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REVERSE-MUTATION AND ADAPTATION IN LEUCINELESS NEUROSPORA

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Mutations affecting specific steps in biochemical syntheses have been produced in *Neurospora crassa* by x-rays and ultra-violet radiation.[1] In crosses with wild-type molds, these mutations segregate in a Mendelian fashion and hence involve the alteration of single determinant factors. Since many of the mutants seem to completely lack a specific synthetic ability it was not certain whether they involved small chromosomal aberrations such as deficiencies, or the inactivation of genes which still self-duplicate. Up to the present time no demonstration of back-mutation, which would afford indirect evidence on this question, has been made for any of the biochemical mutants of *Neurospora*. On the basis of the evidence presented below we conclude that reverse-mutation to the wild-type allele does occur.

Strain 33757, which originated from a culture treated with ultra-violet light, is unable to synthesize the amino acid, leucine, an inability which has been shown by Regnery[2] to be caused by a difference from wild type in a single factor. In the course of the development of an assay method for leucine by the use of strain 33757 it was noted that the weights of some cultures were unusually high and did not bear the typical relationship to leucine in the medium which characterizes the growth of this mutant strain. The phenomenon was called adaptation.[3] Genetic and physiological studies have been made on three independently adapted strains of 33757.[4] Adapted strains α and β were derived as follows from a leucineless albino-marked stock of mating type *A* (33757-4637-*A*). Conidia were inoculated into flasks containing 0.25 mg. *l* (+) leucine per 50-ml. medium and were incubated at 30°C. for $8^1/_2$ days. Samples were taken from two cultures which subsequently proved to have unusually high weights, and inoculated into minimal medium. From this time, adapted cultures α and β were

subcultured on minimal medium where they grew like wild type. The third adapted strain, γ, was obtained from a salmon-colored leucineless stock of mating type *a* (33757-*a*) which was heavily inoculated into minimal medium. In one instance growth was observed and, upon subculturing, this adapted strain was prototrophic[5] and grew like wild type.

It should be emphasized that marker genes, for color and for mating type, were not modified in the course of adaptation. Furthermore, among more than 150 adaptations of the leucineless albino stock which we have observed in our studies, none have shown pigmentation. Consequently it is concluded that the adapted strains did not arise through contamination.

Genetic Studies.—In order to test the presumption that adaptation involves a genic mutation, the adapted cultures were first crossed with a leucineless strain of the opposite mating type. Asci were dissected and the spores allowed to germinate on medium containing leucine. The resulting strains were then tested for their ability to grow on minimal medium. The results are given in table 1.

TABLE 1

CHARACTERISTICS OF f_1 CULTURES OBTAINED BY THE GERMINATION OF SPORES DISSECTED IN ORDER FROM WHOLE ASCI SECURED FROM CROSSES OF LEUCINELESS-ADAPTED BY LEUCINELESS

CROSS:	α × 33757-*a*		β × 33757-*a*				γ × 33757-4637-*A*[a]	
SPORE NO.	COLOR[b]	GROWTH[c] ON MINIMAL MEDIUM	COLOR	GROWTH ON MINIMAL MEDIUM	COLOR	GROWTH ON MINIMAL MEDIUM	COLOR	GROWTH ON MINIMAL MEDIUM
1	−	−	+	+	+	−	+	−
2	−	−	+	+	+	−	+	+
3	+	+	+	+	+	−	−	−
4	+	+	+	+	+	−	[d]	[d]
5	+	−	−	−	−	+	−	+
6	+	−	−	−	−	+	+	+
7	−	+	−	−	−	+	+	−
8	−	+	−	−	−	+	−	+

[a] This ascus was not dissected in order.

[b] + refers to salmon conidial pigment, − to albino.

[c] + refers to growth, − to failure to grow. All strains grew on medium to which leucine was added.

[d] This spore did not germinate.

In view of the 1:1 ratios obtained in these asci, leucine-independence in the adapted strains must be due to a chromosomal and not a non-Mendelian cytoplasmic factor. These crosses do not, however, supply any information about the relationship of the leucineless locus in strain 33757, l_1, to the gene for leucine-independence, *L*.

