[13] Lederberg, J., *Genetics*, **32**, 505–525 (1947).

[14] It should be noted that these data were secured by the plating of undiluted $h-$ cultures. When such cultures are diluted and plated the $h-$ bacteria form microcolonies which are not visible to the naked eye. Mutations to the $h+$ condition occur in these microcolonies and in some cases this results in the formation of visible $h+$ colonies. These plate mutations obscure the characterization of the number of $h+$ organisms originally present in the $h-$ culture. They can be eliminated by omitting asparagine from the minimal medium. Asparagine was present in the medium used for the experiments reported in this paper. Although some plate mutations occur under the crowded conditions formed by plating undiluted cultures, their number is insignificantly small. Even after 5 days less than 5 per cent of all $h+$ colonies are formed by plate mutation, a source of error well within that attributable to random sampling (Ryan, F. J., and Schneider, L. K., *Genetics*, in press). It is interesting to note that a variance analysis of the number of $h+$ colonies on plates of 10^{-6} dilutions of different $h-$ cultures indicates that they form a homogeneous population ($P = 0.54$; P for the variance in the number of $h+$ colonies on plates of different samples of the same culture $= 0.51$). This is independent evidence that 10^{-6} dilutions of $h-$ cultures contain no $h+$ organisms but that $h+$ colonies formed on plates of such dilutions are derived from events occurring on the plates.

[15] The spontaneous mutations may conceivably be to a condition of susceptibility to respond to the testing conditions. This notion is operationally the same as mutation to the ability the testing conditions demonstrate.

[16] Ryan, F. J., *Cold Spring Harbor Symposia Quant. Biol.*, **11**, 215–227 (1946).

[17] Lederberg, J., and Tatum, E. L., *J. Biol. Chem.*, **165**, 381–382 (1946); see also reference 12. Under the conditions of this experiment microcolony mutation adds an insignificantly small number of $h+$ colonies.

[18] Lindegren, C. C., and Lindegren, G., *Science*, **102**, 33–34 (1945).

[19] Carpenter, C. M., Bahn, J. M., Ackerman, H., and Stokinger, H. E., *Proc. Soc. Exp. Biol. Med.*, **60**, 168–171 (1945); Sullivan, M., Stahly, G. L., Burkeland, J. M., and Myers, W. G., *Science*, **104**, 397–398 (1946).

[20] Monod, J., *Ann. Inst. Past.*, **72**, 879–890 (1946).

[21] van Loghem, J. J., *Zbl. Bakt. I. Abt. Orig.*, **57**, 385–387 (1911).

[22] Fildes, P., Gladstone, G. P., and Knight, B. C. J. G., *Brit. J. Exp. Path.*, **14**, 189–196 (1933).

[23] Doermann, A. H., *Arch. Biochem.*, **5**, 373–384 (1944).

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*A METHOD FOR SELECTION OF BIOCHEMICAL MUTANTS OF NEUROSPORA**

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Numerous investigations in recent years have firmly established the value of mutants of the mold Neurospora as tools for research in biochemistry and genetics.[1, 5, 6] Although various means have been used for inducing mutations,[4, 5, 6] the technique of isolation as described by Beadle and

Tatum[4] has been used by most investigators. This method involves isolation of single ascospores, one from each perithecium, derived from a cross of which one parent was treated. By this method at least 97 per cent of the cultures obtained from the single spore isolations are unchanged wild type strains. In order to obtain specific mutants or a diversity of mutants, it is therefore necessary to isolate a very large number of single ascospores.

The present investigations were undertaken for the purpose of developing a method for obtaining desired mutants with less effort than that required by the previous method of single ascospore isolations.

