 | 2 | 12 | 0 |
| SM-A × 8a | 12 | 2 | 8 | 2 |
| SM-A × 1a | 16 | 0 | 0 | 16 |
| S × 10575 | 22 | 0 | 0 | 22 |
| S × E5029 | 24 | 0 | 0 | 24 |

tinamide (Mitchell, 1950). The analysis of asci shown in table 4 was based mainly on tests using 3-inch tubes of minimal medium and minimal plus nicotinamide (2.5 $\gamma$/ml.), although some of the cultures were tested in flasks. Progeny of the cross S (suppressor) × 10575 were tested in tubes of minimal medium and minimal plus L-tryptophane (50 $\gamma$/ml.). In these crosses the suppressor exhibits the behavior of a single gene whose action repairs in some way the function of the mutant gene which types 1, 2, and 3 have in common, *i.e.*, the mutation which distinguishes these strains from wild type, but whose action is ineffective in repairing the functions which strains 10575 and E5029 have lost by mutation.

The influence of the suppressed mutant nuclei which are evidently present in the 3R(anthran.) culture is not certainly known. It seems possible that the presence of a few such nuclei changes the phenotype of the strain from type 3 to type 1, but an attempt to create a type 1-like culture by artificially mixing suppressed-mutant conidia with type 3 conidia failed. In this attempt, several groups of flasks containing minimal medium or minimal supplemented with phenylalanine, anthranilic acid, tryptophane, or nicotinamide were inoculated with a conidial suspension from a type 3 strain, and simultaneously with a dilution series of suppressed-mutant conidia. It was found that those groups receiving a relatively concentrated inoculum of the suppressed-mutant conidia were phenotypically wild, while those with a dilute inoculum were like type 3.

## DISCUSSION

Restated very briefly, the principal experimental facts are these:

(1) Crosses of types 1, 2, and 3 to wild type produce asci each of which has two mutant and two wild-type spore pairs, but the mutant offspring differ, in many cases, from the mutant parent, and in numerous asci the two mutant spore pairs differ from each other.

(2) Intercrosses among the three types produce no wild-type progeny, but many of the asci have three or four phenotypically different kinds of spore pairs.

(3) A culture which is nutritionally like the type 1 strains has been obtained from a type 3 strain by taking the latter strain through repeated transfers to anthranilic acid agar slants. Approximately 5 per cent of the progeny from a cross of this type 1-like culture to strain 1a were phenotypically wild, but subsequent testing showed that they were actually suppressed mutants.

It is by no means certain that the alteration in phenotype of the type 3 strain referred to in the third observation listed above is a result of the heterocaryosis which evidently exists in the 3R (anthran.) culture. If the assumption is made, however, that heterocaryosis is the cause, then one might ask if the seemingly odd progeny from the various crosses listed in tables 2 and 3 might not be due to the presence, in varying proportions, of suppressed-mutant nuclei in the ascospore cultures. The inadequacy of this interpretation is indicated by several experimental facts. First, several transfers to anthranilic acid agar were required before the type 3 strain assumed the nutritional characteristics of a type 1 strain. The need for conditions which favored the selection of certain types of nuclei is indicated. The ascospore cultures listed in tables 2 and 3, on the other hand, displayed their characteristic phenotypes after they had been transferred directly from the minimal agar plates on which they were allowed to germinate, to slants of nicotinamide agar.

Secondly, there is no evidence that ordinary type 1 or type 3 cultures contain any wild-type or suppressed-mutant nuclei, for in 15,000 ascospores plated from two different crosses between the two types, no wild-type offspring were found. Wild types were similarly absent from the dissected asci from intercrosses among types 1, 2, and 3.

A third point against the heterocaryosis hypothesis is found in the similarity which exists between the two members of any spore pair. It has been mentioned that the growth responses of the two members of any spore pair were found to be essentially identical. If these responses were dependent on spontaneous suppressor mutations and the heterocaryosis resulting from these mutations, good agreement within spore pairs would not be expected. If the growth responses were a reflection of genetic constitution, on the other hand, one would expect to find good agreement within pairs.

Finally, if heterocaryosis were responsible for the phenotypes, it seems unlikely that it would be possible to get a strain which would breed true. It was found, however, that in the 3a × 3A cross listed in table 3 all offspring were of type 3. The 3a parent in this case came from a cross of 3A × wild type 8a.

Attempting to account for the experimental observations by assuming an allelic series in which the different alleles lead to altered nutritional requirements is similarly unsatisfactory, for if two strains carrying allelic mutations were crossed, one would expect to recover only two phenotypes (those of the parental strains) from each ascus. In the present case, many of the asci listed in tables 2 and 3 contain representatives of three different types, and two asci have spores of four different types.

It appears, then, that types 1, 2, 3, and 4 have in common a mutation which prevents their growth on minimal medium, and that this primary effect is modified by the action of a number of other genes so that the various types differ qualitatively in their responses to phenylalanine, anthranilic acid, tryptophane, and nicotinamide. They therefore appear to be blocked at different steps along the biosynthetic pathway. Actually there are quantitative differences as well, for there is considerable variation within each type in the amount of growth produced on any substrate, as shown by the standard errors in table 1. It seems likely that if enough crosses were analyzed, a more or less continuous spectrum from type 4 to suppressed mutant or wild type would be indicated.

The analyses which have been made are not sufficiently extensive to permit definite conclusions as to the number of modifying genes involved or the precise effect of any of them. Such conclusions must await the accomplishment of a large amount of genetic work, coupled with enzyme studies. The present work clearly demonstrates, however, that one may be misled if one attempts to assign the basic effect of a genetic mutation to a particular biosynthetic step on the basis of growth response alone.

## SUMMARY

A genetic analysis of several tryptophane- or nicotinic acid-utilizing strains of Neurospora has provided evidence that these strains have in common a mutation which prevents their growth on minimal medium, but that their responses to various growth substances are influenced by the action of a number of modifying genes. The influence of the modifiers is such that different strains which carry the same primary mutation appear to be blocked at different biosynthetic steps along the pathway which leads to the formation of tryptophane and nicotinic acid. A suppressor of the primary mutation has also been found.

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