The demonstration of this relationship depends on crosses made between prototrophic strains from the f_1 and wild type. In performing this cross, the f_1 was used rather than the parent adapted strain to avoid the

confusion that might arise from the heterocaryotic[6] persistence of leucineless nuclei in the adapted culture. Barring mutation, a culture derived from a single ascospore is genetically homogeneous. The spores secured from these crosses are classified in table 2. The most extensive test was performed on the progeny of β where there were 13 asci in which every spore germinated, 5 asci in which at least one member of all four pairs of spores germinated so that each of the 4 chromatids could be accounted for, 66 spores from asci with incomplete germination and 83 spores isolated at random. In addition some data were secured on the progeny of crosses between wild-type and the prototrophic f_1 strains secured from α and γ. A total of 300 single-spore cultures was examined and every one proved to be prototrophic.

TABLE 2

CLASSIFICATION OF THE ORIGIN OF SINGLE-SPORE CULTURES FROM CROSSES OF PROTOTROPHIC f_1 CULTURES WITH WILD TYPE. ALL CULTURES PROVED TO BE LEUCINE-INDEPENDENT

(f_1 FROM CROSS OF (SEE TABLE 1))	×	WILD TYPE	NO. OF ASCI WHOSE 8 SPORES GERMINATED	NO. OF ASCI WITH ALL CHROMATIDS ACCOUNTED FOR (4–7 SPORES)	NO. OF SPORES FROM INCOMPLETE ASCI	NO. OF SPORES ISOLATED AT RANDOM
$\beta \times$ 33757-a (spore 1)		15300-A	0	3	10	0
$\beta \times$ 33757-a (spore 2)		15300-A	1	0	1	0
$\beta \times$ 33757-a (spore 3)		15300-A	12	2	47	83
$\beta \times$ 33757-a (spore 4)		15300-A	0	0	8	0
$\alpha \times$ 33757-a (spore 2)		15300-A	0	4	2	0
$\gamma \times$ 33757–4637-A (spore isolated at random)		43–14-A	0	3	7	0
			—	—	—	—
TOTAL			13	12	75	83

If leucine-independence in the adapted leucineless strains were due to mutation at a locus distinct from l_1, crosses between adapted and wild-type strains should have had in their progeny a recombination class of leucineless. In the 25 asci and among the 118 additional spores listed in table 2 there were no recombinations. Since these numbers test for 109 chances for recombination, of which none were fulfilled, the genes involved are probably alleles.

Physiological Studies.—The physiological behavior of the adapted strains confirms the hypothesis of reverse mutation. The rate of progression of the adapted strains and their prototrophic f_1 progeny on an agar surface is the same as that of wild-type strains[7] from which they were originally derived (table 3). The addition of leucine did not stimulate or retard growth in either case. After $8^1/_2$ days in 50 ml. of liquid medium containing 1 per cent sucrose, wild-type strain 1-A, albino strain 4637-A and adapted

strain β gave mycelial crops weighing between 109 and 114 mg. Thus, in their growth, as in their genetic behavior, leucineless-adapted cultures are indistinguishable from wild type. They probably represent back-mutations of the l_1 locus to the wild-type allele.

TABLE 3

RATE OF GROWTH IN MM. PER HR. OF LEUCINELESS-ADAPTED COMPARED WITH THAT OF WILD TYPE AT 25°C. EACH RATE IS THE AVERAGE OF MEASUREMENTS ON TWO GROWTH TUBES

STRAIN	MINIMAL AGAR SUPPLEMENTED WITH: 0	7.5 γ $l(+)$ LEUCINE PER ML.	2 MG. *dl* LEUCINE PER ML.
L-A	4.3	4.1	4.2
R977-a	4.0	...	...
4637-*A*	4.1	...	...
15300-*A*	4.4	4.2	...
α	4.2	4.4	4.3
β	4.2	4.1	4.2
Leucine-independent spore 1 from f_1 of β (see table 1)	4.2	4.2	4.3