It was obviously desirable to develop a technique for eliminating a large proportion of wild type strains from those isolated and tested. Such a technique has been developed by Fries, for Ophiostoma.[7] An attempt was made to apply a modified form of the Fries technique to Neurospora by removing non-mutated strains by continuous filtration of a suspension of irradiated microconidia that were allowed to germinate and grow on a medium that would support growth of wild type strains but not mutant strains. This procedure was not a success due to a rapid loss in viability of the microconidia used, and because of clogging of the filter by growing mycelia. The few mutants that were obtained by this method occurred as heterocarotic mixtures with wild types.

A successful method has been developed, based on a visual selection of mutants among ascospores germinating on a minimal medium that will support wild type growth but not the growth of biochemical mutants. By this procedure about 80 to 95 per cent of the wild type spores can be discarded without testing.

A major consideration in developing a method of selecting specific mutants is concerned with the effect of a single mutation on the total metabolic pattern of the new strain. There is now ample evidence that the growth of some mutants is inhibited by metabolites which do not affect wild type strains. An outstanding example that has been described in detail is that of the large group of lysineless mutants of Neurospora.[8] These mutants are all inhibited by arginine. It was therefore thought desirable to make the isolation on minimal medium supplemented only with the metabolite that the desired mutant requires for growth. The new method of obtaining mutants by isolating germinating ascospores has been subjected to three types of experiments in order to provide evidence as to its practicability.

1. Germinating mutant ascospores were picked from crosses between known mutants and wild type.

2. A sample of miscellaneous mutant strains (i.e., requiring an amino acid, vitamin, purine or pyrimidine) was picked from a cross of irradiated wild type and untreated wild type.

3. Using appropriate media, specific types of mutants were picked from a cross of irradiated wild type and untreated wild type.

Experimental.—Media: In the experimental work to be described, all crosses were made on the synthetic medium described by Westergaard and Mitchell.[9] This medium supports the formation of a much larger number of perithecia than does corn meal agar.

The "minimal" medium utilized has been described by Beadle and Tatum.[4] The "complete" medium, however, has been modified using Neurospora extract rather than yeast extract and malt extract. This was prepared from Neurospora strain, Abbott 4A, grown with aeration for 3.5 days in 15 liters of minimal medium. The mycelium was collected, chopped and suspended in 4 parts of water. After 20 hours' autolysis under toluene at 37°C., the suspension was autoclaved and filtered. Hydrolyzed casein was added to the filtrate to a concentration of 0.5 mg. %. This preparation was used either as a liquid or solidified with 1.5% agar.

Selection Method: Petri plates (100 mm.) containing 25 ml. of agar medium[9] were inoculated by spreading a suspension of conidia of one mating type of Neurospora over the surface. Following formation of protoperithecia (4 to 6 days) crosses were made by adding a suspension of conidia of the opposite mating type. In cases where irradiation was carried out, the conidia of the second mating type were obtained from a six-day-old culture of wild type grown on minimal medium. These spores were suspended in sterile distilled water and exposed for 4 minutes at a distance of 3.5 in., to radiation from an "Americanaire" ultra-violet lamp (American Sterilizer Co.). More than 95 per cent of the conidia were killed by the treatment. The rayed conidia from one test tube culture were spread over two plates of protoperithecia, producing on incubation, from 1000 to 2000 perithecia. After about 11 days spores were emitted spontaneously and samples were collected on plates containing 25 ml. of medium solidified with 4 per cent agar. Collections were made by inverting the plate containing perithecia over the second plate and thereby allowing the spores to fall on the agar surface. Examinations under the microscope were made at intervals and the collection stopped when the spores were, roughly, 1 mm. apart. The time required to obtain such a distribution varied from 5 to 30 minutes. The plate was then covered and placed at once in an oven at 60°C. for 30 minutes in order to activate the ascospores. This treatment serves also to kill conidia which are usually present. After 12 hours' incubation at 25°C., the wild type ascospores had produced mycelia covering an area about 1–2 mm. in diameter while the mutants were of the order of one-tenth to one-twentieth the size of wild type. Since each perithecium sheds spores into a localized region, the presence of clusters of small forms facilitated detection of mutants. Only one mutant ascospore was taken from a cluster to avoid excessive duplication. The presumed mutant ascospores were picked up on small blocks of agar by use of fine pointed platinum-iridium spatula. They were then placed in a small test tube con-

taining the desired medium. The cultures obtained in this manner were then tested and the mutants identified in much the same manner as that described by Beadle and Tatum.[4]