Are the Mutations Induced?—In order to determine whether the back-mutations were induced an examination was made of the frequency of adaptation in liquid medium containing various amounts of leucine. Adaptations were identified by the arbitrary method previously described.[3] Those cultures which possessed unusually high weights, more than 3σ above the mean of the other members of a series, were classified as adapted. The data are shown in table 4. The frequency of adaptations depended not only upon the temperature but was higher in low leucine concentrations than in high leucine concentrations. Both of these differences are significant by χ^2 test. Since the dependence upon leucine concentration appears to be an example of chemically induced mutation it is important to examine closely the events that take place during the $8^1/_2$-day period.

TABLE 4

EFFECT OF LEUCINE CONCENTRATION ON THE FREQUENCY OF ADAPTATIONS OF 33757-4637-*A* DURING $8^1/_2$ DAYS IN 50-ML. LIQUID MEDIUM

TEMP:	25°C.			30°C.			
MG. $l(+)$LEUCINE	NO. OF CULTURES	NO. OF ADAPTATIONS	% ADAPTATION	NO. OF CULTURES	NO. OF ADAPTATIONS	% ADAPTATION	AV. % ADAPTATION
1.00	40	0	0	20	0	0	0
0.75	40	1	3	20	3	15	7
0.50	40	0	0	20	3	15	5
0.25	40	3	8	20	5	25	13
Av. % adaptation			3			14	

The leucine concentration expressed in table 4 is the initial concentration. As growth proceeds, the medium is depleted of leucine by the mold. When

the final weight is reached, bioassay of the medium proves the exhaustion of leucine. The medium will, at that time, support the growth of wild type *Neurospora*, or of leucineless *Neurospora* if further leucine is added. Thus, leucine alone is exhausted. The time at which this takes place depends upon the initial leucine concentration. On 0.25 mg. of *l* (+) leucine the final weight of about 7.5 mg. is attained in about 3 days. On 1.00 mg. *l* (+) leucine almost 6 days are required for the development of the final weight of 37 mg. During these times the mass of mycelium is increasing and with it, presumably, the number of nuclei in which back-mutation has a chance to occur.

In order to minimize the importance of the difference in time a long-term experiment was designed in which cultures of 33757-4637-*A* started their growth on 0.25 and 0.5 mg. *l* (+) leucine per 50 ml. at 30°C. The frequency of adaptation was observed between 6 and 45 days after inoculation, during which time all cultures were exposed to subthreshold leucine concentrations. Table 5 shows the results obtained. The same criterion was

TABLE 5

EFFECT OF LEUCINE CONCENTRATION ON THE FREQUENCY OF ADAPTATIONS OF 33757-4637-*A* BETWEEN 6 AND 45 DAYS IN 50-ML. LIQUID MEDIUM AT 30°C.

MG. *l*(+)LEUCINE	NO. OF CULTURES	NO. OF ADAPTATIONS	% ADAPTATION
0.50	43	5	12
0.25	38	16	42

used for identification of adaptations. It will be observed that even during this period there is a significantly higher frequency of adaptation in the cultures which began to grow in the presence of low leucine concentrations. Indeed, the frequency is so high that the average weight of all cultures, adapted and non-adapted, is higher (25 mg.) in the 0.25 mg. leucine series than in the 0.50 mg. leucine series (20 mg.). On the spontaneous mutation hypothesis one would expect the number of adaptations to be greater in those cultures with the higher nuclear populations, the high leucine series. We find the reverse to be true—the frequency of adaptations is an inverse function of mycelial mass.

The orthodox viewpoint is that adaptive mutations are not directed by the environment of the cell but occur in a given percentage of the population per unit time, and may be selected by the environment. We have shown that adaptation, in this instance, is a mutation phenomenon. Thus far we have assumed that when a mutation to leucine-independence takes place it will be regularly selected for in the absence of leucine and result in an adaptation. However, this is not the case. Although adaptations are the result of mutation, every mutation does not yield an adaptation as the evidence presented below will indicate.

Heterocaryons.—When a back-mutation occurs in a leucineless mycelium and the mutated nucleus multiplies a heterocaryon[6] is formed consisting of a mixture of leucineless and wild-type nuclei. The phenomenon of selection in such heterocaryons was studied by artificially preparing heterocaryons between leucineless and prototrophic strains and growing them on different concentrations of leucine. Figure 1 shows the behavior of a heterocaryon between the adapted strain β, and the leucineless strain, 33757-4637-*A*, which gave rise to it. Growth was measured on the surface of agar in growth tubes containing no leucine. The leucineless strain showed, of course, no growth. The prototrophic f_1 control grew at the

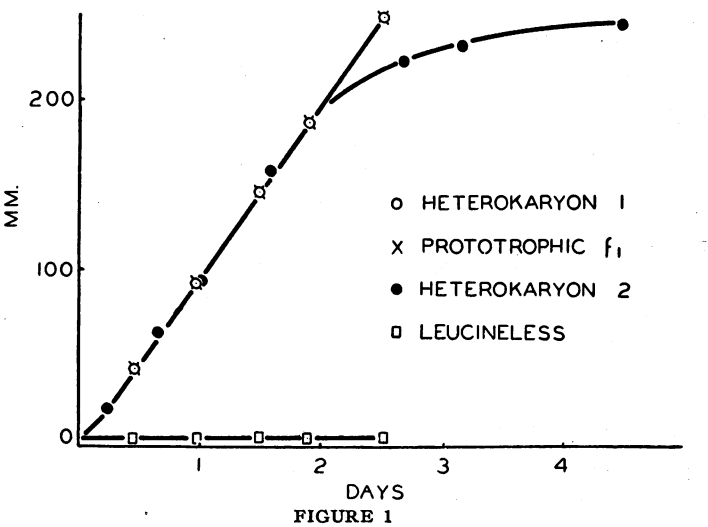

FIGURE 1

Growth rate of a heterocaryon of leucineless and adapted *Neurospora* on minimal medium.

wild-type rate of 4.2 mm./hr. The heterocaryon grew at exactly the same rate, as though the wild-type nuclei it contained had outgrown the leucineless nuclei. On medium containing a limiting concentration of leucine the prototrophic f_1 control still grew at a rate of 4.2 mm./hr. (Fig. 2). However, the heterocaryon grew at the same rate as the leucineless strain, 2.2 mm./hr. It appears as though the wild-type nuclei failed to outgrow the leucineless in the presence of a limiting leucine concentration. In other words, on minimal medium the heterocaryon grows like wild type but in the presence of leucine it grows like leucineless.

To prove that such behavior would be characteristic of hyphae known to be heterocaryotic, minimal agar plates were inoculated with a mixture of leucineless 33757-4637-*A* and of adapted strains α or β. After the mycelium had grown over an area ca. 4 cm. in diameter, tips of single hyphae

were isolated[6] and transferred to media containing or lacking leucine. The plate from which these isolations were made was also kept for further observation. Hyphae isolated to minimal medium continued to grow (as did the parent mycelium on the agar plate) demonstrating that they contained nuclei of the adapted genotype. Since hyphal tips were transferred at random, such nuclei must also have been present in those hyphae inoculated into tubes of leucine-containing medium. The conidia formed in these tubes, however, did not grow when tested on minimal medium. Therefore, in the presence of leucine, hyphae which originally contained some leucine-independent nuclei gave rise to conidia which were purely