Selection of Known Mutants: In order to test the method of visual selection of mutants from germinating ascospores, individual crosses were made between wild type and four different known mutants. Ascospores from the perithecia of these crosses were collected on plates of minimal agar and an attempt was made to pick the mutant types. Since in these plates the ratio of mutants to wild type strains was approximately one to one, the task was much simpler than it would be using rayed material where the incidence of mutants is of the order of 2 per cent. Data from this experiment are given in table 1.

TABLE 1

THE VISUAL SELECTION OF MUTANTS FROM CROSSES BETWEEN KNOWN MUTANT AND WILD TYPE STRAINS

MUTANT REQUIREMENT	NO. PICKED	CULTURES OBTAINED	VIABLE SPORES (%)	NO. OF MUTANTS	MUTANTS (%)
Patothenic Acid	45	16	36	16	100
Uridine	55	45	82	45	100
Adenine	55	39	71	38	97
Lysine	45	20	44	18	90

The data of table 1 demonstrate that the method of visual selection of mutants is basically sound.

General Selection of Mutants: A cross was prepared in four plates, using protoperithecia of *Neurospora crassa* 6a and irradiated conidia of 3A. These strains were newly reisolated from a cross of Em5256A and Em 5297a. Sphores were shed 11 days after the cross was made and collections on plates of minimal agar were begun at this time. These collections were made each day for 7 days with one final sample taken on the 14th day. The shedding of spores was nearly complete at this time. Approximately 20 germinated spores were picked from each plate at each collection and transferred to tubes of casein, Neurospora extract, agar medium. All of the resulting cultures were tested on minimal medium and those that grew in 3 days were discarded. The remaining cultures were tested on the classification mixtures previously described:[4] (1) vitamins; (2) amino acids; (3) yeast extract and (4) minimal. An additional mixture was also introduced to facilitate classification of the more common types of mutants previously obtained. This mixture contained methionine, 2.5 mg. %; adenine, 5 mg %; cytidine, 3 mg. %; lysine, 10 mg. %; tryptophane, 5 mg. %; and succinic acid, 10 mg. %.

Four kinds of mutants were obtained; those which did not grow on minimal medium but grew when supplied yeast extract or specific known compounds; those which grew very slowly on minimal and complete

media; those which covered the surface of the agar in the tubes but grew no further regardless of the supplement given; and those which were obviously morphological (mostly colonial) mutants. Mutants of the last two categories are not reported here. In addition, mutants collected from the same plate on the same day and which required the same known compound were considered to be duplicates. Only one representative was tabulated. Actually very few cases of such duplications were found.

The results of this experiment are summarized in table 2. Two mutant types are recorded: slow growing mutants and biochemical mutants. The requirements of the majority of the biochemical mutants are also given. In many cases these mutants grew only on the yeast extract classification mixture and their specific requirements were not determined. These are listed as unknown mutants.

TABLE 2

SELECTION OF SLOW GROWING AND BIOCHEMICAL MUTANTS

PLATE NO.	NO. PICKED	CULTURES OBTAINED	NO. SLOW MUTANTS	NO. BIOCHEMICAL MUTANTS	TYPES OF BIOCHEMICAL MUTANTS
1	112	71	3	6	Threonine, arginine methionone, cytidine, unknown (2)
2	163	61	9	8	Proline, cytidine, *p*-aminobenzoic acid (2), unknown (4)
3	114	62	3	8	Cytidine, methionine, arginine, adenine (2), unknown (3)
4	123	55	12	12	Lysine, methionine, adenine + methionine, tryptophane (2), unknown (2)
Total	512	249	27	34	

It may be observed from the data in table 2 that a wide variety of mutants was obtained from the four plates. Biochemical mutants which did not grow on minimal medium but did grow in the presence of an appropriate supplement constituted 13 per cent of the cultures that were obtained.