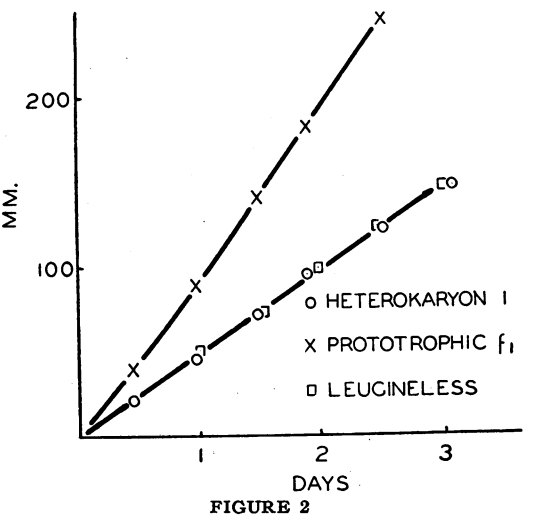

FIGURE 2

Growth rate of a heterocaryon of leucineless and adapted *Neurospora* on a limiting concentration of *l* (+) leucine (0.0075 mg./ml.).

leucineless. Such behavior was characteristic of combinations of leucineless with wild-type strains obtained independently of the leucineless mutation. The similarity of adapted and wild-type strains in this respect is further evidence for the identity of the adapted and wild-type strains at the *L* locus.

This phenomenon is under further study but the following evidence may be reported briefly here. Hyphae were isolated in a similar manner from heterocaryons in which the leucineless and wild-type nuclei bore different marker-genes for conidial color. Such heterocaryotic hyphae behaved similarly to those described above. Furthermore, whenever a leucineless culture was selected from a heterocaryon, the color markers of the wild type could not be demonstrated. This is in favor of the hypothesis that

the wild-type nuclei in such combinations were selected against in the presence of leucine. The disappearance of color markers which characterized leucineless nuclei demonstrated a selection in favor of wild type when heterocaryons were grown on minimal medium. However, in some instances when heterocaryons were studied in growth tubes on minimal medium, they grew like wild type for a time but then slowed down and sometimes stopped (Fig. 1). This behavior helps to interpret the results of the long-term growth experiment in liquid medium.

In liquid cultures when a back-mutation to leucine independence occurs and a heterocaryon is formed the independent nuclei may overgrow and form a complete adaptation. On the other hand, the heterocaryon may begin to grow and then stop as the back-mutated nuclei are inactivated by the leucineless. This behavior was repeatedly observed during growth in liquid medium. In a culture which had reached the maximum growth for its leucine level a new patch of growing mycelium often appeared on the surface of the clot. This new growth may continue and overgrow the whole culture, or, after its initiation, it may stop, forming what was previously termed a "partial" adaptation.[3] Presumably we were observing heterocaryon selection.

The interpretation we offer for the higher frequency of adaptation in low leucine concentrations is in terms of the size of the mycelial mass involved rather than directly in terms of the leucine content of the medium. The greater the mycelial mass, the larger the population of leucineless nuclei in the midst of which a leucine-independent nucleus arises by mutation and the greater the chance that this mutant nucleus will be inactivated. Although we cannot duplicate directly the introduction of a single leucine-independent nucleus into a leucineless mycelium we have studied the growth of heterocaryons in liquid medium. Figure 3 shows the weights assumed after $8^1/_2$ days by a heterocaryon of β and 33757-4637-*A* and by 33757-4637-*A* on different leucine concentrations.

As the leucine concentration rises the amount of mycelium, and hence the number of leucineless nuclei, similarly increases. However, the final weight of the heterocaryon decreases with leucine concentration. Apparently the greater the number of leucineless nuclei the more rapidly the leucine-independent nuclei are inactivated and the sooner growth ceases. These experiments, then, provide evidence for our interpretation of the frequency of adaptations on different leucine concentrations. An adaptation is due to the growth of leucine-independent nuclei which arose by mutation but whether the increased growth will be significant depends upon whether conditions favor the inactivation of the leucine-independent nuclei. Large mycelial masses with many leucineless nuclei are more unfavorable for escape of the prototrophic growth than small mycelial masses. We do not believe that our experiments allow any conclusion as to the rôle of

leucine in the induction of mutation at this locus. Such mutations probably occur spontaneously but their expression depends upon the leucine content of the environment through its effect on mycelial mass.

Discussion.—Because of the heterocaryon selection the back-mutation of locus l_1 is not a favorable object for the study of mutation rates. For example, the effect of temperature shown in table 4 may be due to its influence either on the mutation rate or on the selection efficiency or both. Any calculation of mutation rate would yield a minimum value, at best, because many, if not most, mutations can never express themselves as adaptations because of the rapid inactivation of mutant leucine-independent nuclei.

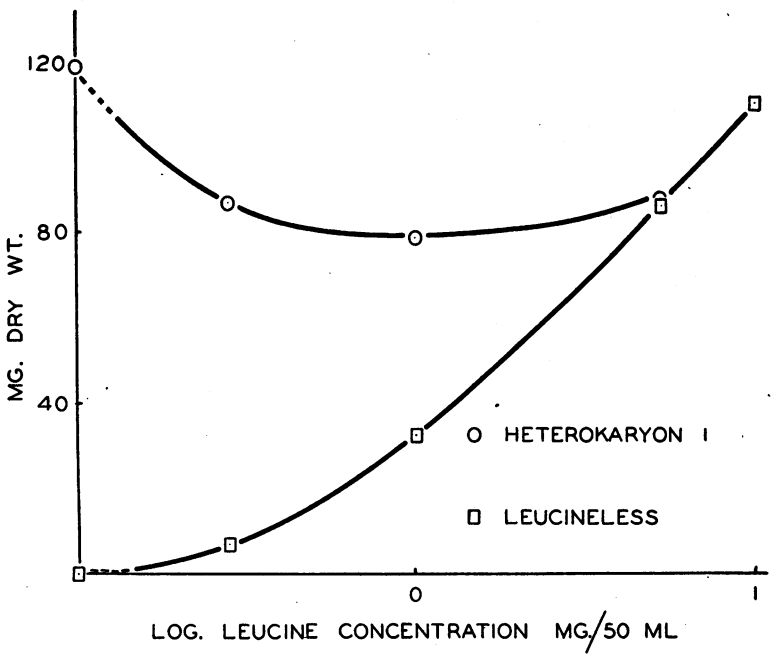

FIGURE 3

Final growth of a heterocaryon of leucineless and adapted *Neurospora* on different concentrations of *l* (+) leucine in liquid medium at 25° C.

On the basis of the selection phenomenon it is to be expected that back-mutations of locus l_1 would not persist in stock cultures maintained on leucine. This probably explains the fact that in our experience no culture in stock tubes has ever adapted. Likewise in nature the heterocaryon selection phenomenon may help to maintain mutant types.

In bacteria nutritional mutants are known to adapt and lose their requirement.[8] In this way they seem to gain a new function. In the light

of recent evidence it is conceivable that nutritional mutants in bacteria are formed by an inactivation of a gene.[9] From this state it may later back-mutate. We conceive of this process in *Neurospora* as follows:

$$L \underset{\text{back-mutation}}{\overset{\text{mutation}}{\rightleftharpoons}} l_1$$

The wild-type allele, L, is present in the wild-type stock and controls some step in the synthesis of leucine. Under the influence of ultra-violet light, or otherwise,[10] this gene can mutate to the inactive state, l_1. We conceive that, where L makes an active enzyme involved in leucine synthesis and self-duplicates as well, l_1 is also self-duplicating and could possibly make a "defective enzyme." The self-duplicating inactive gene l_1 spontaneously back-mutates to L. We believe it to be established, with a fair degree of certainty, that the leucineless mutation in *Neurospora*, l_1, is not a chromosomal rearrangement or deficiency but a modification of a gene to a still self-duplicating particle.[11]

Summary.—The leucineless mutant of *Neurospora* is a true gene mutation. The adaptation to leucine-independence, which this mutant sometimes undergoes, is due to back-mutation to the wild-type condition at the leucineless locus. This is demonstrated by the genetic behavior of the adapted strain in crosses with leucineless and by the genetic behavior of the leucine-independent f_1 progeny in crosses with wild type. Moreover, the adapted and wild-type strains are physiologically identical.