Selection of Specific Mutants: Several experiments were designed to select specific mutants from 20 plates prepared as described in the previous section. The first of these was concerned with selection of mutants requiring histidine. No histidineless mutants have been obtained previously in Neurospora. Ascospores were collected on minimal medium and, after germination, probable mutants were transferred to minimal medium containing 5 mg. % histidine monohydrochloride. Resulting cultures, 47 from 115 spores picked, were tested on minimal. One failed to grow and it was subsequently established to be a histidineless mutant. This mutant fails to grow in the presence of hydrolyzed casein, yeast extract or Neurospora ex-

tract in the concentrations usually used in complete medium. It seems probable that mutants of this type occurred but were eliminated in previous tests because of their failure to grow on the complete medium used.

In a second experiment an attempt was made to select for three types of mutants simultaneously. These were: a frequently occurring type requiring adenine; a type that has occurred only infrequently, requiring pantothenic acid; and a specific mutant, not previously found, that cannot condense indole and serine to form tryptophane. Mutants of the third type might be expected to lack the enzyme which links serine to indole. The presence of this enzyme in wild type Neurospora has been reported by Umbreit, *et al.*[10] In order to minimize the selection of other mutants the ascospores were collected on plates of minimal agar supplemented with the following metabolites: charcoal treated, acid hydrolyzed casein, 100 mg. %; riboflavin, 0.5 mg. %; thiamin, 0.25 mg. %; pyidoxine, 0.25 mg. %; cytidine, 3.75 mg. %; indole 5.0 mg. %. These substances were added to minimize the picking of undesired mutant strains. Presumably mutants having these metabolites as growth requirements would be indistinguishable from wild type strains on the plates.

Of 381 ascospores picked from these plates, 265 grew when placed on a medium of minimal agar supplemented with 5 mg. % adenine, 5 mg. % tryptophane and 0.5 mg. % pantothenic acid. On testing the cultures that grew in this medium, it was found that there were no adenine or pantothenic acid mutants but that one tryptophane mutant had been selected. This mutant required tryptophane for growth but did not grow when supplied indole. It was consequently one of the mutants for which the selection was made. No adenine mutants were found in this experiment in spite of the fact that they have occurred at fairly high frequencies in other experiments. It was subsequently found that indole strongly inhibits the growth of adenine mutants, and it is probably because of this previously unknown fact that no adenineless mutants were obtained. Such an inhibition is not surprising in view of the inhibition of growth of bacteria and yeast by the structurally related benzimidazole, investigated by Wooley.[11]

In a third experiment, ascospores were collected on a medium containing autoclaved fresh liver extract and transferred to tubes containing filter sterilized liver extract. From a total of 145 spores picked, 75 grew when transferred to unheated liver extract medium. One of these cultures failed to grow on minimal medium and has been classified as requiring a heatlabile substance in liver.

Experiments to select for mutants requiring hydrolyzed pantothenic acid or indoleacetic acid were unsuccessful as was an attempt to select a mutant lacking the enzyme urease. In the last case, spores were collected on a medium containing urea as the sole nitrogen source.

Discussion.—It is evident from the experimental data that considerable

progress has been made in developing an improved technique of selecting biochemical mutants in Neurospora. Even with the rigorous discarding of duplicate mutants, 13 per cent of the cultures obtained were found to have special growth requirements. Thus, the present method markedly increases the ease with which such mutants can be obtained.