The incidence of adaptations is significantly higher in the presence of low concentrations of leucine than in the presence of high concentrations. This apparent chemical induction of mutations has its explanation in the fact that a back-mutation in the leucineless strain always results in the formation of a heterocaryon. In a heterocaryon between the leucineless and the adapted or wild-type strains, the leucineless nuclei have an advantage in the presence of leucine. Whether a back-mutation will result in an adaptation depends upon whether conditions favor selection against the leucine-independent nuclei.

[1] Beadle, G. W., *Physiol. Rev.*, **25**, 643–663 (1945); Beadle, G. W., and Tatum, E. L., *Am. Jour. Bot.*, **32**, 678–686 (1945).

[2] Regnery, D. C., *Jour. Biol. Chem.*, **154**, 151–160 (1944).

[3] Ryan, F. J., and Brand, E., *Ibid.*, **154**, 161–175 (1944).

[4] The experimental procedures used in these studies have been described previously. See references 1 and 3 and Ryan, F. J., Beadle, G. W., and Tatum, E. L., *Am. Jour. Bot.*, **30**, 784–799 (1943).

[5] We propose to designate as a prototroph any strain which has the nutritional requirements of the "wild type" from which it was derived irrespective of how it became

prototrophic. (For *Neurospora crassa* see Butler, E. T., Robbins, W. J., and Dodge, B. O., *Science*, **94**, 262 (1941).)

[6] Beadle, G. W., and Coonradt, V. L., *Genetics*, **29**, 291–308 (1944).

[7] Different "wild-type" stocks undoubtedly carry gene differences which can modify such physiological characteristics as growth rate and degree of conidiation.[4] These differences may segregate to different stocks. Therefore, it is not strictly correct to speak of the wild type as an entirely distinctive genotype. However, in every mutant studied the nutritional requirement is determined by a single gene, although the genetic background may, to a certain extent, modify the details of its expression. It would be desirable to use "isogenic" strains, obtained by repeated back-crossing, but the biparental inheritance of *Neurospora* makes very difficult the elimination of any gene differences that may be linked to mating-type alleles.

[8] Roepke, R. R., Libby, R. L., and Small, M. H., *Jour. Bact.*, **48**, 401–412 (1944).

[9] Gray, C. H., and Tatum, E. L., these PROCEEDINGS, **30**, 404–410 (1944).

[10] The leucineless mutant, 33757, was obtained from a culture of *Neurospora* which had been treated with ultra-violet radiation. However, since parallel studies on spontaneous mutation were not carried out[1] it cannot be proved that the mutation $L \rightarrow l_1$ was induced and did not occur spontaneously.

[11] Strain 4637-*A* is known to carry a translocation (McClintock, B., *Am. Jour. Bot.*, **32**, 671–678 (1945)) which is closely linked to albino. It reduces crossing over in a region of the sex chromosome (Doermann, A. H., *Arch. Biochem.*, **5**, 373–384 (1944)). The prototrophic f_1 strain used in these experiments, although wild type in color, may have carried the translocation. The adaptation could have been due to mutation to leucine independence at a locus other than l_1 since, in the cross of the f_1 with wild type, reduction of crossing over might have prevented the appearance of recombinations. There are two types of evidence which militate against these assumptions. First, we found no reason to believe that the f_1 by wild-type cross produced the lethal classes which would be expected if a translocation were involved. Second, the physiological identity of adapted strains with wild type speaks for the identity of the leucine-independent gene and the wild-type allele of l_1.

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*VARIETIES AND MATING TYPES IN PARAMECIUM BURSARIA. I. NEW VARIETY AND TYPES, FROM ENGLAND, IRELAND, AND CZECHOSLOVAKIA**

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Paramecium bursaria consists of a number of varieties (Jennings,[1] Jennings and Opitz[2]). Members of any one variety do not, as a rule, mate with members of other varieties. In each variety there is a definite number of mating types. Animals of diverse mating types conjugate readily; animals of the same mating type usually do not mate together. Variety I contains four mating types designated A, B, C, and D. Variety II has eight mating types designated E, F, G, H, J, K, L, and M. Variety