In the selection of specific mutants three were obtained out of seven attempted. The type requiring adenine probably was not obtained because of inhibition by indole. Since the selection experiments were carried out on a small scale, success in all the selections attempted would be unlikely. Thus, 20 Petri dishes containing a total of 20,000 perithecia would have approximately 300 mutants represented, assuming a mutation frequency of 1.5 per cent. Even if all 300 mutants were represented in the ascopores that were picked, the probability of obtaining a particular mutant that occurred infrequently would not be large. By increasing the number of plates of perithecia, the probability of selecting a particular mutant would, of course, be increased.

It has been previously suspected that some types of mutants have been selected against because of the presence of inhibitors in the "complete" medium used for the isolations. Information obtained in this experimental work suggests that such cases are fairly frequent. At least three mutants have now been found that will not grow on the "complete" medium. These require for growth histidine, tryptophane and methionine, respectively. Thus, it is possible that many groups of mutants have not been obtained because of growth inhibition by substances in the medium used for isolations. It is therefore clear that data on the frequency of occurrence of mutants with different growth requirements should be interpreted with this source of error in mind.

Summary.—1. A new method has been developed for isolating biochemical mutants of Neurospora.

2. The method has been applied successfully to the selection of some specific mutants not previously obtained. The importance of the use of a simple medium for selecting mutants has been discussed.

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[1] Beadle, G. W., *Ann. Rev. Physiol.*, **10**, 17–42 (1948).

[2] Beadle, G. W., *Physiol. Rev.*, **25**, 643–663 (1945).

[3] Horowitz, N. H., *J. Biol. Chem.*, **171**, 255–264 (1947).

[4] Beadle, G. W., and Tatum, E. L., *Am. J. Bot.*, **32**, 678–686 (1945).

[5] Horowitz, N. H., Houlahan, M. B., Hungate, M. G., and Wright, B., *Science*, **104**, 233–234 (1946).

[6] McElroy, W. D., Cushing, J. E., and Miller, H., *J. Cell. Comp. Physiol.*, **30**, 331–346 (1947).

[7] Fries, N., *Nature*, **159**, 199 (1947).
[8] Doermann, A. H., *Arch. Biochem.*, **5**, 373–384 (1944).
[9] Westergaard, M., and Mitchell, H. K., *Am. J. Bot.*, **34**, 573–577 (1947).
[10] Umbreit, W. W., Wood, W. A., and Gunsalus, I. C., *J. Biol. Chem.*, **165**, 731–732 (1946).
[11] Wooley, D. W., *Ibid.*, **152**, 225–232 (1944).

A PREDICTABLE MUTATION IN BACTERIA

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In the bacterium, *Sarcina lutea*, a color mutation has been found which has a number of interesting characteristics. The mutation is stable and does not revert to the original form either spontaneously or under any treatment thus far attempted. It may be produced at any time by either of two different methods of induction, one environmental, the other chemical. It also occurs spontaneously at a low rate.

If the following four species of bacteria, *Bacillus subtilis*, *Proteus vulgaris*, *B. megatherium* and *S. lutea* are grown together in nutrient broth for twenty-four hours and then reisolated by the dilution method, five different strains instead of four are recovered. In addition to the four original species a new non-pigmented form is always obtained. The new type is similar to *S. lutea* in all morphological features except in its color which is white instead of yellow. This new form is here called *S. alba* and is considered to be a mutant form of *S. lutea*.

The above experiment was repeated 15 times and in every case the same mutant form was obtained. About three times as many yellow colonies as white ones are found when the diluted mixture is plated out. All 15 lines of the white mutant have remained stable for at least 50 transfers at two-day intervals while some of the first to be obtained have been cultured for over a year.

Evidence of the stability of the white mutant is as follows. If *S. lutea* and *S. alba* are grown together in nutrient broth and then plated out both white and yellow colonies are recovered. *S. alba* was inoculated into a medium which consisted of an autoclaved forty-eight-hour nutrient broth culture of *S. lutea*. The *S. alba* grew in this medium without any reversion to the yellow form.

Experiments were performed to determine whether a mixture of all four organisms is necessary to produce the white mutation. Each of the original species was left out in turn and mixtures of three species were tested. A